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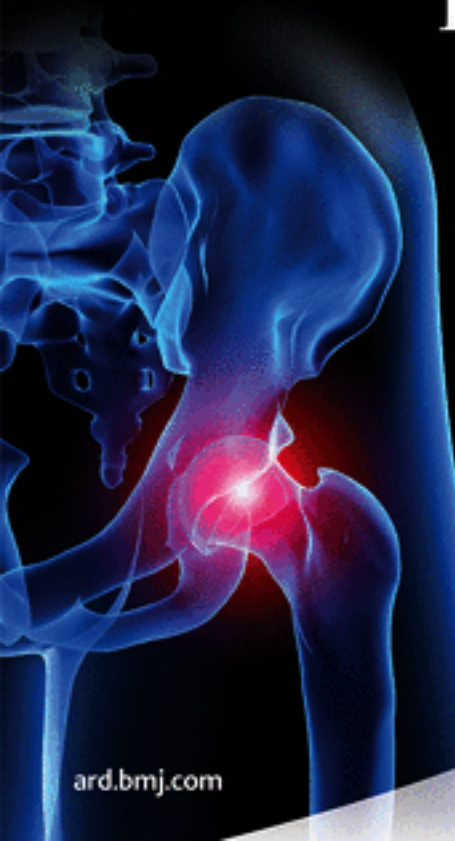

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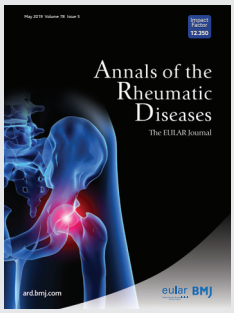

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Time to change the primary outcome of lupus trials

Frederic A Houssiau[1,2]

In *ARD*, a group of outstanding investigators report the results of another lupus trial missing its primary endpoint, namely the Phase III CHABLIS-SC study aimed at testing the efficacy of blisibimod,[1] composed of a tetrameric BAFF/BLyS domain fused to a human IgG1 Fc region. Interestingly, blisibimod displayed unequivocal effects on biomarkers, such as reduction of circulating B cells, serum immunoglobulin titres or anti-DNA antibodies and increase in complement levels, changes in line with its mode of action and known to correlate with improved clinical outcome. Moreover, although the trial was not intended to demonstrate renal efficacy, an interesting reduction of proteinuria was noticed. This paradox raises the possibility that the failure of CHABLIS-SC stems more from the choice of the primary efficacy endpoint than from the drug itself, the more so as BAFF/BLyS was proven to be an appropriate target in four previous clinical trials, namely the belimumab BLISS-52,[2] BLISS-76[3] and BLISS-SC[4] and the tabalumab ILLUMINATE-2[5] studies, all showing the same effects on biomarkers and a significant, although modest, clinical efficacy. Of note, while the primary outcome (SRI-6) failed in the CHABLIS-SC trial, a trend (p=0.056) in favour of blisibimod was demonstrated when a modified endpoint was used, which takes into account, besides SRI-6, achievement of a steroid dose reduction between weeks 40 and 52 compared with day 1.

The main goal of this editorial is to propose a 'U loop' in the choice of the primary outcome measure for lupus trials. So far, and quite logically, the stress has been placed on achieving less disease activity, as measured by one of the many existing indices, such as SELENA-SLEDAI, SLEDAI-2K, BILAG, BICLA, ECLAM or SRI. Tongue in cheek, their numbers somehow indicate that none of them performs so well… My heretical proposal is to use steroid reduction as a pragmatic primary outcome measure, indirectly reflecting improved disease control! I hear you shouting that this is too far-fetched, but this is exactly why an editorial should be written, not just for summarising and contextualising a study.

I anticipate that choosing a low steroid target as primary endpoint will hardly be accepted by regulatory agencies who label drugs based on their proof of clinical efficacy. Yet, it could be argued that a trial would be considered positive if patients assigned the study drug achieve clinical results comparable with patients randomised to the placebo arm (actually a standard of care arm), with less steroid exposure. The minimal clinically meaningful difference could then become a percentage of steroid reduction, for example, 50%, provided this result is sustained during a sufficiently long period of time, for example, 6 months. Actually, this is what the CHABLIS-SC trial reports. Figure 2 of the paper is quite illustrative in this respect: a similar percentage of patients assigned the study drug and the placebo achieved SRI-6 response at week 52, but the mean daily dose of prednisone was strikingly lower in the blisibimod group, with twice more patients achieving a dose ≤7.5 mg/day.

At a first glance, imposing steroid reduction as primary endpoint raises an ethical concern, a potential medical problem and a methodological issue. Regarding the ethics, it is not possible to taper steroids in patients who do not improve. This concern can be solved by foreseeing escape mechanisms, patients unable to comply with a predefined stringent steroid taper being censored as failure and promptly rescued as *per* good clinical practice. If the study drug is a wonder molecule, it should reduce signs and symptoms despite steroid reduction. Steroid withdrawal symptoms might constitute a medical issue in patients taking steroids on the long term. This said, the proposal is not to stop steroids but to achieve some steroid spare. The target might actually differ for patients taking steroids at screening compared with those in whom steroids have been started for treatment of a flare. The methodological issue deals with a real paradigm shift, namely to switch from analysis of response at a given time point (the timing is yet another debated issue) to regular monitoring of a *per* protocol steroid tapering, which is applied except if physician's global assessment of disease (PhGA) activity indicates worsening disease. At the bedside, PhGA drives the steroid dose much more than other scores, which are not used in daily practice, nor for assessment of disease activity, neither for treatment decisions. In other words, this pragmatic approach would parallel clinical practice, steroids being reduced except if patient's condition does not improve. Taken together, the steroid regimen should be very strictly controlled, starting with maximum 20 mg predniso(lo)ne/day, promptly tapered over a few days/weeks. Patients would be evaluated on a regular basis and those who fail the tapering regimen would be declared as treatment failure and treated as needed. At the end of the trial, the percentages of patients achieving a predefined steroid target would be compared.

Before my proposal be turned down, I would like to stress the many reasons why such a primary endpoint would be most welcome. First, a drug allowing steroid reduction would be a major step forward from a patient's perspective. Second, since damage accrual in lupus is mainly due to steroid use,[6] there is little doubt that reducing the cumulative dose of steroids will reduce morbidity and hopefully mortality in the long term. Third, it should be stressed that two recent Phase II lupus trials that included steroid tapering in their co-primary endpoint (together with disease improvement) reached their target! Thus, anti-IFNAR anifrolumab was shown superior to placebo in the Phase II *MUSE* trial, which used as primary efficacy endpoint a composite of the SLE Responder Index (SRI-4) at week 24 with a sustained reduction in steroids from weeks 12 through 24 (<10 mg prednisolone/day and less than or equal to the dose received at week 1).[7] For the sake of exactness and fairness, the trial would have been conclusive even without the oral steroid taper requirement. In the Phase 2B *AURA* lupus nephritis trial, voclosporine (VCS), a new calcineurin inhibitor, was tested against placebo on MMF background. A very stringent steroid tapering was made compulsory to be considered as a responder (sustained reduction of prednisolone ≤10 mg/day between weeks

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16 and 24, besides specific renal targets, such as a uP:C ratio ≤0.5 mg/mg). The results speak for themselves with twice more complete renal remission rates in the MMF/VCS combination group compared with MMF.[8] Such a difference would probably have been missed if the steroid regimen had been left to physician's/patient's decision and if a steroid target had not been included in the primary endpoint.

In line with these success stories and with the post hoc analyses of the CHABLIS-SC study, my minimal suggestion is to systematically include a steroid target in the primary endpoint to unmask the true efficacy of a study drug. My bold proposal goes a step further, namely to use steroid reduction as primary outcome measure.

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Jan Gösta Waldenström and rheumatology

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On 2 September 1943, Jan Waldenström (1906–1996) successfully submitted a paper to *Acta Medica Scandinavica* describing two patients with a new disease. The discovery was to make him world famous.[1] This year marks the 75th anniversary of macroglobulinaemia and it coincided with the 10th biennial international workshop of Waldensm's macroglobulinaemia, discussing advances in the genetic basis, pathogenesis and treatments of the disease.[2] Jan Waldenström (JW) would have enjoyed this workshop immensely, sharing the information that over 95% of the patients had somatic mutations affecting the MYD88 gene on the second chromosome as well as the impressive advances in treatment. Attending this excellent meeting brought back memories of my time as Waldenström's PhD student and triggers me to compose this vignette, focusing on connexions between the interests of my mentor and rheumatology. For a more comprehensive account of Jan Waldenström's legacy, I recommend Robert Kyle's superb obituary, published in *Blood*, a journal JW was attached to from its start.[3]

After the successful defence of his landmark PhD thesis on acute intermittent porphyria,[4] Waldenström's interest focused on haematology. He worked in Uppsala, a university where the study of proteins had prominence. There, The Svedberg had developed the ultracentrifuge and Arne Tiselius the free electrophoresis and Robin Fåhreus the elevated sedimentation rate (ESR). Conditions with ESR caught JW's special attention. An early witness is a paper from 1937, analysing five cases diagnosed with uveoparotitis, a rare condition most prevalent in women and then considered by many to be a form of tuberculosis. He noticed several multiorgan manifestations, the similarities with von Mikulicz disease and Boeck's sarcoid, the presence of high ESR, unspecific Wassermann reaction, absence of proof of tuberculosis, presence of xerostomia and frequent central nervous system manifestations.[5] Today, the diagnosis could have been IgG4-related disease in several if not all of these patients. This early paper also shows a keen interest in inflammatory systemic conditions.

In the early 1940s, JW collected serum from some 100 patients with long-standing ESR exceeding 120 mm and had the samples analysed by the new technique of free boundary electrophoresis by KO Pedersen in the department of physical chemistry. In 1943, he described three cases from this population characterised by repeated bouts of declive purpura, leaving spots of brown discolouration, mild anaemia and, on the whole, good general health. Two of the three women also had dry eye problems and one had dry mouth and swollen parotid glands. He named the condition 'purpura hyperglobulinemica'.[6] Similar patients were soon identified by others and labelled 'Waldenström's purpura hyperglobulinemica', citing his Swedish-language communication that contained only a brief summary in English. A more comprehensive later report presented new cases, detailed case histories and a colour illustration of the typical skin changes (figure 1), and discussed the systemic nature of the condition in depth. Other organ manifestations included lymphadenopathy, uveoparotitis, Sjögren's syndrome and systemic lupus erythematosus (SLE). The serum albumin concentration remained normal in line with the benign nature of the condition.[7] JW was surprised that this English paper was hardly ever cited.[8]

In 1949, Jan Waldenström was appointed as the first professor and chairman of internal medicine at Malmö General Hospital, the new second teaching hospital of Lund University. In Malmö, he continued investigating what he now called gammapathies. The new technique of paper electrophoresis refined by Carl-Bertil Laurell dramatically simplified identification of patients with hypergammaglobulinaemia and serum electrophoresis became a routine test. In collaboration with Sten Winblad, sera were also routinely examined for presence of antibodies to bacterial antigens by a package called 'total serology'. Combined, this led to the distinction between polyclonal reactive and monoclonal malignant conditions, perhaps the most important of all scientific contributions made by JW.[9]

In 1950, JW was invited to speak at the first post–World war II German congress of Gastroenterology. There, he presented a few cases of a new form of active chronic hepatitis, predominantly in young women with high ESR, very high concentration of gammaglobulin and prominence of plasma cells in the liver. Some but not all of the patients developed cirrhosis.[10,11] A similar observation was presented at a meeting in the USA by Kunkel *et al*.[11] This condition was also soon observed by other investigators and known under several names. One that has survived is chronic active hepatitis. Sheila Sherlock has summarised the clinical spectrum of the disease based on 115 of her own cases and emphasised the systemic nature which is characteristic of an autoimmune disorder.[12] Ulcerative colitis, skin rashes, glomerulonephritis, pulmonary infiltrates and Hashimoto's thyroiditis were common. Antinuclear antibodies were found in 40% and rheumatoid factor in 70% of her cases.

In Malmö, JW soon emerged as a charismatic leader, equally popular among patients, medical students and staff, and highly respected by Malmö's

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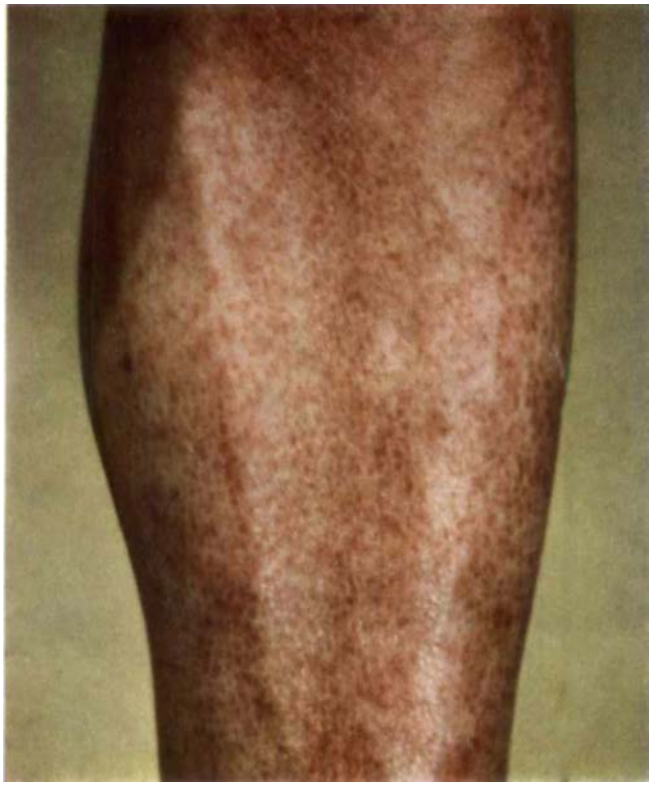

Figure 1 Skin discolouration in the leg in purpura hypergobulinemica. From Waldenström.[7]

ambitious hospital administrators. Within a few years, the department, although frugally staffed, became the leading academic internal medicine unit in the country. JW was a firm believer in the blessing of unfragmented internal medicine, although expecting members of the staff to select an area of special expertise within it. Rheumatology was only established as a specialty in Sweden in 1969, and the first generation of rheumatologists were specialists in internal medicine. But in Malmö, autoimmune disorders like SLE were *speciallité de la maison*. Talbott and Ferrandis' 'Collagen Diseases' was obligatory reading.[13] The book from 1956 still rests on my shelf.

International visitors were frequent guests and fellows came to work with the famous professor. Patients with rare or unclear disease were referred to him from all over Sweden. One example which directly affected me as junior house officer was two cases with extremely low gammaglobulins and antibody deficiency disease, labelled as adult acquired hypogammaglobulinaemia. JW had interviewed the women who came from different hospitals in the country and not simultaneously. In spending good time talking to them, he happened to find out that both had roots in Visseltofta, a village 100 miles to the north of Malmö. I was given the task to find out if they had common ancestors. After some months of searching in old church registers, this in fact turned out to be the case, hinting at a possible genetic aetiology. JW was of course pleased to see the pedigree, and when I presented a brief report with his and my name, he said "Fine, send it to The Lancet". Unfortunately, he erased his own name from the manuscript probably to do me a special favour. The paper was accepted without changes.[14] The observation was later supported by a larger report.[15] The disease now is named common variable immunodeficiency and genomic technology including next-generation sequencing reveals its complex genetic basis and explains links to autoimmunity.[16] The *Lancet* paper

Figure 2 Morris Ziff and Jan Waldenström in Malmö in the 1980s.

was my first publication as internist and it opened the way to USA where I was to become a rheumatologist.

Several of JW's international contacts and visitors were prominent in rheumatology. Henry Kunkel, Morris Ziff, Eric Bywaters, Barbara Ansell, Norman Talal, Eng Tan, Bob Winchester and Ralph C Williams are some names that come to mind (figure 2). JW never passed New York without visiting Henry Kunkel at the Rockefeller Institute where he enjoyed making ward rounds. A paper titled "Forty years with the gammaglobulins"[17] gives further personal proof of JW's close ties with rheumatology, which certainly facilitated my path into the specialty. My own visits with Henry Kunkel's small group usually included a seminar where the presenter was allowed to use the blackboard but not to show slides. The group then had lunch and after lunch went to the library and browsed through the new journals of the day. Electronic journals had not been born.

Although best known for the discovery of macroglobulinaemia, he must also be credited for the distinction between polyclonal reactive and monoclonal malignant hypergammaglobulinaemia. In Malmö, JW initiated a large study of families with SLE.[18] The topic of his last PhD student was polymyalgia rheumatica (19). We can justify the epithet "honorary rheumatologist".

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CLINICAL SCIENCE

Normal mortality of the COBRA early rheumatoid arthritis trial cohort after 23 years of follow-up

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ABSTRACT

Objectives Mortality in patients with rheumatoid arthritis (RA) is higher than in the general population. We investigated mortality in the COBRA-trial cohort after 23 years follow-up, compared with a reference sample of the Dutch population.

Methods The COBRA-trial randomised patients with early RA to sulfasalazine monotherapy (SSZ, n=79) or a combination of SSZ, low-dose methotrexate and initially high, step-down prednisolone (COBRA, n=76). We compared the mortality in the COBRA-trial up to 2017 to a reference sample of the general population in the Netherlands (standardised mortality ratio, SMR), and its relation to early prognostic factors through stepwise Cox regression.

Results Duration of follow-up in patients alive was mean 23 (range 22–24) years. In total, 44 patients died (28%, SMR=0.80 [95% CI 0.59 to 1.06]); 20 of 75 COBRA patients (27%, SMR 0.75 [0.47 to 1.14]) and 24 of 79 SSZ patients (30%, SMR 0.85 [0.56 to 1.25]); p=0.61). In the reference sample of the general population, 55 people (36%) died. 5 factors were significantly associated with increased mortality hazard: damage progression at 28 weeks; high Health Assessment Questionnaire (HAQ) score and absence of HLA-DR 2 or 3; disease duration from start of complaints was also significant, but showed an uninterpretable pattern.

Conclusions This prospective trial cohort study of early RA is one of the first to show similar mortality compared with the general population after 23 years of follow-up. It confirms that early, intensive treatment of RA has long-term benefits and suggests that treating to target is especially important for patients with poor prognosis.

Key messages

What is already known about this subject?
- Mortality in patients with rheumatoid arthritis (RA) is higher than that of the general population, especially in patients with disease duration >10 years.

What does this study add?
- This prospective cohort study in patients with early RA is one of the first to show a normalisation of RA mortality after 23 years of follow-up.
- Several well-known prognostic factors were related to mortality.

How might this impact on clinical practice or future developments?
- The study confirms that early and intensive treatment of RA has long-term benefits and suggests that treating to target is especially important for patients with poor prognosis.

INTRODUCTION

Mortality in patients with rheumatoid arthritis (RA) is higher than that of the general population.[1] In recent studies, the adverse effect of RA appears to have decreased, but in some there is an indication that long follow-up (>10 years) is necessary for the full adverse effect to become apparent.[2 3] Whether early and intensive treatment can improve this mortality is still uncertain.

The COBRA (Combinatietherapie bij Reumatoide Artritis) multicentre, double-blind randomised trial compared the combination of sulfasalazine (SSZ), methotrexate (MTX) and prednisolone (COBRA) to SSZ monotherapy. COBRA combination therapy was superior to SSZ in disease control (activity and damage) with less adverse events.[4] Subsequently, patients in the COBRA study arm retained better disease control in the 5 years following the trial independent of subsequent therapy;[5] after 11 years, these patients had a numerically lower mortality and similar prevalence of comorbidity compared with patients originally in the SSZ arm.[6]

The present study extends mortality follow-up in this COBRA trial cohort to 23 years and explores associations between mortality and well-known prognostic factors.

METHODS

The COBRA double-blind clinical trial ran between 1993 and 1995. A total of 155 patients with early and mostly DMARD naïve RA (disease duration median 4 months, maximum 2 years; 22% prior treatment with antimalarials) were randomly allocated: 76 patients received COBRA combination therapy and 79 patients SSZ monotherapy. COBRA combination therapy comprised SSZ (2 g/day), MTX (7.5 mg/week) and prednisolone (60 mg in the first week tapered to 7.5 mg in week 7). Prednisolone was withdrawn after 28 weeks, MTX after 40 weeks. Patients in the active control group received SSZ (2 g/day) and double placebos.

In addition, all patients received folic acid (1 mg/day), calcium (500 mg/day) and if necessary 25-hydroxyvitamin D (400 IU/day). Patients who experienced a flare started treatment with the drug that was most recently withdrawn. After study completion at 56 weeks, further treatment decisions were at the discretion of the treating rheumatologist,

but physicians were asked not to (re)start prednisolone or MTX for another 6 months to prevent unblinding. All patients gave written informed consent for the study including follow-up.

In 2017, we retrieved mortality data of the COBRA cohort through scrutiny of the centralised Dutch mortality register of the Centrum of Familiegeschiedenis (CBG); where necessary, we contacted the rheumatologist for missing data.

We compared the mortality in the cohorts to each other and to a hypothetical reference sample of the general population in the Netherlands matched for age, gender and calendar period of start of follow-up. In more detail, to form the reference sample, we created a hypothetical 'non-RA twin' for each trial participant, that is, a person of the same sex and age. We then applied the Dutch population level yearly actuarial death rates to this sample until the end of study follow-up . Statistics Netherlands (Centraal Bureau voor Statistiek) provided these rates for the years 1994 to 2016. Finally, we calculated the standardised mortality ratio (SMR): observed deaths divided by the expected deaths in 2017. SPSS for Mac V.24.0 (SPSS, Chicago, Illinois, USA) performed the statistical analyses.

To compare our results with that of recent literature, we first performed a scoping search on Pubmed for systematic reviews with various combinations of the keywords: arthritis; rheumatoid; epidemiology and mortality (online supplementary appendix 1). We found two dated 2013[1] and 2016.[7] Then, we searched for full size publications published in or after 2010 (the closing year of the first review) with these same keywords and found 532 hits. From these, we selected the seven articles that compared mortality of inception cohorts of patients with RA to that of the general population and a follow-up exceeding 10 years. From the literature lists of the selected articles, we found two additional abstracts (no new full-size articles). We contacted the authors but full-size publications were not yet available.

We performed exploratory stepwise forward and backward Cox regression with the following variables as possible hazards: smoking, education level, disease duration defined from start of complaints, from first clinic visit and from diagnosis; disease activity score (DAS-44 joints); functional disability (Health assessment questionnaire); rheumatoid factor; Sharp van der Heijde damage score; rheumatoid factor (anti-citrullinated protein antibody (ACPA) not available at that time); presence of HLA-DR1, 2, 3 or 4; treatment group; change in DAS44 at 16 weeks; change in damage score at 28 and at 56 weeks. We also explored models that included only baseline data, only routine baseline data (ie, excluding HLA-DR) or only routine baseline and follow-up data.

To study representativeness of the trial cohort, we retrieved an unpublished analysis performed in Maastricht (one of the including centres) before publication of the main results. In this analysis, we checked the screening log of 1051 consecutive patients seen in the rheumatology outpatient department while the trial was running. The screening was applied to all patients visiting the clinic and set up to quickly rule out ineligible patients, so the form only needed to be completed until the first exclusion criterion was met. The exclusion sequence was: (1) no RA; (2) age; (3) prior treatment; (4) disease duration; (5) severe comorbidity; (6) American College of Rheumatology (ACR) criteria; (7) inactive disease.

RESULTS

Follow-up was nearly complete with only 1 out of the 155 patients missing (from the COBRA group); this patient was already missing in earlier reports. Mean follow-up in patients alive was 23 (range 22–24) years. In total, 44 patients (28%, SMR=0.80 [95% CI 0.59 to 1.06]) of the cohort died during follow-up; 20 of the 75 patients of the COBRA-group (27%, SMR 0.75; [0.47 to 1.14]) and 24 out of 79 in the SSZ-group (30%, SMR 0.85 [0.56 to 1.25]). The difference in mortality was not significant (p=0.61). In the reference sample of the general population, 55 of 154 persons (36%) died. The mortality rates for COBRA over SSZ moved closer together over time (figure 1).

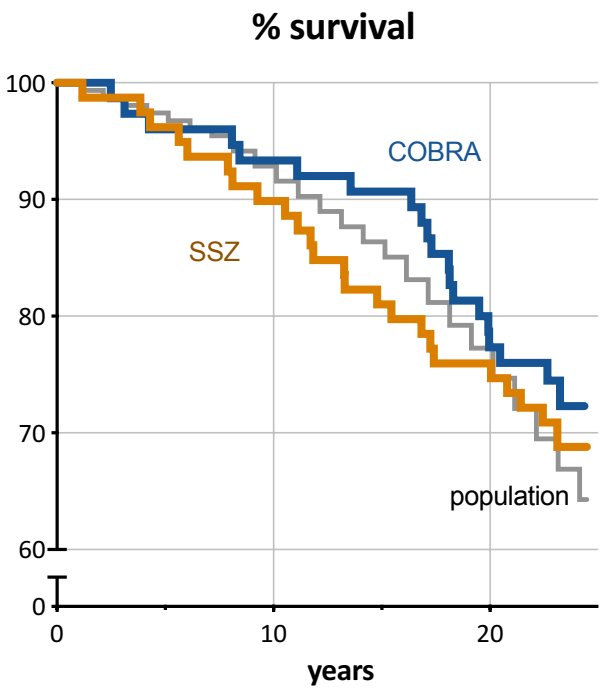

Figure 1 Survival curves of the COBRA trial cohort by treatment. COBRA, n=75 (1 patient missing); SSZ, n=79. Survival of the reference cohort from the general population in grey. Note that all living patients have now been followed for 22–24 years, so the proportion of survivors equals the proportion at risk.

In exploratory stepwise forward Cox regression, five factors were significantly associated with increased mortality hazard: damage progression at 28 weeks; high HAQ score; shorter disease duration from start of complaints and absence of HLA-DR 2 or 3 (online supplementary appendix 1). The same factors were selected in backward stepwise regression (data not shown). In the models restricted to baseline variables, only the presence of HLA-DR1 or 4 was associated with an increased hazard; when only routine baseline variables were offered, none were associated with increased hazard.

In the models restricted to routine and follow-up data, shorter disease duration from start of complaints and damage progression at 28 weeks were associated with an increased mortality hazard (data not shown).

Plots of survival curves of subgroups split at the median or binary value of each factor showed results consistent with the regression results except for disease duration (online supplementary appendix 2). Survival risk did not consistently decrease with increasing duration, making the results for this factor difficult to interpret.

Examination of the screening log of over 1000 patients visiting the outpatient clinic revealed that most were excluded for the following, sequentially applied criteria: no RA (39%), age (23%), prior treatment (17%) and disease duration (7%). Only

25 patients (2.4% of all patients and 3.9% of patients with RA) were excluded for serious comorbidity.

DISCUSSION

This 23-year follow-up study shows normal mortality of the COBRA early RA trial cohort compared with that of the general population of the Netherlands. In fact, it is numerically lower than the reference cohort, with a CI still compatible with a 6% increase. The difference between the original trial groups was not significant. Compared with previous studies with follow-up exceeding 10 years, our results are the first to suggest that early treatment of RA may actually normalise mortality.

The exploratory Cox regression revealed interesting results: the impact of some, but not all traditional prognostic factors was confirmed. Of interest is also the number of factors showing statistical significance in this small sample size, and the strong effect of early damage progression, in the absence of an effect of baseline damage in this early RA cohort. These results must be seen in the light of the characteristics of early RA in the period 1993–1995, including high baseline damage and still substantial progression of damage; caution is also advised in view of the small sample size, and the fact that the overall survival curve suggests the constant hazard assumption may not be met. Nevertheless, the data suggest that it is worthwhile to intensively monitor patients with RA with poor prognostic indicators (high initial HAQ, rapid development of radiographic damage) and to strictly apply 'treat to target' goals.

A recent meta-analysis by Dadoun *et al*[1] covering the last 50 years showed that mortality in patients with RA remains higher than that of the general population, although the difference has decreased over the past decades. Minichiello *et al*[7] reviewed the literature with a focus on severity of RA and noted more improvement on life impact than on mortality.

We updated the existing systematic reviews by studying new published literature in or after 2010 and we see similar results as presented by the systematic reviews (table 1). In the largest study, Holmqvist *et al*[2] documented the mortality of RA in four different inception cohorts from 1997 onwards, covering over 80% of the Swedish population. Compared with the general population, they noted a decreased mortality rate in the first years, followed by increased rates after about 8 years of RA. The mortality increase of the cohort starting between 1997 and 2001 was somewhat lower (HR 1.09) compared with that seen in other, smaller RA studies (SMR/HR 1.23–1.66; table 1). In those studies, the increase in mortality was not always statistically significant.

We also found improvement of the increased mortality in several recent studies (ie, more recent than the Dadoun review) with a shorter follow-up.[8–14] Radovits *et al*[3] and Abasolo *et al*[9] deserve separate mention: although the mean follow-up was less than 10 years, the range was wide and extended to 20 years or more. The former study reported an increased mortality risk only after 10 years of follow-up, whereas in the latter the increased risk was immediately apparent.[3]

Finally, Kiadaliri *et al*[15] had a different study focus that supports the above evidence: they studied the extreme of the distribution of RA-associated mortality, that is, cases where RA was reported as cause of death. They compared rates over time in 31 countries in databases of the WHO and the United Nations, and noted a decline overall, but with large disparities between countries.[15]

Altogether it appears that in the long term the increase in longevity seen in the general population is matched and perhaps surpassed by increases seen in the RA population, but a detrimental difference remains. It is likely that the improved prognosis is the sum of earlier detection and treatment, more aggressive treatment, and better handling of (especially cardiovascular) comorbidity. Differences between studies can be attributed to differences in study population (incidence or prevalence cohorts; trial or clinic; geographical location, management and follow-up duration). Our study represents a favourable extreme, showing normalisation of mortality in an inception trial cohort. Importantly, it confirms our earlier findings suggesting that initial treatment of RA with glucocorticoids does not lead to an excess of harm.

Strengths of our study include initially tightly protocolled treatment and a nearly complete follow-up that spans 23 years. Weaknesses include the lack of power due to the small sample size and lack of data on treatments and cause of death. However, our previous follow-up study already documented a pattern of comorbidity comparable to the general population, without differences in treatment or comorbidity between the

Table 1 Overview of recent studies on RA mortality in inception cohorts with more than 10 years of follow-up, and one recent systematic review*

First author (ref)	Year	Inception period	Follow-up (years)		Sample size		SMR/ HR (95% CI)	
			End year	Duration	Patients with RA	Control		
Holmqvist *et al*[2]	2017	1997–2001	2015	≥14	17 512	78 847	HR	1.09 (1.01 to 1.18)
		2002–2006						1.02 (0.94 to 1.10)
		2007–2011						0.95 (0.86 to 1.05)
		2012–2015						0.77 (0.63 to 0.95)
Sparks *et al*[17]	2016	1976	2012	36	964	9499	HR	1.40 (1.25 to 1.57)
Van Nies *et al*[18]	2010	1993–1995	2008	≥13	108	108	SMR	1.35 (0.95 to 1.93)
		1996–1998			174	174		1.23 (0.91 to 1.67)
		1999–2006			402	402		0.49 (0.31 to 0.77)
Gwinnutt *et al*[19]	2017	1990–1994	2010–2014	20	602	602	SMR	1.25 (1.11 to 1.42)
Kapetanovic *et al*[20]	2011	1985–1989	2008	19	183	183	SMR	1.23 (0.97 to 1.55)
Masi *et al*[21]	2017	1974–1992	2015	≥13	54	216	HR	1.66 (1.12 to 2.46)
Lassere *et al*[22]	2012	1990–1994	2004	≥10	113	113	SMR	1.31 (0.93 to 1.80)
Systematic review*								
Dadoun *et al*[1]	2013	1955–2010		–	51 819		Meta–SMR	1.47 (1.19 to 1.83)

*The other systematic review of Minichiello *et al*[7] did not yield a Meta-SMR.
SMR, standardised mortality ratio.

treatment groups.[6] In addition, recorded causes of death are notoriously unreliable when compared with autopsy results.[16] Also, the peculiarities of a trial cohort hinder generalisability: the patients were selected for active RA disease and had several unfavourable prognostic characteristics related to RA (bad RA, worse prognosis than the patient with average RA) but were also selected for lack of severe comorbidity and perhaps other generally favourable factors associated with trial participation (better health, better prognosis than the general population). However, examination of a large screening log suggests that comorbidity was not an important reason for exclusion.

In conclusion, this is the first study with follow-up of more than 20 years that strongly suggests normal mortality of patients with RA in a trial cohort, and no difference between patients initially treated with a combination of conventional disease modifying drugs (including glucocorticoids) or SSZ monotherapy right from the start of the disease.

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EPIDEMIOLOGICAL SCIENCE

Prevotella copri in individuals at risk for rheumatoid arthritis

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ABSTRACT

Objectives Rheumatoid arthritis (RA) has been associated with a relative expansion of faecal Prevotellaceae. To determine the microbiome composition and prevalence of *Prevotella* spp. in a group of individuals at increased risk for RA, but prior to the development of the disease.

Methods In an ongoing cohort study of first-degree relatives (FDRs) of patients with RA, we identified 'FDR controls', asymptomatic and without autoantibodies, and individuals in pre-clinical RA stages, who had either developed anticitrullinated peptide antibodies or rheumatoid factor positivity and/or symptoms and signs associated with possible RA. Stool sampling and culture-independent microbiota analyses were performed followed by descriptive statistics and statistical analyses of community structures.

Results A total of 133 participants were included, of which 50 were categorised as 'FDR controls' and 83 in 'pre-clinical RA stages'. The microbiota of individuals in 'pre-clinical RA stages' was significantly altered compared with FDR controls. We found a significant enrichment of the bacterial family Prevotellaceae, particularly *Prevotella* spp., in the 'pre-clinical RA' group (p=0.04).

Conclusions *Prevotella* spp. enrichment in individuals in pre-clinical stages of RA, before the onset of RA, suggests a role of intestinal dysbiosis in the development of RA.

Key messages

What is already known about this subject?

- A high relative abundance of *Prevotella copri* has been identified in patients newly diagnosed with rheumatoid arthritis (RA), suggesting a role of gut microbiota dysbiosis in the aetiopathogenesis of the disease.

What does this study add?

- This is the first study to describe a significantly altered microbiota, particularly a *Prevotella* spp. enrichment, already in individuals in pre-clinical stages of RA, compared with controls.

How might this impact on clinical practice or future developments?

- Our results, together with previous studies in patients with early RA and recent mechanistic studies, support the mucosal origins hypothesis and the role of intestinal dysbiosis in the development of RA.
- Intestinal dysbiosis could act as an early environmental modulator and may be a target of future preventive interventions in individuals at risk of RA, before the onset of the disease.

INTRODUCTION

The aetiopathogenesis of rheumatoid arthritis (RA) is thought to result from a multistep process, where environmental factors induce a pathological activation of the immune system in susceptible individuals.[1] Recent studies have suggested that the initial steps of the pathological autoimmune response originate in mucosal sites, rather than in the joints.[2] Intestinal dysbiosis has been suggested to have a causal role in the pathogenesis of RA and has been shown to trigger arthritis development in genetically susceptible mice.[3–6] *Prevotella copri* has been identified as highly enriched in the gut microbiota of patients newly diagnosed with RA and an increased immune response to this organism has been demonstrated in patients with RA suggesting a role of *P. copri* in the disease onset.[7–9] Sequence homology between RA-specific autoantigens and epitopes from proteins of *P. copri* have been reported, supporting the molecular mimicry hypothesis, although exact mechanisms remain uncertain.[8] Considering these observations, intestinal dysbiosis involving *Prevotella* spp. may be a risk factor for RA and a potential therapeutic target. However, to formally establish a causal role of intestinal dysbiosis in RA development, longitudinal studies prior to the onset of RA are required to demonstrate that the presence of *Prevotella* spp. precedes the development of RA. The aim of this study was thus to characterise the microbiota and determine the prevalence of *Prevotella* spp. in individuals during the pre-clinical phases of RA, before the development of clinically apparent RA.

MATERIALS AND METHODS

Study design and study population

First-degree relatives of patients with RA (RA-FDRs) have an increased risk of developing RA compared with the general population.[10 11] The SCREEN-RA study is an ongoing cohort study of RA-FDRs, comprising subjects without a diagnosis of RA at enrolment, described in detail elsewhere (online supplementary text).[12]

We performed a nested case–control study within SCREEN-RA cohort to analyse the intestinal microbiota in individuals in pre-clinical phases of the disease. We identified participants in 'pre-clinical RA' stages based on the European League Against Rheumatism terminology for pre-clinical phases of RA.[13] Operationally, we combined two pre-clinical

RA stages for statistical power reasons: (1) 'systemic autoimmunity associated with RA' defined by anticitrullinated protein autoantibodies positivity and/or rheumatoid factor (RF) positivity,[14] and/or (2) 'symptoms and signs associated with possible RA' as defined by the Connective Tissue Disease Screening Questionnaire with or without undifferentiated arthritis (see online supplementary text for details).[15–17] We included a control group, namely 'FDR controls', namely RA-FDRs without any autoantibodies or symptoms associated with possible RA.

Participants were contacted by telephone to explain the objectives of the study and invited to provide stool samples for microbiome analysis. We included individuals with complete clinical information at the time of the stool sampling. We excluded participants who had undergone antibiotic therapy within the last 3 months, with a known history of inflammatory bowel disease and/or gastrointestinal tract surgery. The protocol was approved by the ethics committee and all participants signed an informed consent before providing a stool sample.

Sampling, DNA extraction and amplicon sequencing analysis to analyse the faecal microbiota

The DNA Genotek OMNIgene·Gut Stool Microbiome Kit was used to collect, store and ship the samples.[18] Stool samples processing and culture-independent analyses were performed. After DNA extraction, the variable region 4 (V4) region of the 16S rRNA gene was amplified using barcoded primers (F515/R806) and sequencing was performed on an Illumina MiSeq as previously described[19] (details in the online supplementary text).

Statistical analysis

Controls and individuals in pre-clinical stages of RA were matched by sex, age and tobacco at the sampling stage. Based on our a priori hypothesis, the primary outcome of the study was the prevalence of bacteria from the family of Prevotellaceae, particularly *Prevotella* spp. Based on the mucosal origins hypothesis of RA,[2] we postulated that the relative prevalence of Prevotellaceae in the stool of individuals in pre-clinical stages of RA would be increased compared with FDR controls. Statistical analyses of community structures were performed. We used *linear discriminant analysis (LDA) effect size (LEfSe)*, an algorithm to compare the relative abundance of the different features between groups, as previously described.[19 20] We performed subgroup analyses, dividing the group of 'pre-clinical stages of RA' into 'systemic autoimmunity associated with RA' and 'symptoms and signs associated with possible RA'. We further explored the general characteristics association with Prevotellaceae abundance.

RESULTS

Study population

Among the 1067 RA-FDR participants in the SCREEN RA cohort, 183 (17%) were invited to provide stool samples, based on a priori inclusion criteria and the matching algorithm. A total of 133 RA-FDRs sent stool samples and could be analysed. General characteristics were balanced between the two groups (table 1).

Table 1 General characteristics at stool collection (133 participants)

Characteristics	FDR controls n=50	Pre-clinical RA stages† n=83
Age (years), median (IQR)	55 (47–62)	58 (50–66)
Female sex, n (%)	39 (78)	74 (89)
Current smoking, n (%)	11 (22)	16 (19)
Past smoking, n (%)	26 (55)	29 (41)
Pack years smoked, median (IQR)	0.4 (0.4–0.7)	0.4 (0.4–0.7)
Current alcohol, n (%)	22 (47)	29 (41)
Body mass index, median (IQR)	24 (22–27)	24 (22–27)
Swollen joints on examination, median (IQR)•	0 (0–1)	1 (0–3)*
Tender joints at examination, median (IQR)	0 (0–1)	1 (0–2)*
ACPA positivity, n (%)	0 (0)	38 (46)*
RF positivity, n (%)	0 (0)	28 (34)*
Shared epitope (one or two copies), n (%)	32 (65)	42 (53)

*P value<0.05, Kruskal-Wallis test for continuous variables and Fisher's exact test for categorical variables.
†Pre-RA group includes individuals with 'systemic autoimmunity associated with RA' and with 'symptoms and signs associated with possible RA'. An isolated asymptomatic swollen joint was not sufficient to be classified as being in a 'pre-clinical stage of RA'.
ACPA, anti-citrullinated protein autoantibody; FDR, first-degree relative; RA, rheumatoid arthritis; RF, rheumatoid factor.

Microbiota analysis

The comparison of microbial diversity in the faecal microbiota within individuals and between individuals, that is, alpha and beta diversity, respectively, of the FDR control and the pre-clinical RA groups did not reveal significant differences (see online supplementary figures S1–S3). We used the LEfSe method to analyse potentially more specific differences in microbiota composition between FDR controls and individuals in the 'pre-clinical stages of RA'.[20] Indeed, we found statistically significant differences in the relative abundances of bacterial taxonomic groups between the participants in pre-clinical stages of RA development and FDR controls (figure 1, LDA score >2, p<0.05). The family Prevotellaceae was the group of bacteria with the highest LDA score and was significantly enriched in individuals in 'pre-clinical stages of RA' (LEfSe p=0.040).

In a subgroup analysis, the family Prevotellaceae was enriched particularly in participants with 'systemic autoimmunity associated with RA' compared with 'FDR controls' (online supplementary figure S4; LEFSe p=0.019), and no significant difference was found between individuals in the two groups of pre-clinical stages of RA (online supplementary figure S5), which allowed us to analyse them together.

We then specifically analysed the relative abundance of the family Prevotellaceae and associated taxa to evaluate whether all individuals of the pre-clinical RA phases display an enrichment of Prevotellaceae or whether an enrichment is observed only in some individuals (figure 2). This analysis confirmed that a larger proportion of individuals within the pre-clinical RA group compared with FDR controls (53% vs 30 %) had significant levels of Prevotellaceae (>1%), but Prevotellaceae are not present in all individuals. The general characteristics of individuals with high relative abundance (>1%) of Prevotellaceae were not different compared with individuals with no Prevotellaceae or lower relative abundance, but for a higher prevalence of RF positivity (online supplementary table S2). Furthermore, besides *P. copri*, other *Prevotella* spp. in other operational taxonomic units contribute to the Prevotellaceae enrichment in 'pre-clinical RA' (online supplementary figure S6).

DISCUSSION

The present study focused on the prevalence of *Prevotella* spp. in the stool of individuals at risk for RA during pre-clinical phases of the disease. The microbiota of individuals in pre-clinical RA stages was significantly altered compared with FDR controls. In

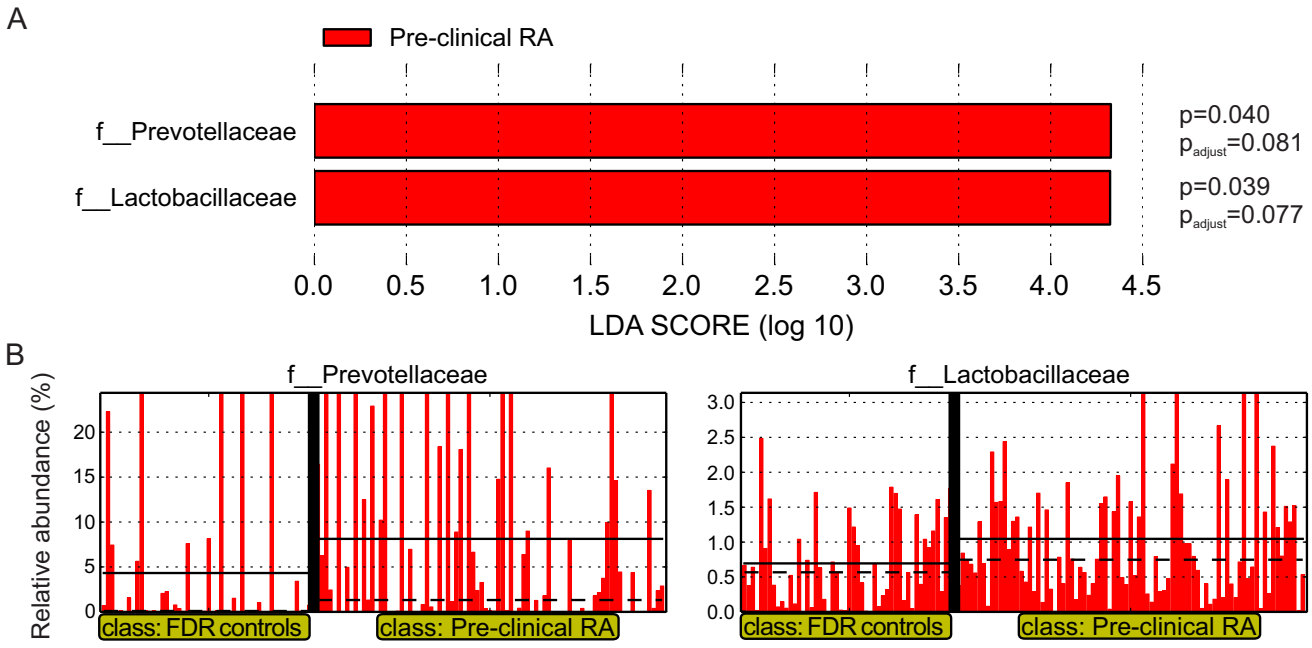

Figure 1 Linear discriminant analysis (LDA) effect size (LEfSe) evaluates the different relative abundance of bacteria. The faecal microbiota composition of a subset of participants of the SCREEN-RA cohort was compared using 16S rRNA gene sequencing. (A) Bacterial families identified using LEfSe (LDA >2, p<0.05). Red bars: bacterial taxa enriched in the preclinical RA group. P_{adjust}: p values with Bonferroni adjustment. (B) Relative abundance (range 0 to 1) of the bacterial families Prevotellaceae (left panel) and Lactobacillaceae (right panel) in individual samples of the two groups. The thick horizontal dashed line in each graph shows median relative abundance and the solid line indicates mean relative abundance. FDR, first-degree relative; RA, rheumatoid arthritis.

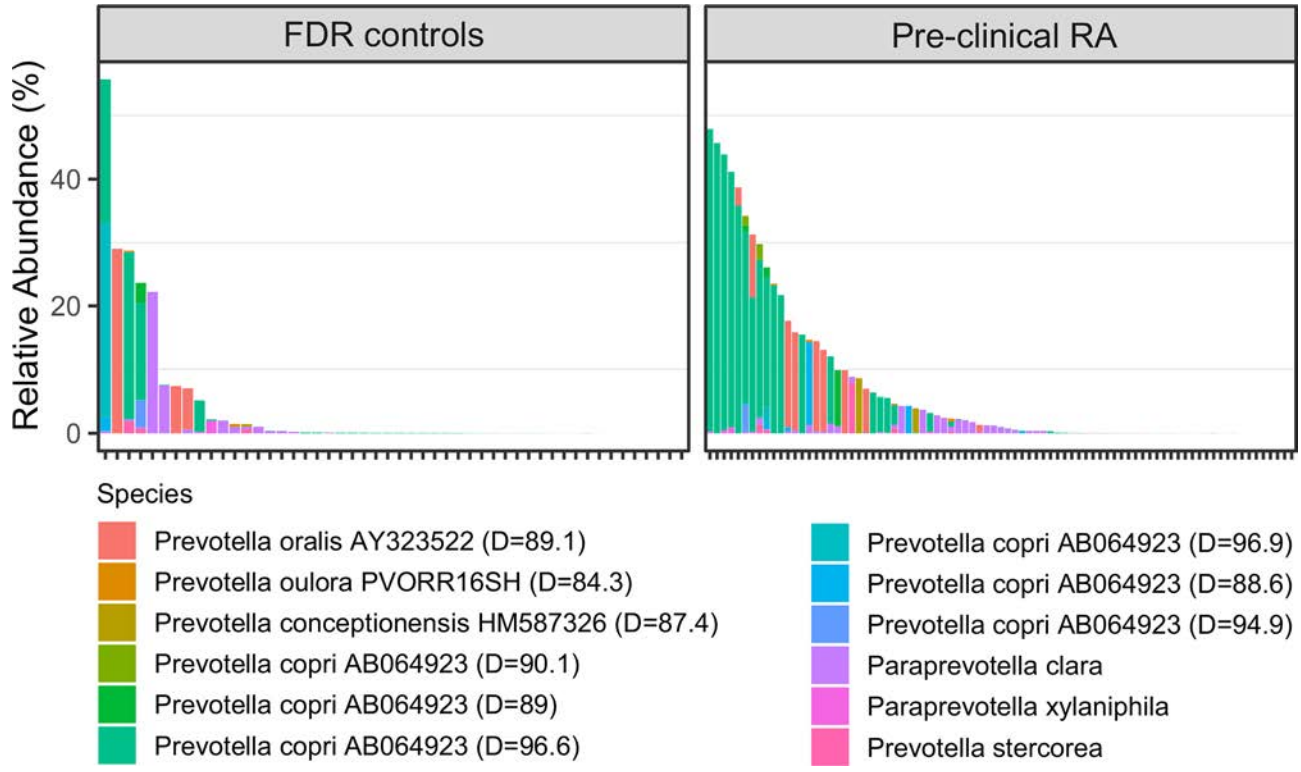

Figure 2 Relative abundance of species belonging to the Prevotellaceae family in individual samples. The samples are ordered by decreasing cumulative relative abundance of operational taxonomic units (OTUs) assigned to the taxonomic level of *Prevotella* species. OTUs assigned only to the level of family or genus are not displayed. for each listed OTU, the closest related taxonomically described species is listed. 'D' indicates the sequence similarity between them. FDR, first-degree relative; RA, rheumatoid arthritis.

particular, the relative abundance of bacteria of the Prevotellaceae family and associated taxa were enriched among individuals in pre-clinical stages of RA and differed significantly from controls, in particular in individuals with 'systemic autoimmunity associated with RA', which is consistent with the mucosal origins hypothesis of RA development.[2]

A previous study analysed the microbiome of faecal samples of American patients with new-onset untreated RA and detected high abundance (>5%) of *P. copri* in 75% (33 of 44) compared with only 21.4% (6 of 28) of healthy individuals.[9] This finding was not replicated in a study involving Chinese patients with RA.[21] Cross-sectional studies in patients with RA do not allow making causal inferences, as this association could be due to differences in behaviours between patients and controls. Our study describes an increased relative abundance in *Prevotella* spp. in individuals in 'pre-clinical RA stages', using participants enrolled in a FDR-RA cohort. While this is still not a longitudinal study, the demonstration of a larger proportion of individuals in pre-clinical stages of RA with a significant abundance of Prevotellaceae strengthens the case for an involvement of *Prevotella* spp. in the RA aetiopathogenesis. However, longitudinal studies are needed to determine the specific role of intestinal dysbiosis and whether *P. copri* or other *Prevotella* spp. trigger systemic autoimmunity or drives the development of symptoms associated with RA.

Our study had limitations. The demonstration of a specific immune response against *P. copri* during pre-clinical stages would have strengthened our findings. In patients with RA, an increased humoral and Th1 cellular immune response against *P. copri* has been demonstrated.[7 8] The microbiome study of the family members with RA and a replication of our results in a new-onset RA population would have further reinforced internal consistency. Our results, together with previous studies in patients with established RA and recent mechanistic studies, support the mucosal origins hypothesis and the role of *Prevotella* spp. dysbiosis in RA development.

In conclusion, we demonstrated that individuals at risk for RA with systemic autoimmunity and/or symptoms associated with RA have an enrichment of *Prevotella* spp. compared with FDR controls. Our findings support the mucosal origins hypothesis in the development of RA. Intestinal dysbiosis could act as an early environmental modulator and may be the target of future preventive interventions.

Correction notice This article has been corrected sinceit published Online First. The equal contributor statement has been added and the correspondence details updated.

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Contributors DAR, TRL, AF and TS designed the study. DAR, ER, CL and AF were involved in patient recruitment, samples and data collecting. TRL, AG and TS were involved in samples processing and analysis. DAR, AF, TS and TRL were involved in statistical analyses and interpretation of data. All authors were involved in writing the manuscript and approved the final version. The first authors and corresponding authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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TRANSLATIONAL SCIENCE

Porphyromonas gingivalis experimentally induces periodontis and an anti-CCP2-associated arthritis in the rat

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ABSTRACT

Objectives Association between periodontal disease (PD) and rheumatoid arthritis (RA) has been extensively described, but direct evidence of causal involvement of PD in RA is missing. We investigated the priming role of oral *Porphyromonas gingivalis* (*P. gingivalis*) in PD and subsequent RA and we assessed biomarkers of bone resorption and arthritis development in rats.

Methods Lewis rats were orally exposed to either *P. gingivalis*, *Prevotella intermedia* or control gel for 1 month and then followed for 8 months. The onset and development of PD was assessed by serology, gingivitis severity and micro-CT (µCT). We investigated arthritis development using circulating proinflammatory markers, anticyclic citrullinated peptide (CCP), anticitrullinated protein antibody (ACPA), ankle histology and µCT.

Results PD was only observed in the *P. gingivalis* treated rats, as early as 1 month postexposure. Joint and systemic inflammation were detected only in the *P. gingivalis* group after 4 and 8 months. At 8 months, inflammatory cell infiltrate was observed in ankle joints and paralleled cortical erosions and overall cortical bone reduction. Furthermore, anti-CCP2 correlated with local and systemic bone loss.

Conclusions In our long-term study, PD induced by oral exposure to *P. gingivalis* triggered seropositive arthritis, with systemic inflammation and bone erosions. This is the first in vivo demonstration of arthritis induced by oral priming with *P. gingivalis*.

Key messages

- Exact mechanisms explaining the association between periodontal disease and rheumatoid arthritis remain unknown.
- *Porphyromonas gingivalis*, but not *Provatella intermedia*, induces periodontis and ankle joint inflammation at clinical, biological and histological levels with bone loss and erosion.
- Our data reinforce the role of *P. gingivalis* in arthritis induction.
- This model shares some similitude with rheumatoid arthritis: involvement of periodontis and anticyclic citrullinated peptide 2 development.

INTRODUCTION

Periodontal disease (PD) and rheumatoid arthritis (RA) are two inflammatory diseases that share many features including local inflammation-induced bone loss.[1] Despite clinical association between PD onset and development of RA, few studies have investigated the direct mechanisms. One of the suspected mechanisms in the bacteria-induced PD leading to RA is the development of antibodies at the site of inflammation against citrullinated proteins. Among all the bacteria inducing PD and found during RA,[2] *Porphyromonas gingivalis* (*P. gingivalis*) was the first identified to induce citrullination. *P. gingivalis* and gingival citrullinated proteins were previously detected in gingival biopsies from patients with RA, who had also high blood concentrations of anti-*P. gingivalis* antibodies.[3] In addition, correlations between circulating anti-*P. gingivalis* antibodies and anti-citrullinated protein antibodies (ACPA) were also reported.[3] Nevertheless, involvement of *P. gingivalis* in RA onset is still controversial.[4 5] The presence of *P. gingivalis* or PD exacerbates experimental arthritis, and experimental arthritis exacerbates PD.[6 7] A major limitation in these studies is the lack of a demonstrated specific role of a particular PD-inducing bacterium in RA, since any generic proinflammatory stimulation can worsen arthritis burden independently of the role of PD associated organisms. Therefore, we have investigated the effects of oral exposure to *P. gingivalis* during onset and development of arthritis and compared its effect with *Prevotella intermedia* (*P. intermedia*), another gram-negative bacteria also associated with PD.[8]

METHODS

Thirty female Lewis rats were randomly divided into three groups (10 per group) and orally exposed to a carboxymethylcellulose sodium salt gel containing *P. gingivalis* W50 (ATCC #53978; PG group), *P. intermedia* (ATCC#25611; PI group) or the gel alone (CTRL group).[9] Baseline was defined as the first day of exposure. Animal experiments were performed accordingly to the Animal Research: Reporting of In Vivo Experiments guidelines for the use of laboratory animals. Five rats per group were sacrificed after 1 month and the others at 8 months. Rat in vivo monitoring,[10] periodontitis validation, biochemistry assays and determination,[11] microcomputed analysis and bone histology

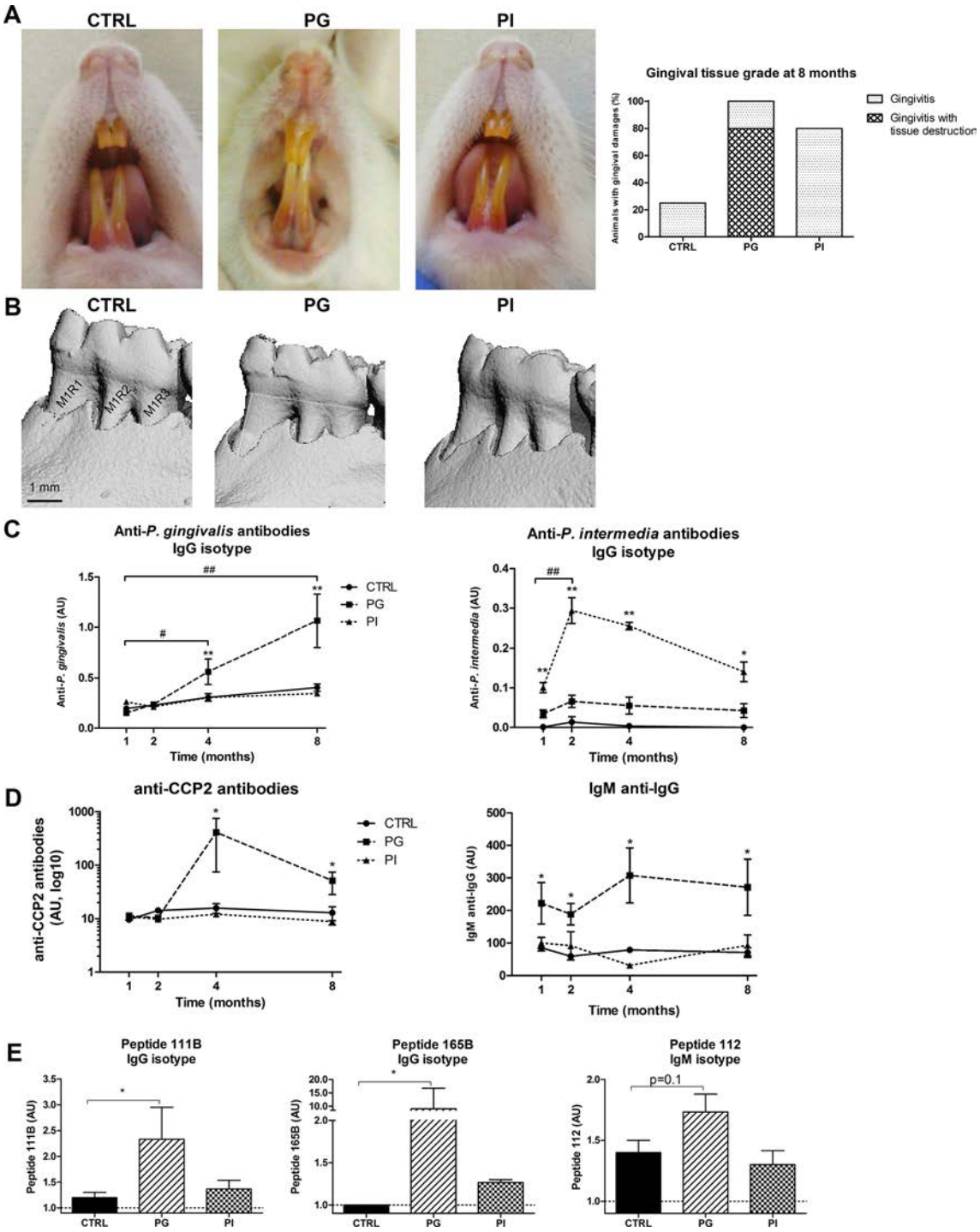

Figure 1 Periodontitis was observed in rats exposed to PG group. (A) Representative pictures of rat mouth: healthy in CTRL (left), gingival missing in PG (middle) and gingival inflamed in PI (right) groups, with gingival tissue scoring (right panel). (B) µCT pictures of mandibles of CTRL (left), PG (middle) and PI (right). CTRL and PG pictures are representative, while the PI rat pictured was the only one with a mandible erosion (≤300 µm). Other PI rat mandibles were similar to CTRL rat mandibles. Scale bar: 1 mm. (C) Immunisation was confirmed with serology assays: anti-*P. gingivalis* antibodies detection in the PG group and anti-*P. intermedia* antibodies in the PI group. (D) Strong induction of anti-CCP2 antibodies from 4 months and IgM anti-IgG from 1 month only in PG. (E) Three citrullinated peptides recognised by sera only in PG group. ELISA plates were coated with 30 citrullinated peptides and blocked with BSA. Sera were diluted at 1/80. After washing, peroxidase-conjugated antimurine IgG was added. The OD was read at 405 nm. The background OD was obtained by adding each serum to a well without peptide. Values are means with SEM. Statistics: group effect: *p<0.05, **p<0.01; time effect: $^{\#}$p<0.05, $^{\#\#}$p<0.01. ABC, alveolar bone crest; AU, arbitrary units; BSA, bovine serum albumin; BS/BV, bone surface/bone volume; BV/TV, bone volume/tissue volume; CCP-2, circular citrullinated protein peptide 2; CEJ, cement–enamel junction tangents; CTRL, control group; Ig, immunoglobulin; M, molar; µCT, micro-CT; OD, optical density; PG, *Porphyromonas gingivalis* exposed group; PI, *Prevotella intermedia* exposed group; R, root.

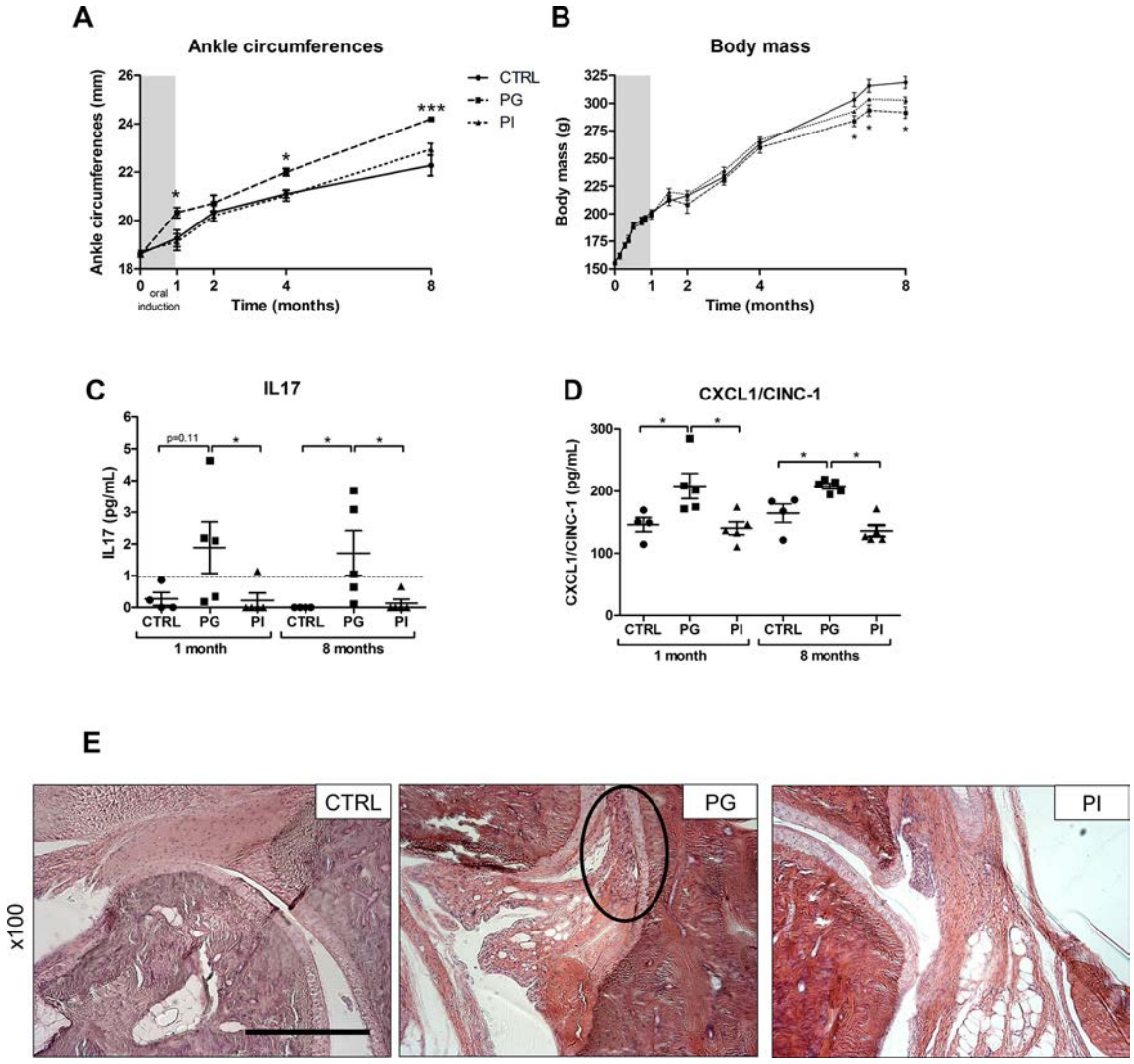

Figure 2 Only *Porphyromonas gingivalis* induced all the hallmarks of prearthritis followed by joint inflammation. (A) Ankle circumferences measured with digital callipers increased only in the PG group. (B) Animal body mass was recorded and reported a low weight in the PG group. (C) Serum IL-17 and (D) CXCL1/CINC-1 were highest in the PG group at 1 and 8 months. (E) Inflammatory infiltrate in ankle joints was observed (circle) with H&E staining only in the PG group. Scale bar: 500 µm. Statistics: group effect: *p<0.05, ***p<0.001; time effect: #p<0.05. AU, arbitrary units; CCP-2, circular citrullinated protein peptide 2; CTRL, control group; CXCL1/CINC-1, CXC ligand 1/cytokine-induced neutrophil chemoattractant-1; IL-17, interleukin 17; PG, *P. gingivalis* exposed group; PI, *Prevotella intermedia* exposed group; ×100, 100 times magnification.

methods[12] were described in the supplementary methods section. Non-parametric tests were performed with p values under 0.05 considered as statistically significant.

RESULTS

Seropositive periodontitis with alveolar bone crest regression and mandibular bone erosions developed within 8 months only in the PG group

Gingival erythema was observed in the PI and PG groups, while mandible tissue destruction was recorded only in the PG group (figure 1A). One or more large bone erosions (≥600 µm) were only observed in the PG group, mostly localised to the mesial area around the first root of the first molar (M1R1) (figure 1B; online supplementary figure S1A-C). Intra-alveolar osteopenia was only reported in the PG group (online supplementary figure S1D-E; <0.05). Anti-*P. gingivalis* and anti-*P. intermedia* antibodies were increased in the serum at 4 and 8 months compared with 1 month in PG and PI groups (figure 1C; $p<0.01$). Anti-CCP2 antibodies were detected after 4 months in the PG group (figure 1D; $p<0.05$) with a trend to correlate with anti-*P. gingivalis* serology (online supplementary S1F; $r=0.90$, $p=0.08$). Additionally, comparative immunoglobulin (Ig)M anti-IgG concentrations were higher in the PG group from the end of oral exposure to the end of the experiment (figure 1D; $p<0.05$). To assess the specificity of anti-CCP2, we detected anti-citrullinated peptide antibodies from 30 peptides citrullinated forms of the beta chain of fibrinogen and detected a positivity for three peptides (figure 1E; $p<0.05$). However, the uncitrullinated control peptides were also reactive. Following confirmation of PD induction, we investigated the presence of arthritis.

Clinical, biological and histological markers showed joint inflammation in the PG group

Ankle circumferences were higher in rats from the PG group than in other groups at the end of oral infection and also at 4 and 8 months (figure 2A; $p<0.05$ for months 1 and 4 and $p<0.001$ for month 8) following normalisation to body mass (which was lower in the PG group at 7 and 8 months)

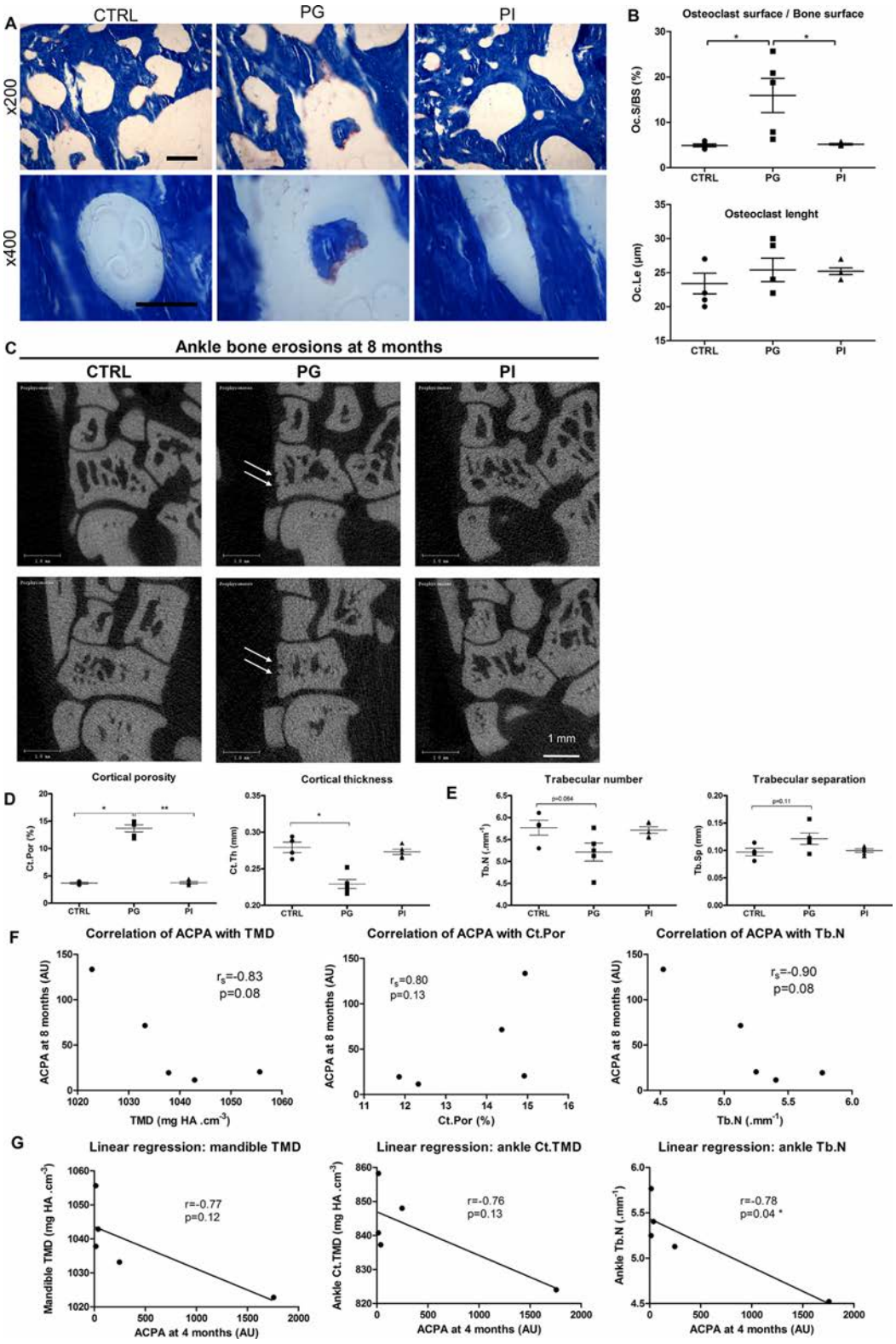

Figure 3 (A) Increased staining of TRAcP+osteoclasts (purple) in the PG group. (C) Representative µCT 2D images of ankle erosions (white arrows) in the PG group at 8 months. Osteoclast activity and microarchitecture parameters alterations were enhanced in the PG group. Scale bar: 100 µm. (B) Osteoclast analysis included Oc/S/BS and Oc.Le. (C) µCT analysis for cortical and trabecular navicular bone. (D) Cortical µCT analysis included Ct.Por and Ct.Th. (E) Trabecular analysis included Tb.N and Tb.Sp. (F) Correlation assessment of anti-CCP2 with local and systemic bone loss at 8 months, together with prediction of anti-CCP2 at 4 months. Correlations were tested at 8 months between anti-CCP2 and mandibular TMD, ankle Ct.Por and ankle Tb.N in the PG group only. Spearman correlations: r_s. (G) Linear regression (coefficient: r; *p<0.05) at 4 months provided insights in anti-CCP2 prediction to (D) mandible TMD, (E) ankle Ct.TMD and (F) ankle Tb.N. Statistics: group effect: *p<0.05, **p<0.01. CCP, citrullinated cyclic peptide; CTRL, control group; Ct.Por, cortical porosity; Ct.Th, cortical thickness; µCT, micro-CT; Oc/S/BS, osteoclast surface/bone surface; Oc.Le, osteoclast length; PG, *Porphyromonas gingivalis* exposed group; Pi, *Prevotella intermedia* exposed group; Tb.N, trabecular number; Tb.Sp, trabecular separation; TMD, tissue mineral density; TRAcP, tartrate-resistant acid phosphatase.

(figure 2B; p<0.05). From month 1 to month 8, interleukin (IL)-17 and CXC ligand 1/cytokine-induced neutrophil chemoattractant-1 (CXCL1/CINC-1) concentrations were higher in the PG animals (figure 2C–D; p<0.05). Finally, histological assessment of ankles for 8 months demonstrated the presence of inflammatory cell infiltrate in the PG rats (figure 2E). Therefore, arthritis was observed at the ankle joint after PD induction by *P. gingivalis*. We then asked whether bone erosion could be detected.

P. gingivalis induced osteoclast activity, bone erosions and quantitative bone loss in the ankle

Tartrate-resistant acid phosphatase+osteoclast number was elevated in the ankles of the PG rats (figure 3A–B). Bone erosions were detected in the cortical layer of the ankle bones of the PG rats as measured by micro-CT (µCT), especially the navicular bone. These changes were not found in the PI or CTRL groups (figure 3C). PG rats showed significantly higher cortical porosity (Ct.Por) and lower cortical thickness (Ct.Th) (figure 3D; p<0.05), and although the data did not reach significance, displayed reduced trabecular number (Tb.N) and increased trabecular separation (Tb.Sp) (figure 3E; p=0.06 and 0.11). At 8 months, anti-CCP2 correlated with a strong trend (given the small number of experimental animals) with the mandibular tissue mineral density (TMD) loss (figure 3F left), ankle Ct.Por (middle) and ankle Tb.N (right). Thus, higher levels of anti-CCP2 were associated with alveolar bone loss and ankle bone loss. Moreover, anti-CCP2 levels at 4 months were predictive of mandibular and joint bone loss at 8 months (mandibular TMD changes (figure 3G left) ankle Ct.TMD (figure 3G middle) and ankle Tb.N (figure 3G right); r=−0.78, p=0.04).

DISCUSSION

Epidemiological associations between PD, induced by periodontopathogenic bacteria, and rheumatoid arthritis (RA) have been largely described, but reproducible animal models to investigate the direct relationship between PD and RA are desperately needed. Herein, we define a novel model of PD-induced RA and that oral exposure to *P. gingivalis* induces severe PD, leading to elevations of anti-CCP2, IL-17 and CXCL1 levels and subsequent synovial inflammation and bone destruction. We also show that oral exposure to *P. gingivalis* induces deep mesial erosions of M1R1, which is the major local consequence of oral exposure to *P. gingivalis*, consistent with prior reports.[13] Finally, compared with *P. intermedia*, exposure to *P. gingivalis* induced overt PD in Lewis rats, while *P. intermedia* resulted in mild gingivitis with small erosions on mesial periodontal bone.

The contribution of *P. gingivalis* to protein citrullination in the pathogenesis of PD and subsequent anti-CCP2 production in RA has already been documented, but the data is conflicting.[14] In our model, anti-CCP2 was detected in the serum 4 months after PD induction, and at the same time as anti-*P. gingivalis* antibodies. The identified citrullinated epitope detected was similar to those recently observed in a model of T cell immunisation to PAD in mice.[11] These recognised peptides encompassed resides 420–479 of the beta chain of human fibrinogen. Moreover, expression of these two markers was correlated at all times only in the PG group, demonstrating its specificity to *P. gingivalis* exposure. However, we were not able to demonstrate specificity against citrullinated sequence compared with non-citrullinated sequence, as previously reported in rodents.[15] Our rat model also mimics the results observed in human RA disease where IgM anti-IgG and anti-CCP2 are elevated and correlated with anti-*P. gingivalis* antibodies.[16]

Rat ankle bone loss following oral *P. gingivalis* exposure was observed after 8 months of initial *P. gingivalis* exposure. *P. gingivalis* induced is comparable to the bone loss observed during other experimental RA models such as the rat adjuvant-induced arthritis model.[17] As in patients with RA, bone loss was mostly related to osteoclast activation. PG rats bone was less impacted when compared with the cortical bone. The animals with greater alterations in bone parameters were those with earlier anti-CCP2 positivity. Therefore, we successfully correlated anti-CCP2 levels with ankle bone loss. Moreover, anti-CCP2 at 4 months could efficiently predict reduction in bone mass, confirming that ACPA is not only a good biomarker, but might also be directly involved in the mechanisms leading to bone loss. *P. gingivalis* exposure led to anti-CCP2 production and subsequent bone resorption. This is consistent with previous studies of ACPA administration to healthy mice where ACPA showed high affinity to the bone marrow site[18] and led to a reduction in bone mass.[19] In line with this findings, marginal jawbone loss was associated with presymptomatic ACPA-positive subjects.[20] Finally, ACPA increased the expression of CXCL1/CINC-1 in mouse osteoclasts cultures.[18] Therefore, our PG rat model mimics this sequence of RA development. Biofilms in PD are composed of several strains, with *P. gingivalis* and *P. intermedia* considered as the most aggressive organisms in these films. However, involvement of other bacteria from the red complex associated to *P. gingivalis* was not excluded. Given the role of *P. gingivalis* as a major cause of PD and PD-induced arthritis, the consequences of exposure to *P. gingivalis* and co-infection with other oral pathogens remain to be investigated.

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Contributors GC, MRG, VBB, SP and HM designed the study. GC, MRG, IA and RC performed the experiments. GC, LM, IA, SP and HM analysed the data. GC and HM wrote the manuscript. All authors approved the final version.

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TRANSLATIONAL SCIENCE

PTPN14 phosphatase and YAP promote TGFβ signalling in rheumatoid synoviocytes

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ABSTRACT

Objective We aimed to understand the role of the tyrosine phosphatase PTPN14—which in cancer cells modulates the Hippo pathway by retaining YAP in the cytosol—in fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA).

Methods Gene/protein expression levels were measured by quantitative PCR and/or Western blotting. Gene knockdown in RA FLS was achieved using antisense oligonucleotides. The interaction between PTPN14 and YAP was assessed by immunoprecipitation. The cellular localisation of YAP and SMAD3 was examined via immunofluorescence. SMAD reporter studies were carried out in HEK293T cells. The RA FLS/cartilage coimplantation and passive K/BxN models were used to examine the role of YAP in arthritis.

Results RA FLS displayed overexpression of PTPN14 when compared with FLS from patients with osteoarthritis (OA). PTPN14 knockdown in RA FLS impaired TGFβ-dependent expression of MMP13 and potentiation of TNF signalling. In RA FLS, PTPN14 formed a complex with YAP. Expression of PTPN14 or nuclear YAP—but not of a non-YAP-interacting PTPN14 mutant—enhanced SMAD reporter activity. YAP promoted TGFβ-dependent SMAD3 nuclear localisation in RA FLS. Differences in epigenetic marks within Hippo pathway genes, including YAP, were found between RA FLS and OA FLS. Inhibition of YAP reduced RA FLS pathogenic behaviour and ameliorated arthritis severity.

Conclusion In RA FLS, PTPN14 and YAP promote nuclear localisation of SMAD3. YAP enhances a range of RA FLS pathogenic behaviours which, together with epigenetic evidence, points to the Hippo pathway as an important regulator of RA FLS behaviour.

Key messages

What is already known about this subject?

► Fibroblast-like synoviocytes (FLS) play an important role in the pathogenesis of rheumatoid arthritis (RA). Several signalling pathways are known to be dysregulated in rheumatoid FLS; however, the contribution of the Hippo pathway remains unexplored.

What does this study add?

► This study shows that the tyrosine phosphatase PTPN14 and the transcription coactivator YAP, known key players in the Hippo pathway, are dysregulated in RA FLS and can modulate the pathological behaviour of FLS in RA.

How might this impact on clinical practice or future developments?

► The study suggests that YAP and potentially other members of the Hippo pathway, which is already being targeted for cancer therapy, could be leveraged as therapeutic targets for novel RA therapies.

INTRODUCTION

Fibroblast-like synoviocytes (FLS) in the synovial intimal lining of the joint play a pivotal role in the pathogenesis of rheumatoid arthritis (RA)[1–4] through invasion of extracellular matrix, secretion of proinflammation cytokines and production of cartilage-degrading matrix metalloproteases (MMPs).

We previously surveyed the expression of protein tyrosine phosphatases (PTPs) in FLS from patients with RA (RA FLS) and reported that PTPσ, PTPκ and PTPα promote the aggressiveness of RA FLS.[5–7] In the same survey, non-receptor protein tyrosine phosphatase 14 (PTPN14, also known as PEZ) was found to be among the most highly expressed PTPs in RA FLS.[8]

PTPN14 is a ubiquitous phosphatase with nuclear and cytosolic localisation and is frequently mutated in various cancers.[9–11] Structurally, PTPN14 includes an N-terminal 'Band 4.1, ezrin, radixin, moesin homology' (FERM) domain, a linker region and a C-terminal catalytic PTP domain. The linker contains two PPxY motifs that drive the interaction between PTPN14 and Yes-associated protein (YAP), a tumour-promoting transcription coactivator that is a downstream effector of the Hippo pathway.[12 13] In cancer cells, PTPN14 acts as a tumour suppressor via sequestration of YAP in the cytoplasm independent of phosphatase activity.[14–17] Among PTPN14 known substrates, protein kinase Cδ (PRKCD) and Ras and Rab interactor 1 (RIN1) are important regulators of endosome-related receptor trafficking,

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suggesting that PTPN14 activity can modulate surface receptor presenting and recycling.[18]

PTPN14 has been previously implicated in promoting transforming growth factor β (TGFβ) signalling through the TGFβ receptor,[9 19] via an unknown mechanism of action. TGFβ is highly expressed in the RA synovium.[20] Although the overall role of TGFβ in RA pathogenesis remains incompletely understood, TGFβ potentiates the proinflammatory action of tumour necrosis factor α (TNF) and interleukin 1β (IL-1) on RA FLS.[21]

Here, we report that RA FLS display TGFβ-dependent overexpression of PTPN14 when compared with FLS derived from patients with osteoarthritis (OA FLS). We propose that in RA FLS, PTPN14 promotes TGFβ signalling via a YAP-mediated mechanism. In addition, we identify the Hippo pathway and YAP as molecules of interest in RA FLS pathogenic action.

MATERIALS AND METHODS

Further information is available in the online supplementary methods.

FLS lines: FLS lines were obtained from arthroplasties (UC SAN DIEGO IRB#140175). Each line was derived from discarded synovial tissue from patients with RA or OA undergoing synovectomy or total joint replacement, as previously described.[22] The diagnosis of RA conformed to the American College of Rheumatology 1987 revised criteria.[23]

Antisense oligonucleotide (ASO) knockdown: Human FLS were grown to 90% confluence and treated with 2.5 µM morpholino antisense oligonucleotides (ASO) (Gene Tools). ASO was replenished in fresh culture media after 3 days and in serum-starvation media after 6 days.

SMAD reporter assays: SMAD reporter assays were performed using the Qiagen's Cignal SMAD Reporter (luc) kit.

Mice: All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the La Jolla Institute (#AP140-NB4) and UC SAN DIEGO (#S16098). C57BL/6 KRN mice were provided by Dr Christophe Benoist (Harvard Medical School, Boston, Massachusetts, USA) and were crossed with NOD mice (Taconic Bioscience) to obtain arthritic offspring (K/BxN mice) whose serum was pooled for use in the K/BxN passive serum-transfer arthritis model.

Statistical analysis: Two tailed statistical analyses were performed as indicated in the figure legends using GraphPad Prism software. A comparison was considered significant if $p < 0.05$.

RESULTS

TGFβ-dependent overexpression of PTPN14 in RA FLS

A comparison between FLS from 11 patients with RA and 10 patients with OA revealed that *PTPN14* is significantly overexpressed in RA FLS than in OA FLS ($p<0.01$) (figure 1A). We also detected significantly increased PTPN14 protein levels in five RA FLS lines compared with five OA FLS lines ($p<0.01$, figure 1B,C). Immunofluorescence (IF) assessment of synovial specimens from patients with RA versus OA showed high expression of PTPN14 in RA (figure 1D). Published data and a survey of ImmGen data suggest that *PTPN14* is expressed prominently in stromal cells and poorly in immune cells.[24 25] In line with this observation, a comparative assessment of *PTPN14* mRNA expression in synovial biopsies from the Pathology of Early Arthritis Cohort (PEAC) -including 87 treatment-naïve patients with RA—showed that *PTPN14* was significantly more expressed in biopsies characterised by a prominent or exclusive FLS presence (fibroid)—which showed limited expression of CD3, CD20, CD138 and CD68—markers of T cells, B cells, plasma cells and macrophages, respectively (online supplementary figure 1)—versus biopsies characterised by prominent immune cell infiltration (non-fibroid) ($p<0.0001$, figure 1E).

We next examined the effect of growth factors on PTPN14 expression in RA FLS and found that TGFβ1 (TGFβ, 50 ng/mL), but not platelet-derived growth factor (PDGF, 50 ng/mL) stimulation enhances *PTPN14* expression in serum-starved RA FLS ($p<0.05$) (figure 1F). RA FLS exhibit an intrinsic upregulation of the mRNAs for TGFβ (*TGFB1*), TGFβ receptor I (*TGFBR1*) and thrombospondin 1 (*THBS1*, encoding an activator of latent TGFβ[26]) when compared with OA FLS.[27] Since *PTPN14* is induced by TGFβ, we assessed whether *PTPN14* expression correlates with *TGFB1, TGFBR1* and *THBS1* in FLS. As shown in figure 1G, the expression levels of *PTPN14* positively correlated with *TGFBR1* in RA (Spearman $\gamma=0.8455$, $p<0.01$) and OA FLS (Spearman $\gamma=0.8364$, $p<0.01$) and *THBS1* in RA FLS (Spearman $\gamma=0.6545$, $p<0.05$) and OA FLS (Spearman $\gamma=0.6727$, $p<0.05$), while there was no correlation between the expression levels of *PTPN14* and *TGFB1* (data not shown). Inhibition of TGFβ signalling using two selective TGFβRI antagonists SB505124[28] and RepSox,[29] reduced *PTPN14* expression in unstimulated RA FLS ($p<0.05$, $p<0.01$, respectively) (figure 1H,I) suggesting that enhanced autocrine stimulation with TGFβ plays a role in the upregulation of *PTPN14* in RA FLS. However, additional unknown pathways likely contribute to enhance PTPN14 mRNA and protein levels in RA FLS in vitro and in the rheumatoid joint.

PTPN14 promotes TNFα-stimulated and IL-1β-stimulated MMP production in RA FLS

We next tested whether PTPN14 regulates the response of RA FLS to TNF and IL-1β, critical pathogenic cytokines in RA.[30] For knockdown PTPN14 in RA FLS, we treated RA FLS with a cell-permeable antisense oligonucleotide (PTPN14 ASO) targeting exon 4 or a control scrambled ASO. Knockdown of PTPN14 in RA FLS after ASO treatment was confirmed by western blotting (online supplementary figure 2). Knockdown of PTPN14 in RA FLS significantly inhibited TNFα-induced expression of *MMP1* (figure 2A left panel) but did not affect expression of *MMP3*, *VCAM1* or *IL6* (data not shown). We also noticed that knockdown of PTPN14 significantly reduced the expression of *MMP13* in RA FLS (figure 2A right panel and data not shown). The effect of PTPN14 ASO on IL-1β-induced *MMP1* induction in RA FLS was non-significant (data not shown). In order to rule out off target effects of PTPN14 ASO, we designed a second ASO targeting PTPN14 exon 2 (PTPN14 ASO 2). Treatment of RA FLS with PTPN14 ASO 2 led to inhibition of TNFα-induced *MMP1* and inhibition of *MMP13* expression identical to the ones obtained with PTPN14 ASO (online supplementary figure 3). Flow cytometry assessment did not show any effect of PTPN14 deficiency on RA FLS survival (online supplementary figure 4). Knockdown of PTPN14 also did not significantly affect RA FLS migration or invasiveness in response to fetal bovine serum (FBS) or RA FLS attachment to cartilage (figure 2B).

PTPN14 promotes TGFβ signalling in RA FLS

As shown in figure 2A, the effect of PTPN14 knockdown was particularly significant on MMP13, an important collagenase in RA, whose expression is regulated by TGFβ signalling in RA FLS and other cell types.[6 31] Due to the proposed role of PTPN14 in TGFβ signalling[9] and since TGFβ is known to potentiate

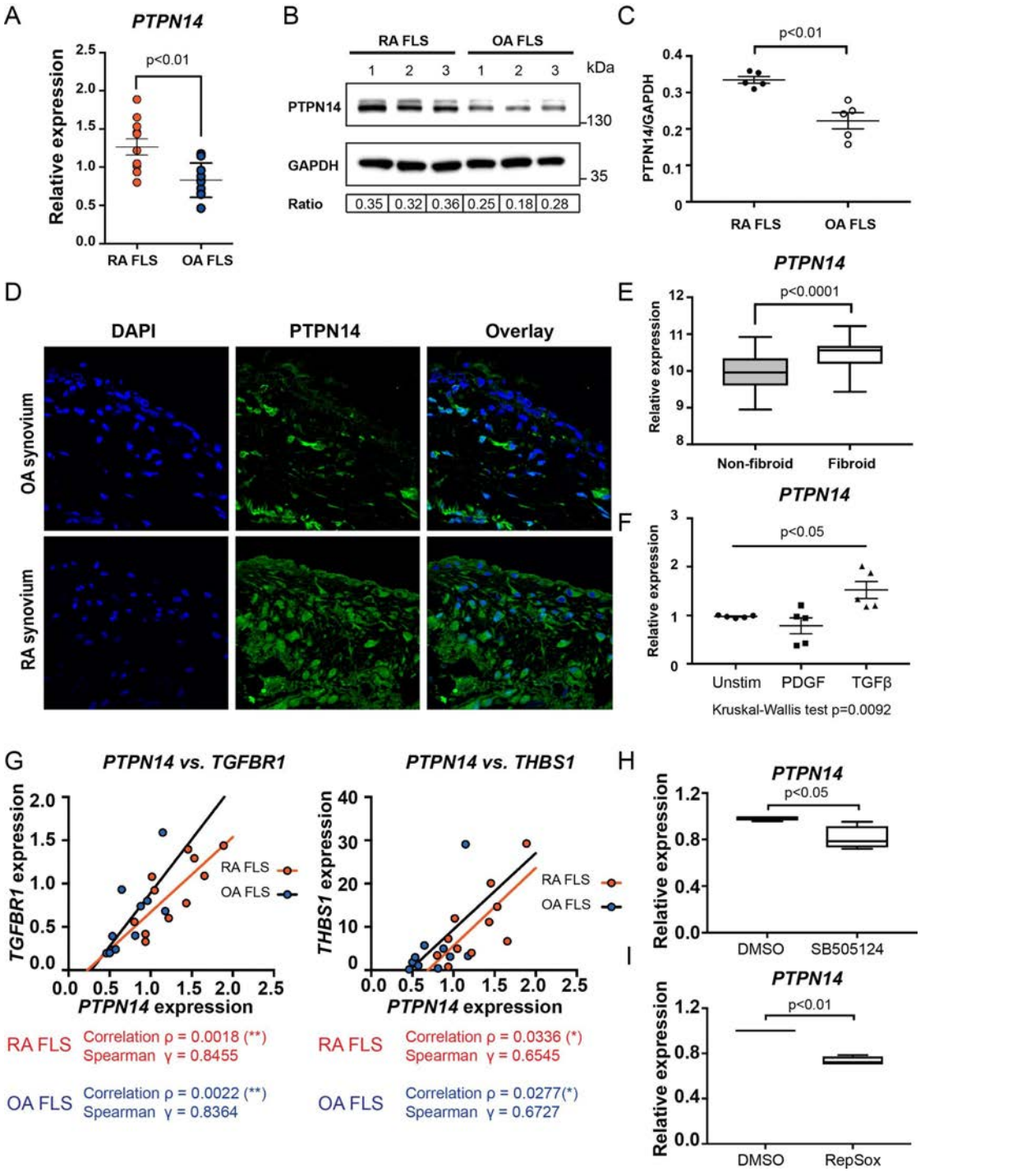

Figure 1 PTPN14 displays TGFβ-dependent overexpression in RA FLS. (A) *PTPN14* mRNA expression was assessed by qPCR in 11 RA FLS lines and 10 OA FLS lines. Results were normalised to *POL2A* using $2^{-\Delta Ct}$ method. Mean±SEM are shown. (B) PTPN14 protein expression levels in 3 RA FLS and 3 OA FLS lines was assessed by Western blotting. (C) PTPN14 protein expression was assessed by western blotting in 5 RA FLS lines and 5 OA FLS lines. Results were normalised to GAPDH. Mean±SEM are shown. (D) IF of synovial sections from patients with OA or RA stained with anti-PTPN14 antibody (green signal) and DAPI (blue signal). Representative images are shown at 60× magnification. (E) *PTPN14* mRNA expression levels measured by RNAseq in 65 non-fibroid vs 17 fibroid RA synovium specimens. (F) RA FLS (n=5) were stimulated with platelet-derived growth factor (PDGF, 50 ng/mL) or transforming growth factor β1 (TGFβ, 50 ng/mL) for 24 hours. *PTPN14* expression was assessed by qPCR. Results were normalised to *GAPDH* using $2^{-\Delta\Delta Ct}$ method. Mean±SEM are shown. (G) The expression level of *PTPN14*, *TGFBR1* and *THBS1* was assessed by qPCR on 11 RA FLS lines and 11 OA FLS lines. Graphs show *PTPN14* vs *TGFBR1* expression or *PTPN14* vs *THBS1* expression for each line. (H–I) *PTPN14* mRNA expression was measured by qPCR performed in triplicate after RA FLS (n=4–5) treatment with 50 µM TGFβRI inhibitor SB505124 (H) or 1 µM RepSox (I) for 24 hours. Results were normalised to *GAPDH* using $2^{-\Delta\Delta Ct}$ method. Box-and-whisker plots (E,H,I) depict median (line within box), 25th percentile and 75th percentile (bottom and top borders) and range of minimum to maximum values (whiskers). Data were analysed using the two-tailed Mann-Whitney test (A,C,E,H,I), the Kruskal-Wallis test with two-tailed Mann-Whitney posthoc test (F) or the Spearman correlation test (G). P value was adjusted for multiple comparisons in (F). FLS, fibroblast-like synoviocytes; IF, immunofluorescence; OA, osteoarthritis; qPCR, quantitative PCR; RA, rheumatoid arthritis.

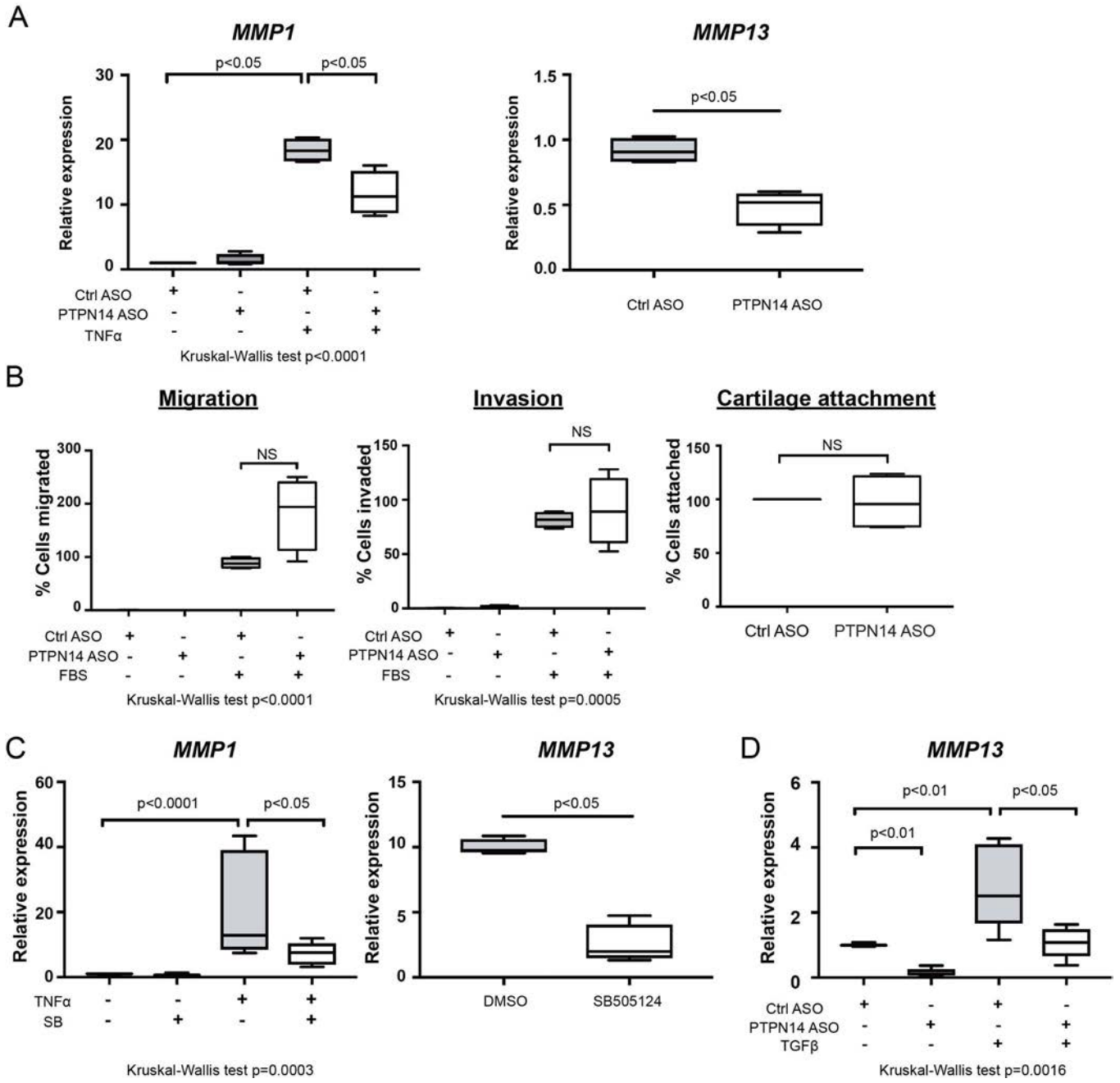

Figure 2 PTPN14 promotes TGFβ-dependent MMP production by RA FLS. (A) RA FLS (n=4) were treated with Ctrl ASO or PTPN14 ASO for 6 days, serum-starved in the presence of ASO for 24 hours and then stimulated with or without TNFα for 24 hours. *MMP1* and *MMP13* mRNA expression was measured by qPCR performed in triplicate. Results were normalised to *GAPDH* using $2^{-\Delta\Delta Ct}$ method. (B.) RA FLS (n=4) were treated with Ctrl ASO or PTPN14 ASO for 6 days and serum starved for 24 hours in the presence of ASO. For the migration assay, treated cells were allowed to migrate for 24 hours in transwell assay in response to 5% FBS. For the invasion assay, treated cells were allowed to invade through matrix-coated transwells for 24 hours in response to 5% FBS. Cells were then fixed with 100% methanol and stained with 0.2% crystal violet. For each well, four non-overlapping area (top, bottom, left and right) were imaged and counted. For the cartilage attachment assay, bovine cartilage fragments were pretreated with IL-1β (2 ng/mL) for 24 hours. Cells were incubated in constant rotation with cartilage fragments for 2 hours and then incubated at 37°C overnight. (C) After serum starvation for 24 hours, RA FLS (n=4) were incubated in the presence or absence of 25 µM SB505124 (SB) and stimulated with or without TNFα for 24 hours. *MMP1* and *MMP13* mRNA expression was analysed by qPCR performed in triplicate. Results were normalised to *GAPDH* using $2^{-\Delta\Delta Ct}$ method. (D) RA FLS (n=5 or 6) were treated with Ctrl or PTPN14 ASO for 6 days, serum starved with the presence of ASO for 24 hours and then stimulated with TGFβ for 24 hours. *MMP13* mRNA expression was analysed by qPCR performed in triplicate. Results were normalised to *GAPDH* using $2^{-\Delta\Delta Ct}$ method. (A–D) Box-and-whisker plots depict median (line within box), 25th percentile and 75th percentile (bottom and top borders) and range of minimum to maximum values (whiskers). Data were analysed using the Kruskal-Wallis test with two-tailed Mann-Whitney posthoc test (A–D) or the two-tailed Mann-Whitney test (right panels in A–C), NS=non-significant. ASO, antisense oligonucleotides; FBS, fetal bovine serum; FLS, fibroblast-like synoviocytes; qPCR, quantitative PCR; RA, rheumatoid arthritis.

the action of TNFα and IL-1β in RA FLS, we hypothesised that the phenotypes observed in RA FLS subjected to PTPN14 knockdown might be at least in part due to blockade of autocrine RA FLS TGFβ signalling. Consistent with this model, inhibition of TGFβRI using SB505124 reduced TNFα-stimulated *MMP1* induction and abrogated *MMP13* expression in RA FLS (figure 2C). We then assessed whether PTPN14 regulates TGFβ-induced *MMP1* and *MMP13* expression in RA FLS and found that knockdown of PTPN14 reduced TGFβ-induced expression of *MMP13* (figure 2D), while no induction of MMP1 was observed after treatment of RA FLS with TGFβ alone (data not shown).

PTPN14-YAP interaction enhances nuclear YAP-mediated TGFβ-SMAD signalling

We next tried to assess the mechanism of action of PTPN14 in TGFβ signalling. We did not observe alterations in TGFβRI expression in cells treated with PTPN14 ASO (figure 3A). Thus, we examined the role of PTPN14 in intracellular canonical TGFβ signalling, mediated by phosphorylated SMAD complexes, which translocate to the nucleus to regulate transcription of target genes.[32] We observed no difference in phospho-SMAD2 (pSer465/467) or phospho-SMAD3 (pSer423/425) levels on TGFβ stimulation between RA FLS treated with control or PTPN14 ASO (data not shown). However, RA FLS treated with PTPN14 ASO showed significantly reduced basal and TGFβ-induced nuclear localisation of SMAD3—but not of SMAD2—when compared with cells treated with control ASO ($p<0.05$) (figure 3B online supplementary figure 5). MMP13 expression has been shown to be regulated by TGFβ through both canonical SMAD-dependent[33] and non-canonical mitogen-activated protein kinase (MAPK)-dependent pathways.[31] RA FLS subjected to knockdown of PTPN14 did not display significantly altered phosphorylation of extracellular-regulated kinase (Erk), C-Jun N terminal kinase (JNK), p38 MAPK, MAPK kinase 3 (MKK3), MKK4, MKK6 and MKK7 (data not shown), suggesting that non-canonical TGFβ signalling is unlikely to mediate PTPN14-driven expression of MMP13 in RA FLS.

As described, PTPN14 is known to regulate signalling via phosphatase activity dependent and independent mechanisms. There are four known PTPN14 substrates identified in cancer cell lines: β-Catenin,[34] p130Cas,[35] PRKCD and RIN1.[18] Substrate-trapping double mutated PTPN14 catalytic domain (D1079A/C1121S) was expressed and substrate trapping experiments[36 37] were carried out with RA FLS lysates, but none of the identified substrates were pulled down by PTPN14 in RA FLS (data not shown). We thus looked at phosphatase activity-independent regulatory mechanisms. It is well documented that PTPN14 regulates Hippo signalling by forming a complex with YAP.[16 17] We confirmed the existence of a PTPN14-YAP physical complex in RA FLS by immunoprecipitation (figure 3C). To assess the mechanism of action of PTPN14 in TGFβ-SMAD signalling, we next examined the effect of PTPN14, a catalytically inactive PTPN14 C1121S mutant and a PTPN14 PPxY motifs Y570F/Y732F mutant —which is unable to bind to YAP[15 17]—in a SMAD reporter assay in HEK293T cells. Consistent with the observations made in RA FLS, overexpression of PTPN14 in HEK293T cell enhanced SMAD reporter activity on TGFβ stimulation (figure 3D). Mutations of the PPxY motifs significantly reduced the SMAD-enhancing activity of PTPN14 while catalytically inactive (C/S) PTPN14 was as effective as PTPN14 WT at promoting the SMAD reporter activity (figure 3D). These data suggest that PTPN14-mediated promotion of TGFβ-induced SMAD activation depends on the ability of PTPN14 to interact with YAP rather than on the phosphatase activity.

The PTPN14-YAP complex enhances YAP cytosolic localisation in cancer cells.[15] Therefore, we asked whether PTPN14 regulates the nuclear localisation of YAP in RA FLS. Immunofluorescence of resting RA FLS showed that >80% YAP was localised to the nucleus in subconfluent (~70% confluent) cells (data not shown). Figure 3E shows that both YAP and PTPN14 were found in resting and TGFβ-stimulated RA FLS nuclear lysates. Immunofluorescence analysis of RA FLS revealed no significant changes in nuclear localisation of YAP in unstimulated vs TGFβ-stimulated and in cells subjected to knockdown of PTPN14 vs cells treated with control ASO (figure 3F online supplementary figure 6A).

In the early embryo, YAP is also known to control TGFβ-signalling by modulating SMAD nuclear/cytosolic distribution.[38] In RA FLS, we found that partial knockdown of YAP using an ASO directed against YAP exon 2 modulated TGFβ-induced SMAD3 nuclear translocation in RA FLS (figure 3G online supplementary figure 6B,6C). To further demonstrate that nuclear YAP is important to sustain TGFβ-dependent SMAD signalling, we carried out SMAD reporter assays by expressing YAP in frame with a nuclear localisation sequence (NLS-YAP), which results in exclusive overexpression of YAP in the nucleus. Figure 3H shows that NLS-YAP significantly enhanced TGFβ-induced SMAD reporter activity. We conclude that in RA FLS PTPN14 and YAP promote nuclear recruitment of SMAD3 during TGFβ signalling.

The Hippo pathway displays epigenetic alterations in RA FLS and modulates TNF signalling and invasiveness of RA FLS *in vitro*

The Hippo pathway has recently emerged as a critical regulator of cancer growth and survival and of multiple important basic cell functions; however, no information is available yet on the role of this pathway in RA FLS. A recent highly integrated analysis of epigenetics marks in RA FLS versus OA FLS has identified multiple pathways that are differentially marked and candidate players in the pathogenic behaviour of rheumatoid FLS.[39] We thus interrogated the available RA-FLS and OA-FLS epigenetic database, inclusive of nine marks—six histone modifications (H3K27ac, H3K4me1, H3K4me3, H3K36me3, H3K27me3 and H3K9me3), open chromatin, RNA-seq and DNA methylation—for epigenetic alterations in the Hippo pathway. Applying the same integrative method and pathway analysis described in Ref. 39, we discovered that the 'Hippo signalling' pathway was significantly enriched in differential epigenetic marks between RA FLS and OA FLS. As shown in figure 4A, the vast majority of known genes belonging to the Hippo pathway (differentially modified genes are highlighted in magenta in the figure), displayed differences in one or more of 5 histone modification (detailed in figure 4B) and/or open chromatin and/or DNA methylation marks. *YAP1* (encoding YAP) was differentially modified in H3K4me1, H3K4me3 and open chromatin regions. Figure 4C shows a genome browser screenshot exemplifying the epigenetic landscape within 300 kb of *YAP1* for one representative couple of RA vs OA FLS lines with boxes identifying differentially marked regions.

Since the above-mentioned findings point to YAP as an important pathogenic factor and a potential mediator of PTPN14 action in RA FLS, we next assessed whether inhibition of nuclear YAP functions alters RA FLS behaviour and/or phenocopies the effect of PTPN14 knockdown in RA FLS. We

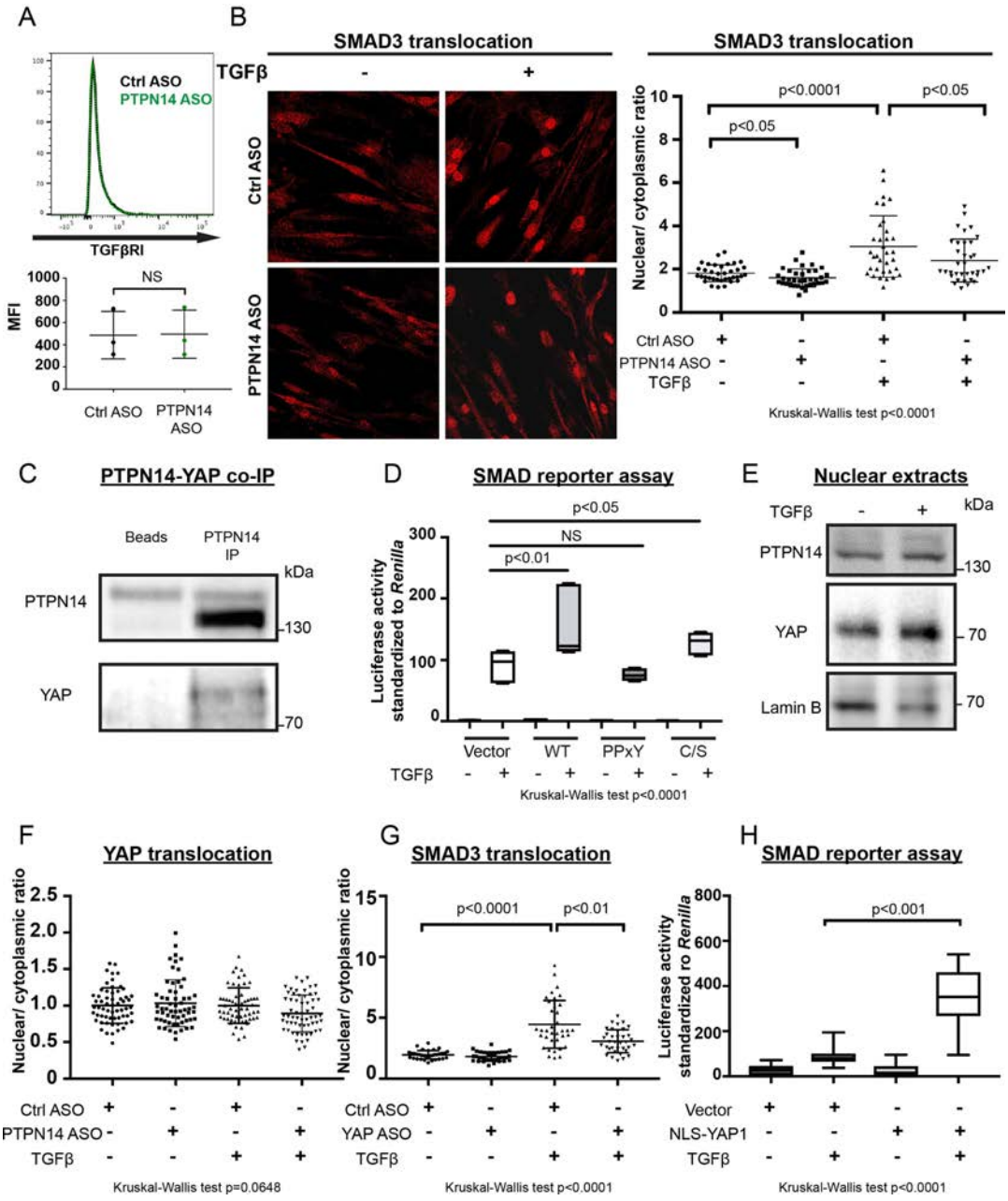

Figure 3 PTPN14-YAP interaction and nuclear YAP enhance TGFβ-SMAD signalling. (A) Upper panel, representative flow cytometry analysis of TGFβRI on RA FLS (n=3) treated with Ctrl (shown in black) or PTPN14 ASO (shown in green) for 6 days. Lower panel, plot shows MFI for each line. (B) RA FLS (n=3) were treated with Ctrl or PTPN14 ASO for 7 days, stimulated with TGFβ (50 ng/mL) for 30 min and then fixed and stained with an anti-SMAD3 antibody. Nuclear/cytoplasmic ratio of SMAD3 signal was calculated using image J for 12 cells from each RA FLS line. Representative images are shown in 60× magnification. Mean±SD is shown. (C) Co-immunoprecipitation of PTPN14 with YAP. Western blotting with indicated antibodies is shown. Panel is representative of three experiments with similar results. (D) TGFβ-induced SMAD activation was assessed via SMAD reporter assay in HEK293T cells. Cells were starved for 24 hours, transfected with empty vector or vectors encoding WT PTPN14, or PTPN14 Y570F/Y732F (PPxY) or C1121S (C/S) mutants and then stimulated with TGFβ (50 ng/mL) for 24 hours. Graph shows ratio of firefly/*Renilla* luciferase signal. (E) Western blotting of nuclear fraction of unstimulated or TGFβ-stimulated RA FLS using anti-PTPN14, anti-YAP or anti-lamin B (as a nuclear loading control). Panel is representative of 3 RA FLS lines with similar results. (F) RA FLS (n=5) were treated with Ctrl or PTPN14 ASO for 7 days stimulated with TGFβ (50 ng/mL) for 30 min, then fixed and stained with an anti-YAP antibody. Nuclear/cytoplasmic ratio of YAP signal was calculated using image J for 12 cells from each RA FLS line. Representative images are shown in 60× magnification. Mean±SD is shown. (G) RA FLS were treated with Ctrl or PTPN14 ASO for 7 days stimulated with TGFβ (50 ng/mL) for 30 min, then fixed and stained with an anti-SMAD3 antibody. Nuclear/cytoplasmic ratio of SMAD3 signal was calculated using image J for 12 cells from each RA FLS line. Representative images are shown in 60× magnification. Mean±SD is shown. (H) TGFβ-induced SMAD activation was assessed via SMAD reporter assay in HEK293T cells. Cells were starved for 24 hours, transfected with empty vector or vectors encoding NLS-YAP and then stimulated with TGFβ (50 ng/mL) for 24 hours. Graph shows ratio of firefly/*renilla* luciferase signal. (D,H) Box-and-whisker plots depict median (line within box), 25th percentile and 75th percentile (bottom and top borders) and range of minimum to maximum values (whiskers); three independent experiments performed in triplicate is shown. (A) Data were analysed using the two-tailed paired t-test. (B,D,F–H) Data were analysed using the Kruskal-Wallis test with two-tailed Mann-Whitney posthoc test, NS=non-significant. P value is adjusted for multiple comparisons in (D). FLS, fibroblast-like synoviocytes; MFI, mean fluorescence intensity; RA, rheumatoid arthritis.[1]

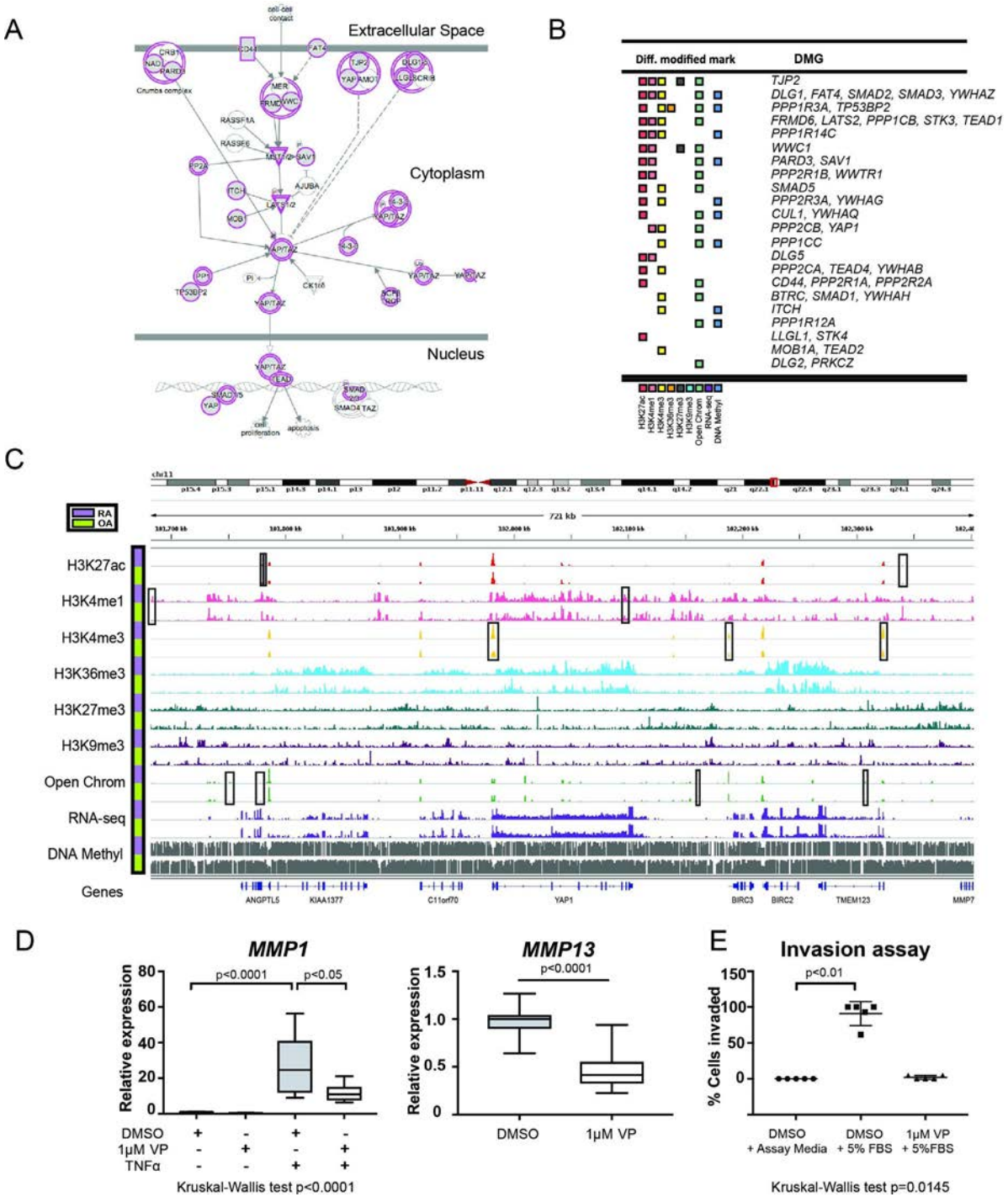

Figure 4 The Hippo pathway displays epigenetic alterations in RA FLS and modulates RA FLS TNF signalling and invasiveness. (A) The ingenuity pathway of Hippo signalling. Differentially marked genes (DMG) between RA and OA FLS are indicated in magenta colour. Legends: triangles for kinases; rectangles for G-protein coupled receptors; circles for transcription regulator; oval for other; double-sided shapes for complexes/groups. (B) Map of differentially modified marks for DMG in the Hippo signalling pathway between RA and OA FLS. (C) Representative epigenomic landscape of *YAP1* including six histone modifications, open chromatin, RNA-seq and DNA methylation. The figure shows an example of relative signal intensity across a ±300 kb region of *YAP1* for each mark in one RA vs one OA FLS lines. Differentially marked regions between RA and OA are indicated by boxes. (D) RA FLS (n=4) were serum-starved for 24 hours and then treated with DMSO or 1 μM VP for 45 min. Cells were then stimulated for 24 hours with 50 ng/mL TNFα in the presence of DMSO or VP. *MMP1* and *MMP13* mRNA expression levels were determined by qPCR performed in triplicate. Results were normalised to *GAPDH* using $2^{-\Delta\Delta Ct}$ method. Box-and-whisker plots depict median (line within box), 25th percentile and 75th percentile (bottom and top borders) and range of minimum to maximum values (whiskers). (E) After 24 hours serum starvation, RA FLS (n=5) were allowed to invade for 24 hours through a matrigel-coated transwell chamber in response to 5% FBS in the presence of DMSO or 1 μM VP. For each well, four non-overlapping area (top, bottom, left and right) were imaged and counted. Plot shows mean±SD per cent invading cells. Data were analysed using the Kruskal-Wallis test with two-tailed Mann-Whitney posthoc test (D,E) or the two-tailed Mann-Whitney test (right panel in D). DMG, differentially marked genes; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FLS, fibroblast-like synoviocytes; OA, osteoarthritis; qPCR, quantitative PCR; RA, rheumatoid arthritis; VP, verteporfin.

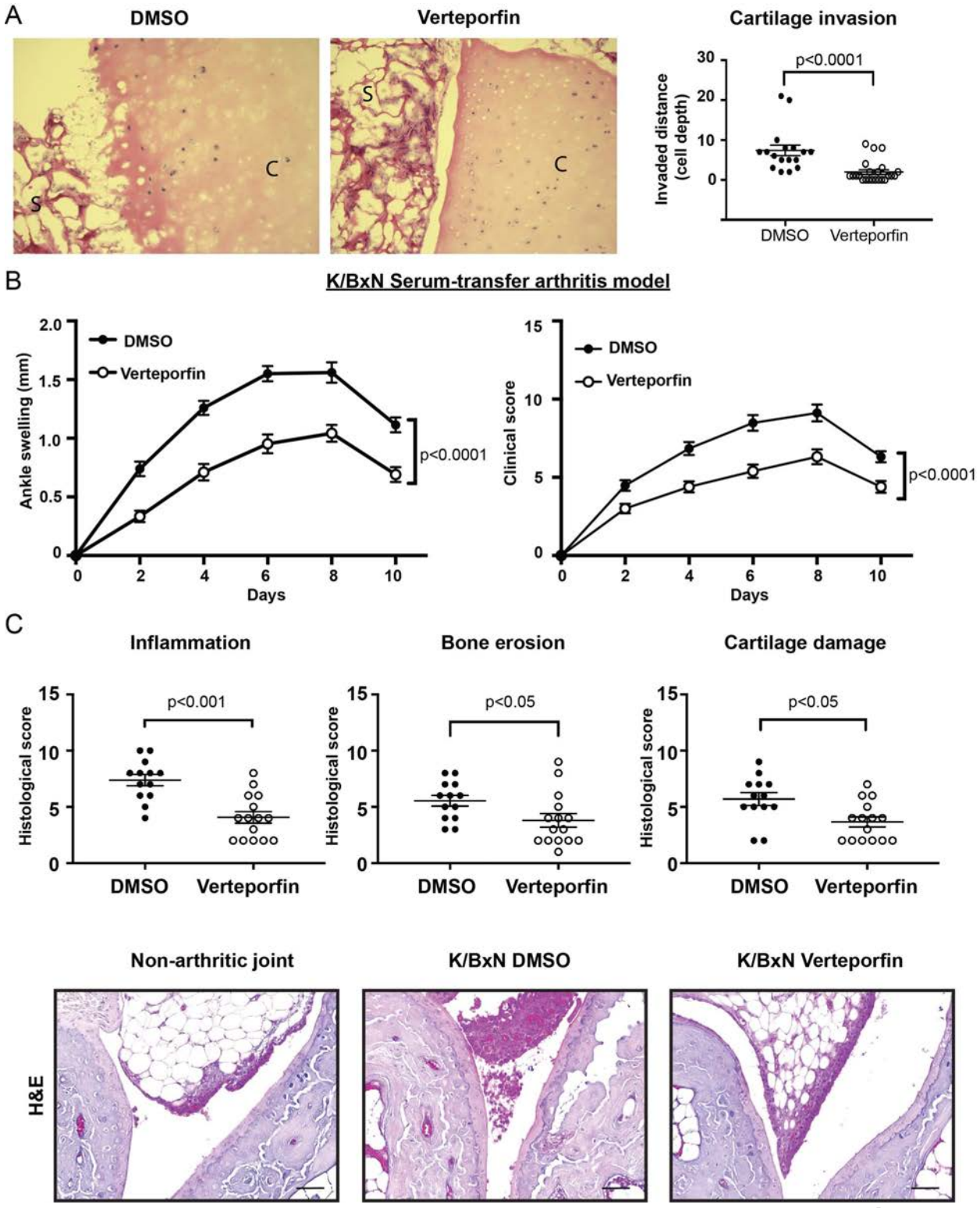

Figure 5 YAP promotes RA FLS invasiveness and arthritis severity in mice. (A) Cartilage fragments were coimplanted with 5×10^5 RA FLS (n=2) subcutaneously in SCID mice. Mice (n=3–4) were injected intraperitoneally (i.p.) with 15 mg/kg VP or vehicle every other day for 34 days. Two days after the last administration, the cartilage was harvested and assessed for FLS invasion. Representative images of cartilage sections viewed at 20× magnification are shown. S=sponge, C=cartilage. Plot shows mean±SD of the depth of cartilage invasion by RA FLS assessed on a minimum of 4 fields/specimen. (B) Eight-week-old female Balb/c mice were injected i.p. with 100 µL of K/BxN serum to induce arthritis, and injected i.p. daily with 50 mg/kg verteporfin (n=28) or DMSO-containing vehicle (n=25) starting on the day of arthritis induction. Ankle thickness and clinical score were assessed every day. Graph shows mean±SEM of mouse ankle swelling (left graph) and clinical score (right graph). (C) After 14 days, joints from mice in (B) were harvested and subjected to histological staining with H&E (for inflammation and bone erosion assessment) and safranin O (for cartilage damage assessment). Inflammation, bone erosion and cartilage damage of the talonavicular joint were scored blindly between 0 and 5 by two people. Plot shows mean±SEM of combined scores. Data were analysed with two-tailed Mann-Whitney test (A,C) or two-tailed Mann-Whitney test using area under curve in (B). DMSO, dimethyl sulfoxide; FLS, fibroblast-like synoviocytes; RA, rheumatoid arthritis.

treated RA FLS with the small molecule verteporfin, an FDA approved drug for photodynamic therapy that has been shown to inhibit YAP transcriptional activity in vitro and in vivo.[40] In line with the observed effect of PTPN14 knockdown, inhibition of YAP with 1 µM verteporfin in RA FLS inhibited TNFα-induced expression of *MMP1* and reduced expression of *MMP13*. However, verteporfin also inhibited TNF-induced *MMP3, VCAM1* and *IL-6* expression compared with cells treated with vehicle (figure 4D and online supplementary figure 7). Moreover, treatment with verteporfin dramatically inhibited RA FLS invasiveness in response to FBS (figure 4E), suggesting that YAP promotes RA FLS pathogenic action through transcriptional and potentially other mechanisms that only partially overlap with the mechanisms regulated by PTPN14.

YAP promotes RA FLS invasiveness *in vivo* and arthritis severity in mice

To further assess whether YAP promotes RA FLS pathogenic behaviour in vivo, we employed the severe combined immunodeficiency (SCID) model of FLS cartilage engraftment.[41] In line with the in vitro observations reported in figure 4, daily administration of verteporfin (15 mg/kg) to cartilage and RA FLS-engrafted SCID mice led to a significant ($p<0.0001$) reduction of in vivo cartilage invasion by RA FLS (figure 5A). In order to further assess the role of YAP in a second synoviocyte-dependent model of RA, we also examined whether treatment with verteporfin affects disease development in the passive K/BxN serum-transfer arthritis model. Figure 5B shows that daily administration of 50 mg/kg verteporfin to K/BxN serum-transferred mice (n=28) led to significant reduction of arthritis severity ($p<0.0001$) compared with control mice treated with DMSO (n=25). Histological assessment of affected joints showed that verteporfin treatment significantly protected mice from bone erosion, cartilage damage and inflammation (figure 5C and online supplementary figure 8).

DISCUSSION

Here, we report that RA FLS display overexpression of PTPN14, which promotes TGFβ canonical signalling. We provide evidence that promotion of SMAD signalling by PTPN14 depends on the formation of a YAP-PTPN14 complex. Although the exact molecular mechanism through which PTPN14 regulates TGFβ-induced SMAD3 translocation in RA FLS remains to be clarified, we speculate that the PTPN14-YAP complex enhances the ability of nuclear YAP to recruit SMAD3 on TGFβ stimulation. YAP has been shown in other cells types to form a complex with SMAD2/3, which promotes nuclear translocation of SMAD complexes.[38 42] Thus, it is possible that a trimolecular PTPN14-YAP-SMAD3 complex is formed in the nucleus of RA FLS. The observation that PTPN14 knockdown only partially recapitulates inhibition of YAP nuclear functions is in line with the fact (evident in figure 3F) that PTPN14 is not necessary for YAP nuclear localisation. The latter is a somehow unexpected finding since in many cancer cell types, the PTPN14-YAP complex prevents nuclear translocation of YAP[15] and RA FLS have been likened to tumour-like cells due to their peculiar aggressive features in vitro and in vivo.[43] However, the partial overlap between PTPN14-mediated and YAP-mediated signalling in RA FLS might also reflect unknown mechanisms of compensation in cells subjected to knockdown of PTPN14. A limitation of our studies of PTPN14 is that some of the changes observed after knockdown in FLS are modest and further studies—eg, in animals with conditional deletion of PTPN14 in FLS when they become available—are needed to confirm that PTPN14 is involved in the pathogenesis of RA and its hierarchical dominance in disease pathogenesis.

Our data also suggest for the first time that the Hippo pathway and nuclear YAP contribute to the aggressive phenotype of RA FLS. Although our reanalysis of the available dataset showed no differences in Hippo pathway transcript levels in resting RA vs OA FLS, the pathway carries an extensive epigenetic signature in RA FLS, which warrants mRNA and protein expression studies in RA and OA FLS subjected to RA-relevant stimulations and in RA synovium. Furthermore, our in vivo data also point to a potential benefit of YAP inhibition to reduce FLS pathogenesis in RA. Since verteporfin has been shown to ameliorate antigen-induced arthritis in rabbits by inducing apoptosis of inflammatory cells,[44] further investigations are warranted to elucidate whether YAP inhibition could also control the immune-mediated arm of RA pathogenesis, thus configuring YAP as a potentially unique target for dual immune and FLS-based RA therapy.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the La Jolla Institute (#AP140-NB4) and UC SAN DIEGO (#S16098). The generation and banking of FLS lines from arthroplasties was approved by the UC San Diego IRB (#140175). Ethical approval for the PEAC cohort was granted by the King's College Hospital Research Ethics Committee (REC 05/Q0703/198).

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CLINICAL SCIENCE

Achieving remission in psoriatic arthritis by early initiation of TNF inhibition: a double-blind, randomised, placebo-controlled trial of golimumab plus methotrexate versus placebo plus methotrexate

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ABSTRACT

Objectives Early initiation of effective treatment favours remission in rheumatoid arthritis, but it remains unknown if the same concept applies to psoriatic arthritis (PsA). Therefore, this study investigated whether the combination of golimumab plus methotrexate (MTX) as a first-line treatment is superior to MTX alone in inducing remission in PsA.

Methods This investigator-initiated, multicentre, double-blind, randomised, placebo-controlled trial included 51 MTX and bDMARD-naive patients with PsA fulfilling the CASPAR criteria and with active disease at baseline (≥3 swollen joint count/tender joint count). Patients were randomised to golimumab (50 mg SC monthly)+MTX (n=26) (TNFi arm) or matched placebo+MTX (n=25) (MTX arm). MTX was started 15 mg/week and increased to 25 mg/week over 8 weeks. The primary endpoint was percentage of patients achieving Disease Activity Score (DAS) remission (<1.6) at week 22. Safety was assessed throughout the study.

Results The primary efficacy endpoint was achieved by 81% in the TNFi arm versus 42 % in the MTX arm (p=0.004). This difference in DAS remission was already observed at week 8. A significant difference in favour of the golimumab+MTX arm at week 22 was also observed for other response criteria such as MDA, ACR20/50/70, disease measures and patient-reported outcomes. The occurrence rates of adverse event and treatment-emergent adverse event were similar in both arms.

Conclusions In patients with early PsA, DAS remission at week 22 was almost doubled with golimumab+MTX versus MTX alone. This double-blind, randomised, placebo-controlled study supports the concept that early initiation of TNFi in patients with PsA favours remission.

Trial registration number NCT01871649.

Key messages

What is already known about this subject?

- Data on early intervention in psoratic arthritis (PsA) is sparse, although two studies exploring the concept in PsA (Baranauskaite, an open-label study) and peripheral SpA (Carron, randomised controlled study) suggested the contribution of early intervention in PsA.

What does this study add?

- The major finding of this study was that early initiation of the combination therapy with golimumab plus methotrexate doubled the number of patients achieving a Disease Activity Score remission when compared with methotrexate alone.
- This was confirmed by additional outcome measures, as well as by larger improvement in clinical disease activity measures and patient-reported outcomes but not function or quality of life.
- Our results extend the findings of the open-label RESPOND study that early intervention in PsA contributes to achieve remission in PsA. Future follow-up will explore if these responses are maintained up to week 50 on methotrexate monotherapy.

How might this impact on clinical practice or future developments?

- Taken together, the superior clinical efficacy and good tolerability/absence of novel safety signals, these results—in line with the results the previously published studies of Baranauskaite *et al* and Carron *et al*—suggest the value of early intervention in PsA rather than the classical step-up approach.

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INTRODUCTION

Psoriatic arthritis (PsA) is a chronic inflammatory arthritis affecting the joints and connective tissue and is associated with psoriasis of skin and nails. Treatment options for PsA have tremendously increased over the last two decades. The initial treatment in most patients consists of conventional synthetic disease-modifying antirheumatic drugs (csDMARDS). Patients with PsA with persistent moderate to high disease activity are eligible for tumour necrosis factor inhibitors (TNFi). In rheumatoid arthritis (RA), there is ample evidence for strategies aiming to reach and maintain remission of inflammation (ie, treat to target).[1–4] Also, the early start of treatment improved outcomes, as the earlier the start of treatment, the higher the remission rates seen.[5 6]

Whether initiation of potent targeted therapies in an early disease phase favours remission in other types of inflammatory arthritis, including PsA, remains unknown. The current treatment paradigm in PsA still consists of a step-up approach with non-steroidal anti-inflammatory drugs (NSAIDs) and/or non-biological DMARDs, mostly methotrexate (MTX) or leflunomide, as a first-line treatment.[7 8] MTX is most commonly used as first-line treatment despite the fact that its potential efficacy is not supported by randomised, placebo-controlled studies.[9] TNFi, which have demonstrated strong efficacy in multiple randomised, placebo-controlled studies in PsA,[10–13] are merely recommended as second-line therapy for patients with PsA failing to respond to first-line therapy.[7 8] More recently, other targeted therapies such as interleukine(IL)-12/ /IL-23 p40 inhibition, IL-17A inhibition and Janus kinase (JAK) inhibition have become available as second-line or third-line options.[14–17]

A couple of studies have started to explore if early initiation TNFi favours remission in PsA. Baranauskaite *et al* investigated the use of early MTX with or without infliximab in an open-label study in patients with early PsA. They showed high response in both arms, with a significantly greater improvement in the MTX plus infliximab arm compared with the MTX alone arm American College of Rheumatology response criteria (ACR20): 86.3% vs 66.7%). Larger differences were seen between the treatment arms with more stringent outcome measures such as ACR50, ACR70 and Minimal Disease Activity (MDA).[18] However, the important limitation of this study was the open-label design and these data have not yet been confirmed in a placebo-controlled setting in PsA. Exploring the same concept in a slightly different population, Carron *et al* investigated the early initiation of TNFi treatment in a placebo-controlled study in a mixed population of patients with early peripheral spondyloarthritis, of which 40% had concomitant nail or skin psoriasis.[19] Patients achieved clinical remission (defined as absence of arthritis, enthesitis and dactylitis) in 75% in the TNFi-treated arm versus 20% in the placebo arm.

Based on this circumstantial evidence that early treatment with TNFi could favour high remission rates in PsA, the current double-blind placebo-controlled randomised study was initiated to investigate whether the combination of golimumab plus MTX as a first-line treatment is superior in achieving remission compared with treatment with MTX alone in patients with PsA who are naive to MTX and TNFi.

METHODS

Study design

This investigator-initiated, randomised, placebo-controlled, double-blind study was conducted at three centres in the Netherlands between September 2013 and September 2017. Patients were randomly assigned in a 1:1 ratio to receive either five injections with golimumab (50 mg subcutaneous monthly) or matched placebo. In both arms, MTX was started at 15 mg/week orally and increased to 25 mg/week over 8 weeks. Statistical minimisation was applied for centre, number of swollen joints and disease duration using a software program ALEA, a validated randomisation tool (NKI, Amsterdam, the Netherlands). The primary endpoint of the study was measured at the end of the 22-week blinded treatment period.

Patients

Patients aged 18–70 years were eligible if they had PsA according to the Classification Criteria for Psoriatic Arthritis (CASPAR) and current active disease, defined as the presence of at least three swollen and three tender joints at baseline.[20] Patients previously treated with MTX or any biological DMARD were excluded. Allowed co-medication included NSAIDs and/or systemic steroids <10 mg/daily at stable dosages from 2 weeks prior to baseline. Local corticosteroids were not allowed within 4 weeks prior to baseline. Three patients used concomitant fumaric acid and one patient used concomitant sulphasalazine (table 1). Key exclusion criteria were the presence of latent or active tuberculosis, malignancy in the past 5 years (other than basal cell

Table 1 Baseline demographics and clinical characteristics of the study patients by treatment arm

	Golimumab+MTX (N=26)	Placebo+MTX (N=25)
Age, years	47.5 (11.8)	45.8 (11.0)
Gender (male:female)	18:8	20:5
Disease duration arthritis, years	0.5 (0.5–1.8)	0.5 (0.4–3.0)
Disease duration skin, years	6.0 (1–20)	11 (4–19)
Prior use of csDMARD (leflunomide)	1	0
Concomitant use of topical psoriasis treatment	6	13
Concomitant use of fumaric acid (N)	1	2
Concomitant use of sulfasalazine (N)	0	1
Concomitant NSAID use at baseline (N)	16	17
Concomitant corticosteroid use at baseline (N)	0	0
DAS CRP	2.3 (1.03)	2.46 (0.87)
Swollen joint count (median (IQR))	7 (4–8.25)	5 (4–9.5)
Tender joint count (median (IQR))	9.5 (4–15.25)	10 (5.5–17)
PASI score (median (IQR))	1.6 (0.32–3.3)	2.3 (0.3–6.8)
No of patients with baseline PASI >2.5	10	10
No of patients with enthesitis	4	7
No of patients with dactylitis	9	8
No of patients reporting inflammatory axial symptoms at baseline	4	2
ESR (mm/h)	20.5 (6.5–33.3)	15.0 (5.0–29)
No of patients with raised ESR (>20 mm/h)	13	14
CRP (mg/dL)	4.5 (1.23–13.3)	7.0 (2–15.9)
No of patients with raised CRP (>5 mg/dL)	14	9
VAS patient global (mm)	44.7 (24.7)	39.3 (23.4)
VAS patient pain (mm)	43.5 (24.2)	41.3 (28.4)
VAS physician (mm)	44.5 (14.5)	47 (19.7)
Morning stiffness (min)	44 (32.5)	42.3 (33.3)
BASDAI	41.0 (18.6)	41.3 (23.3)

Values are mean (SD), N or median (p25, p75).
BASDAI, Bath ankylosing spondylitis disease activity index; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; MTX, Methotrexate; NSAID, non steroidal anti inflammatory drug; PASI, psoriasis activity and severity index; VAS, visual analogue scale on a 0-100mm scale.

carcinoma of the skin), recent severe infections or other severe diseases that may affect patient's participation to the study in the opinion of the investigator.

The study was conducted in compliance with the International Conference on Harmonisation Good Clinical Practice guidelines and the Declaration of Helsinki.

Assessments

The primary efficacy endpoint of this study was the proportion of patients achieving a status of Disease Activity Score (DAS) remission at week 22, defined by a DAS C reactive protein (CRP) score <1.6 (0.54×SQRT(Ritchie Articular Index)+0.065× swollen joint count (SJC)44+0.17×ln(CRP+1)+0.0072×Visual Analogue Scale (VAS) global health+0.45).[21] Secondary endpoints included additional response criteria such as MDA,[22] DAS score low disease activity (LDA) (<2.4), DAPSA LDA and ACR20/50/70 responses. Disease activity measures included 66/68 tender and swollen joint count (TJC/SJC), dactylitis count, Leeds Enthesitis Index including the plantar fascii,[23] Psoriasis acitivity and severity index (PASI) and PASI 75 (≥75% improvement in the PASI score) for subjects with baseline PASI ≥2.5, CRP, ESR and VAS physician. Patient-reported outcomes (PROs) were patient pain and patient global score on a VAS from 0 to 100 mm, morning stiffness duration, and Bath Ankylosing Spondylitis Index (BASDAI). Function and quality of life were assessed using the Short Form 36 (SF36), Health Assessment Questionnaire (HAQ) and Dermatology Life Quality Index (DLQI) scores. All efficacy endpoints were evaluated at week 22 as well as at week 8.

Safety endpoints included adverse events (AEs) and serious AEs (SAEs), and discontinuation or interruption of study treatments because of AEs. Routine laboratory investigations, vital signs and physical examination findings were recorded at screening and at every visit (baseline, weeks 4, 8, 14 and 22).

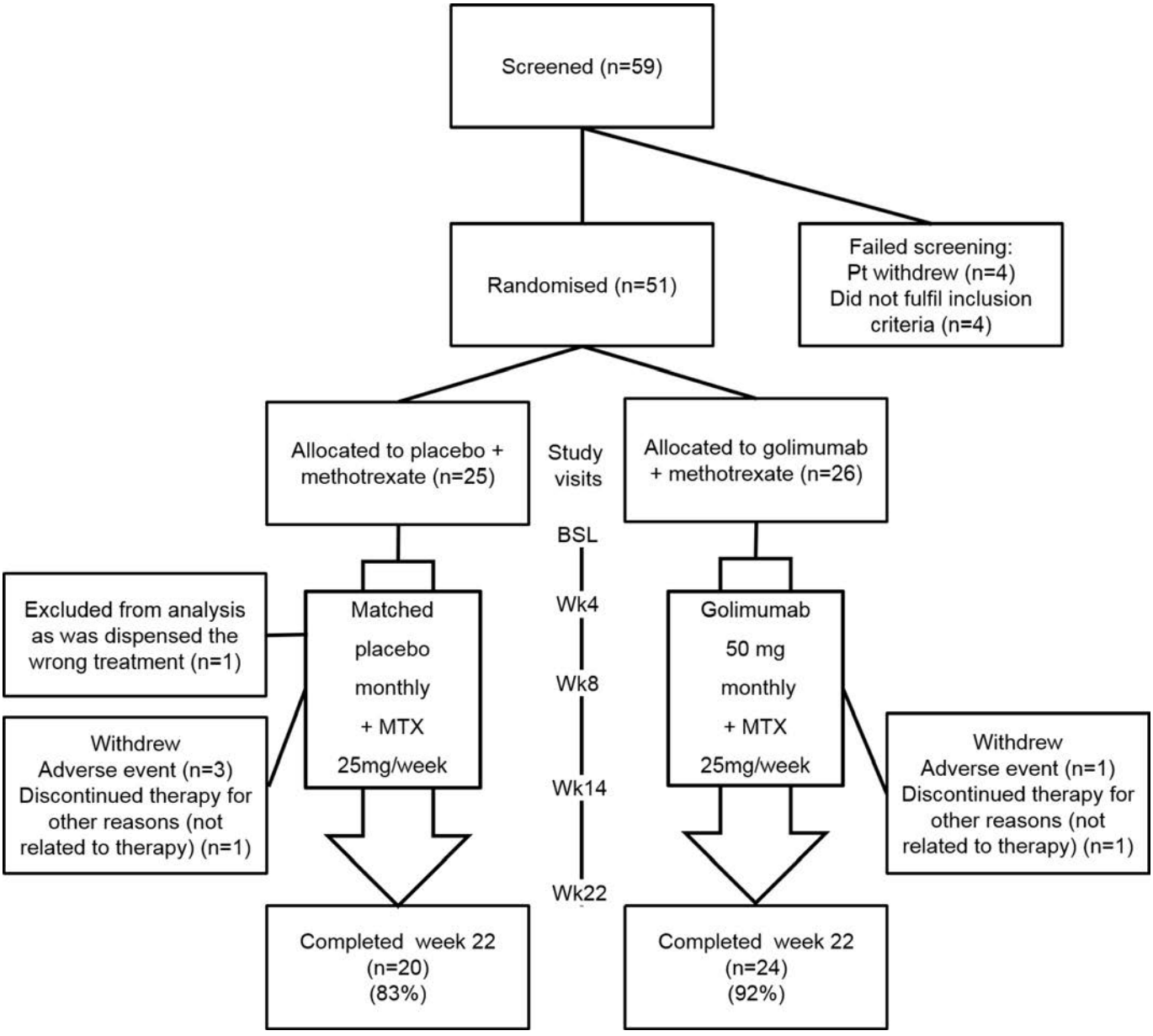

Figure 1 Overview of patient disposition and study design. Patients were randomly assigned in a 1:1 ratio to receive either five injections with golimumab (50 mg SC monthly) or matched placebo. In both arms, methotrexate (MTX) was started at 15 mg/week orally and increased to 25 mg/week over 8 weeks.

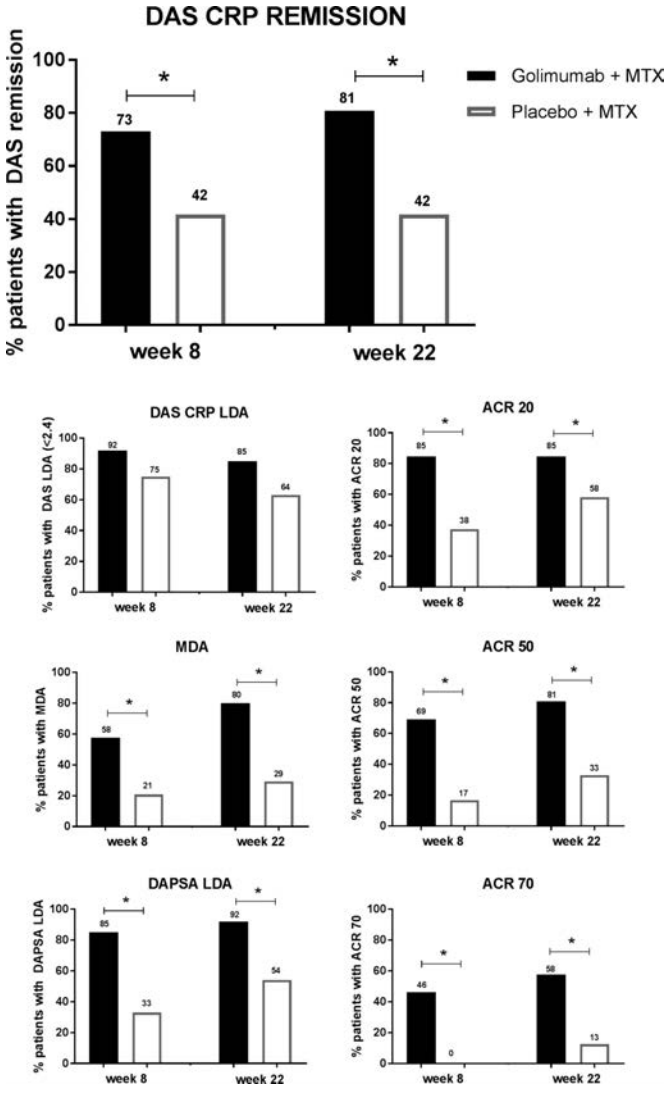

Figure 2 Primary and secondary response measures: upper panel: percentage of patients in DAS CRP remission after 8 and 22 weeks in the golimumab+MTX and the placebo+MTX arm, respectively. Other panels: percentage of patients reaching DAS CRP LDA, MDA, DAPSA LDA and ACR20/50 and 70 responses.

Statistical analysis

The sample size was calculated based on the results of the RESPOND study. This open-label study of Baranauskaite *et al*[18] showed a DAS remission rate of 68.6% in the TNFi+MTX arm versus 29.2% in the MTX arm. Therefore, we estimated an expected 40% difference in response rate between both treatment arms. Considering a two-sided significance level of 0.05 and a power of 80%, the power analysis indicated 24 patients each arm.

Baseline characteristics and safety analyses included all randomised patients who received at least one dose of trial medication (51 patients). For efficacy analyses, one individual with wrong administration of golimumab versus placebo due to protocol violation was excluded from the MTX arm. Therefore, the intention-to-treat population for efficacy included 50 patients. Missing data were handled using non-responder imputation for the primary endpoint as well as all other binary endpoints and using last observation carried forward for continuous variables. Values are reported as mean (SD) or median (IQR) as applicable. At each time point, differences between placebo and golimumab were tested using a χ^2 test for the categorical variables, and an ANCOVA with the baseline variable as covariate for continuous variables. All statistical tests were two sided and p values of <0.05 were considered statistically significant.

RESULTS

Study population and patient disposition

A total of 59 patients were screened at three rheumatology clinics in The Netherlands between September 2013 and September 2017 (figure 1). Fifty-one patients were randomised to receive either golimumab+MTX (n=26) (TNFi arm) or placebo+MTX (n=25) (MTX arm). The baseline characteristics were similar in the two treatment arms(table 1).

Median time since diagnosis was 0.5 (0.5–2) years, most patients (35/50) presented with a polyarticular disease pattern, and the median SJC was 5 (4–8) and TJC 10.[5–15] Twenty patients had a PASI score ≥2.5 at baseline, and enthesitis was present in 11 patients and dactylitis in 17 patients.

Prior to unblinding, one patient from the MTX arm was excluded from all efficacy analyses due to an error at the pharmacy causing the wrong treatment to be administered. The efficacy analyses are therefore based on data of 50 patients: golimumab+MTX (n=26) and placebo+MTX (n=24).

During the 22-week period, in total six patients did not complete the study period as scheduled; reasons reported for drop out were two patients were lost to follow-up due to adverse events (one in the TNFi arm and one in the MTX arm both at week 14 of the study) and four patients withdrew their informed consent (one in the TNFi arm and three in the MTX arm).

All patients completing the 22-week study period received the full 5/5 of assigned study injections. The overall mean dosage of MTX during the full 22-week period was mean (SD) of 19.2 (4.5) mg/week in the TNFi arm and 21.2 (2.4) mg/week in the MTX arm.

Efficacy

The study met the primary efficacy endpoint with DAS remission at week 22 achieved by a greater number of patients in the TNFi arm (21/26;81%) versus the MTX arm (10/24;42%) (p=0.004) (figure 2). This difference in favour of the golimumab+MTX arm was confirmed by other composite response criteria at week 22 (figure 2): TNFi-treated patients reached an MDA in 21/26 (81%) versus 7/24 (29%) in the MTX arm (p<0.001). Although not reaching statistical significance, a similar trend was seen for DAS CRP LDA (85% vs 64%, p=0.072), and a DAPSA LDA was achieved in 92% versus 54% (p=0.001). An ACR 20/50/70 response was achieved by respectively 85%, 81% and 58% in the TNFi arm versus 58%, 33% and 13% in the MTX arm (p=0.039, p=0.001 and p=0.001, respectively). With exception of DAS CRP LDA, statistically significant differences were already seen by week 8 for all these response measures (figure 2).

Disease activity measures, PROs, and measures of physical function and quality of life are listed in table 2.

Significant differences in response on PROs included VAS patient pain, VAS patient global, morning stiffness duration and BASDAI. This effect was already seen at week 8 for VAS global. No significant differences were seen in physical functioning and in health-related quality of life between both arms at week 22. No significant differences were seen in the achievement of PASI75 and DLQI scores.

Safety and AEs

One serious AE occurred in a patient in the MTX arm (cervical spine stenosis, requiring surgery), which was considered not

Table 2 Disease activity and patient-reported outcomes at baseline, week 8 and week 22

	Baseline		Week 8			Week 22		
Efficacy measures	Golimumab+MTX	Placebo+MTX	Golimumab+MTX	Placebo+MTX	P value for group difference	Golimumab+MTX	Placebo+MTX	P value for group difference
DAS CRP	2.1 (1.7–2.7)	2.4 (1.9–2.9)	1.12 (0.7–1.61)	1.8 (1.31–2.34)	0.002	0.91 (0.68–1.36)	1.8 (1.18–2.19)	0.000
Swollen joint count	7 (4–8.3)	5 (4–10.3)	1 (0–3)	4 (1.5–8)	0.003	0 (0–1.25)	2 (0–4)	0.042
Tender joint count	9.5 (4–15.3)	10 (5.3–15.5)	1 (0–4)	5 (3–9.8)	0.019	0 (0–4)	3 (1–5)	0.019
PASI (in group with BSL PASI >2.5)	5.75 (4.0–7.55)	4.95 (3.5–8.45)	0.65 (0–3.05)	2.7 (0.75–4.25)	0.210	0.55 (0–1.9)	0.5 (0–1.95)	0.924
No of patients with enthesitis	4	7	4	3	0.594	2	4	0.209
No of patients with dactylitis	9	8	5	5	0.836	0	1	0.313
ESR (mm/h)	20.5 (6.5–33.3)	15.5 (5–30.5)	2 (2–5)	8 (5–19)	0.003	2 (2–18)	8 (2–13)	0.566
CRP (mg/dL)	4.5 (1.2–13)	7.1 (2.2–16.6)	0.75 (0.3–2.95)	2.9 (1.25–7.75)	0.079	1.1 (1.48–2.85)	3.6 (1.2–7.0)	0.144
VAS patient global (mm)	48(26–59)	36 (25–54)	21 (6–36)	31 (16–46)	0.184	9 (4–32)	31 (14–57)	0.038
VAS patient pain (mm)	44 (29–64)	34 (17–7)	11 (3–24)	30 (16–38)	0.003	6 (2–18)	34 (6–58)	0.001
VAS physician (mm)	48 (37–53)	46 (37–64)	10 (6–25)	33 (19–50)	0.000	4 (1–20)	18 (9–33)	0.047
BASDAI	40.5 (29.9–56.3)	47.1 (19.1–56.9)	36.5 (16.3–59.6)	41.6 (22.5–61.0)	0.287	18.1 (4.9–23)	24.6 (11.7–49.5)	0.022
HAQ	0.38 (0.19–1.0)	0.63 (0.19–1.47)	0 (0–0.3)	0.43 (0.03–0.84)	0.003	0 (0–0.125)	0.25 (0–0.5)	0.403
SF36 PCS	41.1 (35.8–48.1)	43.6 (36.1–48.5)	47.0 (40.9–55.1)	48.8 (45.3–53.0)	0.056	50.1 (43.7–52.2)	50.7 (44.5–52.1)	0.543
SF36MCS	47.9 (40.7–55.4)	51.6 (47.4–56.6)	51.7 (40.7–56.8)	50.3 (44.2–56.5)	0.041	50.7 (40.0–55.5)	50.9 (37.8–52.7)	0.125
DLQI	2 (0–7)	2 (0–5.75)	1 (0–3.5)	1 (0–5)	0.891	1 (0–3)	0 (0–3.5)	0.272

Values are median (p25, p75) or No of patients.
BASDAI, Bath ankylosing spondylitis disease activity index; CRP, C-Reactive Protein; DLQI, Dermatology Life Quality Index; ESR, Erythrocyte Sedimentation Rate; HAQ, Health Assessment Questionnaire; PASI, psoriasis activity and severity index; SF36, Short form 36 Physical Component Score; SF36 MCS, Short form 36 Mental Component Score; VAS, Visual Analogue Scale.

to be study related and did not result in early withdrawal. AEs occurring during the study period are described in table 3.

The incidence in adverse events was similar between arms. In total, 43/50 patients experienced at least one AE during the trial period (range, 1–7), all of which were graded mild to moderate. The most frequent AE involved nausea and occurred in similar incidences in both treatment arms and considered to likely to be treatment related. In 18 patients, an AE led to temporary halt and/or lowering of MTX dosage, and four AEs led to early withdrawal from the trial. No deaths occurred.

Table 3 Adverse event types and incidence up to 22 weeks

	Golimumab+MTX (n=26)	Placebo+MTX (n=25)
Subjects with SAE (non study-drug related)	0	1
Subjects with AE/event leading to lower or quit MTX		
Total	8	11
ALAT elevation	2	6
Nausea/vomiting	4	2
Infection	2	3
No of subjects with other treatment-related AE	21	22
Liver toxicity	2	5
Upper airway infections	5	5
Other infections	3	8
Headaches	1	1
Malaise/tiredness around MTX intake	5	5
Nausea/vomiting	17	13
Other	8	8

MTX, methotrexate; SAE, Severe adverse event.

DISCUSSION

The major finding of this randomised, double-blind, placebo-controlled study was that the combination of golimumab plus MTX as a first-line treatment is superior to treatment with MTX alone in patients with early PsA who are naive to MTX.

When interpreting the data of this study, two factors related to study design should be carefully considered. First, the study was specifically designed to compare the combination of a TNFi+MTX with MTX monotherapy and not to study the efficacy of MTX monotherapy itself. Monotherapy with MTX was chosen as the control arm for the sole reason that this is currently the most frequently used first-line therapy in PsA and is recommended by several guidelines.[8 24] Therefore, MTX reflects current standard of care despite the fact that previous trials of MTX in PsA failed to unequivocally establish efficacy.[9 18] As one of the potential reasons for the lack of efficacy in previous trials was the relatively low dosage of MTX (up to 15 mg/week), we used a more aggressive dosing scheme with a start dose of 15 mg/kg, a rapid dose increase to 25 mg/week over 8 weeks, which resulted in a mean dose of around 20 mg/week over the 22-week study period. Whereas this was aimed to reflect the full potential of MTX in early PsA, the absence of a non-treated placebo arm and the powering (aimed for the golimumab+MTX vs MTX alone) precludes meaningful conclusions on the potential efficacy of MTX as standalone treatment.

Also, we used here golimumab as a prototype TNFi; although not formally demonstrated, there is no scientific or clinical evidence suggesting that the concept demonstrated here would not apply to all TNFi. Whether the concept also applies to other biologic targeted therapies used in PsA (anti-IL-17A, anti-p40, anti-p19) remains to be investigated.

Second, the population included in this trial of patients with early, MTX-naïve PsA differs considerably from previous pivotal

large phase III randomised controlled trials. As expected, disease duration was much shorter (0.5 years in our study vs 6–7 years in the large phase III studies) and, in line with the inclusion criterium of a minimum SJC/TJC of 3 at baseline, both SJC (median 5 vs 12) and TJC (10 vs 21) were lower in this trial in early, MTX-naive disease.[10 16 25] Whereas the population we included here is likely more representative of early untreated PsA, the differences in baseline features do not allow to compare the outcomes between this study and previous pivotal phase III trials.

Within this particular framework of study design, the study met its primary endpoint by demonstrating that almost double the number of patients treated with golimumab+MTX achieved DAS remission at week 22 versus MTX alone. Similar or even more pronounced differences were confirmed by other outcome measurements such as DAPSA LDA, MDA, AR50 and ACR70, as well as by several PROs. Moreover, most of these differences were already observed at week 8. The early and consistent improvement in stringent response criteria in favour of the golimumab+MTX arm confirms and extends the results of the open-label RESPOND study[18] that early initiation of TNFi contributes to achieve low disease activity or even remission in PsA.

The DAS remission was chosen as the primary endpoint as this measure a 'depth of response' instead of a decrease of disease activity as measured by ACR response. We included several secondary endpoints, including the traditional response measures, showing similar results.

Our data raise a number of additional questions. First, clear effects were already seen at week 8, but most outcomes were even more pronounced at week 22. It remains unknown if the responses—in particular the stringent responses such as remission—have already plateaued at week 22 or could even further increase over time. Similarly, it remains to be determined if the combination of TNFi and MTX is only needed for the induction of remission or is also needed to maintain this state of remission over time. To this purpose, golimumab (or placebo) was stopped at week 22 in those patients achieving DAS CRP remission and an extension of the present study will explore if responses are maintained up to week 50 on MTX monotherapy.

Second, the improvement in outcome measurements was paralleled by significant improvement of single disease parameters such as SJC and TJC, but not enthesitis, dactylitis and PASI. This could of course be due to the fact that only a fraction of the patients included in this PoC study had these disease manifestations (table 1) and, accordingly, that the study was underpowered to detect potential differences. Alternatively, MTX could be more effective for these disease manifestations than for pure articular disease, as suggested for skin by the proven efficacy of MTX in psoriasis.[26]

Third, HAQ showed a significantly larger improvement in golimumab+MTX versus placebo+MTX at week 8 but that was not maintained at week 22, with a gradual improvement in HAQ also observed in the MTX alone arm. More intriguingly, there was no difference at all in SF36 and DLQI scores between both treatment arms. Obviously, the study was not powered to this purpose, but the total absence of numerical trends suggest that the improvements in disease outcome measures are not reflected in function and QoL in this population with early disease. Further research is needed to fully explore this disconnect.

Fourth, in this study we did not include MRI or ultrasound to evaluate the presence or absence of active synovitis or enthesitis or their resolution over time. Arthritis and enthesitis was scored by joint and enthesitis counts. These types of assessments would have required a much larger study population. An interesting follow-up question would be if the observed clinical remission in peripheral PsA truly represents a resolution of inflammation without any signs of subclinical inflammation on imaging, and second, if the differences in achieved remission rates also protect from development of structural damage.

Finally, the potential benefit of early initiation of TNFi should be balanced against potential risks. In this study, treatment with either golimumab+MTX or placebo+MTX was well tolerated, only a small number of patients withdrew from the study due to AEs and no treatment-related severe AEs occurred during the study period. The AEs in this study were similar in both treatment arms and were consistent with previous studies with TNFi and MTX (mostly in longer standing disease),[10 13 27 28] without any novel safety signal. However, the study size and duration limits the interpretation of safety and tolerability.

In conclusion, initiation of combination therapy with golimumab+MTX in patients with early, MTX-naive PsA doubled the number of patients achieving DAS remission when compared with placebo+MTX. This was confirmed by additional outcome measures, as well as by larger improvement in clinical disease activity measures and PROs but not function or QoL. Taken together with the good tolerability and absence of novel safety signals, these results—in line with the results of an open-label study in PsA[9] and a randomised controlled trial in pSpA[19]—suggest the value of early intervention in PsA rather than the classical step-up approach.

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Contributors All authors were involved in drafting the article or revising it critically, and all authors approved the final version of the manuscript to be published. LJJvM, MTN, AWRvK and DB were involved in the study conception and design. LJJvM, HMdJ, IF, MGHvdS, MK and AWRvK were involved in the acquisition of study data. LJJvM, IF, HMdJ, MGHvdS, MK, AWRvK and CB were involved in the analysis and interpretation of the study data.

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Competing interests DB is currently an employee of UCB Pharma. LJJvM, IF and HMdJ have nothing to disclose. MGHvdS has been an advisor for Abbvie and Novartis, and received research grants from Janssen, Eli Lily and Novartis. The department of MK is supported by Novartis, Abbvie, Pfizer, Roche, Lilly and BMS, and MK has been an advisor for Novartis and Abbvie. MTN received research grants, consultation and/or speaking fees from Abbvie, BMS, Celgene, Eli Lilly, Janssen, MSD, Mundipharma, Novartis, Pfizer, Roche, Sanofi and UCB Pharma. AWRvK received speaker fees from Celgene, Novartis, Eli Lilly and Janssen, and received research support from MSD and Janssen.

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CLINICAL SCIENCE

Machine learning identifies an immunological pattern associated with multiple juvenile idiopathic arthritis subtypes

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ABSTRACT

Objectives Juvenile idiopathic arthritis (JIA) is the most common class of childhood rheumatic diseases, with distinct disease subsets that may have diverging pathophysiological origins. Both adaptive and innate immune processes have been proposed as primary drivers, which may account for the observed clinical heterogeneity, but few high-depth studies have been performed.

Methods Here we profiled the adaptive immune system of 85 patients with JIA and 43 age-matched controls with indepth flow cytometry and machine learning approaches.

Results Immune profiling identified immunological changes in patients with JIA. This immune signature was shared across a broad spectrum of childhood inflammatory diseases. The immune signature was identified in clinically distinct subsets of JIA, but was accentuated in patients with systemic JIA and those patients with active disease. Despite the extensive overlap in the immunological spectrum exhibited by healthy children and patients with JIA, machine learning analysis of the data set proved capable of discriminating patients with JIA from healthy controls with ~90% accuracy.

Conclusions These results pave the way for large-scale immune phenotyping longitudinal studies of JIA. The ability to discriminate between patients with JIA and healthy individuals provides proof of principle for the use of machine learning to identify immune signatures that are predictive to treatment response group.

Key messages

What is already known about this subject?

► Juvenile idiopathic arthritis (JIA) constitutes a heterogeneous class of diseases unified by the onset of arthritis in childhood. Improved understanding of the immunological architecture of JIA subsets is required for pathophysiology-based diagnosis and treatment.

What does this study add?

► An immune pattern was identified in multiple subtypes of JIA, shared with other paediatric patients with inflammatory diseases and accentuated in patients with systemic JIA and patients with active disease. The use of machine learning on the immune phenotyping data set generated an algorithm capable of discriminating patients with JIA from healthy controls with ~90% accuracy.

How might this impact on clinical practice or future developments?

► Our study serves as a proof-of-principle of immune-directed machine learning in precise diagnosis and personalised therapeutic choice. Future longitudinal studies of larger populations and more extensive immune profiling can implement automated analysis for full value extraction and translation to clinical practice.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most common class of childhood rheumatic diseases. It is characterised by the onset of arthritis with no defined cause prior to 16 years of age and the persistence of symptoms for more than 6 weeks. Evidence exists for both genetic inheritance and environmental triggers, which are currently unknown.[1 2] Elucidation of the precise aetiology and pathogenesis of JIA remains complicated by the clinical heterogeneity among the constituent diseases.[3 4] Patients with JIA were classified into seven subtypes by the International League of Associations for Rheumatology (ILAR) according to clinical features; however, it remains unknown as to whether each subtype has a distinct pathogenesis, and this classification may require revision with more complete understanding of the pathophysiology.[5 6]

Detailed analysis of cellular immunophenotypes and genetic variants associated with JIA subtypes could help improve the current classification system.[7] T cells are central to the pathogenesis of JIA and research has focused on unravelling the dynamic balance between proinflammatory (T helper 17 [Th17], Th1) and anti-inflammatory regulators (regulatory T cells), but the debate on the driving effector CD4 helper subset remains ongoing.[8–10] In particular, the inflammatory nature of interferon-gamma (IFNγ)-producing Th1 cells in an arthritic context has been questioned, with IFNγ-deficient mice developing systemic JIA (sJIA)-like symptoms on immune stimulation.[11]

High-depth immunophenotyping of the innate immune response to stimuli has recently identified an sJIA signature[12]; however, a similar indepth study on the adaptive immune response has been lacking. While most JIA subtypes share a strong similarity with autoimmune diseases, sJIA is often considered an autoinflammatory disease, raising the possibility of distinct immunological drivers of the disease.

Identification of the immunological signature of JIA can substantially improve our understanding of the disease pathophysiology, can lead to better diagnosis and disease classification, and in the future may be used to stratify patients for appropriate therapeutic approaches. Here we performed deep immunophenotyping on a large cohort of unrelated patients with JIA. We found a common inflammatory immune pattern across both patients with JIA and disease controls, driving a distinct immunological profile to that of healthy children. The immunological pattern was identifiable through machine learning and was elevated in sJIA compared with non-sJIA patients, and patients with active JIA compared with patients with inactive JIA, leading to the potential future application of immune-led machine learning in JIA treatment selection trials.

METHODS

Study population and sampling

Patients were recruited through the paediatric rheumatology department of Leuven University Hospital in our single-centre study. Controls were recruited through the general paediatric clinics and assessed as healthy through an interview with parents and review of electronic health records. Patients with JIA were classified via four distinct systems. First, patients were classified based on the ILAR criteria[13] into persistent oligoarticular, extended oligoarticular, rheumatoid factor positive (RF+) polyarticular, rheumatoid factor negative (RF−) polyarticular, enthesitis-related and sJIA. Patients with psoriatic and undifferentiated JIA were not found within our cohort, while patients with oligoarticular arthritis were frequent enough to allow splitting into persistent and extended subcategories. Second, we grouped RF− polyarticular, extended oligoarticular and persistent oligoarticular arthritis into one RF− polygo category distinct from RF+ polyarticular arthritis and sJIA. Third, we used a grouping system that combined polyarticular with extended oligoarticular, resulting in three categories: persistent oligoarticular, combined polyarticular/extended oligoarticular and systemic. Finally, we used a grouping system that first distinguished systemic patients, and then secondarily divided non-systemic patients into antinuclear antibody (ANA)-positive and ANA-negative subsets (using a cut-off titre of 160), resulting in three categories: ANA+ JIA, ANA− JIA and sJIA. For the patient presenting with sJIA with ANA+, this patient was clustered within the sJIA group. Patients were classified as having active versus inactive disease according to the Wallace criteria.[14] Patients with enthesitis-related JIA were assessed using a modification of the Wallace criteria, where patients also needed no active enthesitis and no active axial disease in order for the disease to be considered inactive. Both enthesitis and axial spine involvement were ascertained through a thorough history and clinical examination by the paediatric rheumatology specialist, but no patients were found to have active enthesitis or axial spine involvement at the time of sampling. Patients with JIA were grouped according to their treatment into either untreated patients with JIA (Med0, no medication or non-steroidal anti-inflammatory drugs), steroid-treated patients with JIA (Med1, oral steroids or methotrexate or leflunomide, or a combination) or biologic-treated patients with JIA (Med2, abatacept, adalimumab, canakinumab, etanercept, tocilizumab, with or without additional steroid, leflunomide or methotrexate treatment). Disease controls consisted of two distinct populations: first, a group of 5 patients diagnosed with juvenile-onset systemic lupus erythematosus (SLE); and second, a group of 11 patients with non-arthritis systemic inflammatory diseases (SID). The latter group comprised three patients with undefined systemic autoinflammatory disorders, three patients with periodic fever, aphthous stomatitis, pharyngitis and adenitis, one patient with chronic neurological cutaneous and articular syndrome, one patient with pericarditis and systemic autoinflammation, one patient with deficiency of adenosine deaminase 2, one patient with congenital tufting enteropathy due to a mutation in *EPCAM*, and one patient with Takayasu arteritis.

The distribution of age at time of sampling in cases and controls is shown in online supplementary figure 1. For patients with sJIA, some patients fell outside the normal age range established for healthy controls; however, these young adult patients with sJIA did not differ in immune phenotype from paediatric patients with sJIA. Blood samples from all participants were collected in heparin tubes and rested at 22°C for 4 hours before separation of serum and peripheral blood mononuclear cells (PBMCs) using a lymphocyte separation medium (LSM, MP Biomedicals). PBMCs were frozen in 10% dimethyl sulfoxide (Sigma) and stored at −80°C for a maximum of 10 weeks.

Immune phenotyping

For flow cytometry, thawed cells were stained with antibodies to allow deep immunophenotyping using a panel previously published[15] by our group (online supplementary table 1). Ki67 and FOXP3 staining were performed after treatment with fixation-permeabilisation buffer (eBioscience). Cytokine staining was performed after ex vivo stimulation for 5 hours in 50 ng/mL phorbol 12-myristate 13-acetate (Sigma) and 500 ng/mL ionomycin (Sigma) in the presence of GolgiStop (BD Biosciences). Stimulated cells were surface-stained, fixed and permeabilised with Cytofix/Cytoperm (BD Biosciences), before staining for cytokines. Data were acquired on a BD FACSCanto II and analysed with FlowJo (Tree Star). Additional immune phenotypes were assessed via Meso Scale Discovery using the V-PLEX Cytokine Panel 1 Human Kit. ANA results were collected by reviewing medical records. Patient serum samples attained during routine clinical visits were used to determine ANA titres by the clinical immunology laboratory of the university hospital in an indirect immunofluorescence assay on Human epithelial type 2 cells.

Statistical analysis

Data on 42 immunological parameters (online supplementary table 2), with a focus on cellular subsets within the adaptive immune system, were generated for 43 healthy age-matched controls, 16 disease controls and 85 patients with JIA (online supplementary datasets 1 and 2). Data (phenotypic, flow cytometric and serological) were collated and stored in Microsoft Excel. All data analyses were performed using the VEGAN package[16] in R V.3.1.2 (http://www.r-project.org).[17] The flow cytometry data were expressed as percentages as exported from FlowJo V.7.6.5. Statistical comparison was based on Kruskal-Wallis one-way analysis of variance (ANOVA),[18] followed by Dunn post-hoc test[19] implemented in R, and p values were adjusted with the false discovery rate method[20] (online supplementary codes 1 and 2). Multiparameter analysis was performed using non-metric multidimensional scaling (MDS), with the

vegdist and metaMDS functions in R. Differences on MDS plots were assessed for the MDS1 and MDS2 values across the different individual groups using Kruskal-Wallis one-way ANOVA, followed by Dunn post-hoc test, adjusted with the false discovery rate method.

Machine learning

In order to investigate the ability of machine learning to classify patient groups, the following model selection procedure was performed: the cohort was randomly split into 10 almost equally large groups, so-called folds. Using these groups/folds, tenfold cross-validation was performed in order to assess each method's ability to generalise to previously unseen cases. In every test, hyperparameter selection was performed on a training set consisting of nine folds. Then the best hyperparameters were used to train a model on the nine training folds, and the performance of this model was evaluated using the withheld test fold, which was neither used for training nor for the selection of hyperparameters. This procedure was performed for all 10 folds. Data set training was run using random forests, artificial neural networks and support vector machines, with the capacity of each to explain the data assessed on the basis of superior area under the receiver operating characteristics (ROC) curve.

Random forests were run using hyperparameter selection with out-of-bag estimates on the respective training sets.[21 22] We considered 5 and 11 features per split. The following were the sampling schemes trialled: (1) Optimising for accuracy, we considered the default sampling scheme which took the total number of samples, but the sampling was done with replacement. Moreover, sampling was performed independently of the samples' class memberships. (2) Optimising for balanced accuracy, the balanced random forest sampling scheme[23] was applied: each tree is based on as many positive samples as there are in the training set and exactly as many negative samples. (3) Use of choice of sampling scheme as a second hyperparameter, to select for superior area under the ROC curve: in addition to the schemes above, two more sampling schemes were added, where each tree is based on as many negative samples (smaller class) as there are in the training set and 1.5 and 2 times as many positive samples (larger class). Regardless of the goal criterion, we always constructed ensembles of 10 001 trees. Model selection was performed using R V.3.3.0[24] with the 'randomForest' package.[25]

Artificial neural networks

Hyperparameter selection was performed using ninefold cross-validation on the respective training sets. The following hyperparameters were considered, with 672 combinations:

- Network architecture: we considered networks with one hidden layer consisting of 25, 50, 100 and 200 nodes, as well as networks with two hidden layers with 25, 50 and 100 nodes each (with 7 different architectures in total).
- Number of training epochs: 150 and 300.
- Learning rates: 0.005 and 0.01.
- Momentum: 0.5 and 0.9.
- Dropout: dropout both for input nodes and hidden activations at rates of 0.2 and 0.5.[26]
- Class weights: the larger class was always weighted with 1 in the objective function, while we tried class weights of 1, 1.5 and 2 for the smaller class in order to allow the network to better deal with the unbalanced distribution of classes.
- Weight decay (also known as L2 regularisation): with and without weight decay with a regularisation parameter of 0.001.

For all artificial neural networks we trained, we used rectified linear unit (ReLU) activations[27] for the hidden nodes and a sigmoid activation for the output node along with binary cross-entropy as optimisation objective. Training was performed by stochastic gradient descent using a mini-batch size of 32 samples. All computational experiments were implemented in Python V.3 using the Keras framework[28] as a simple interface to the TensorFlow framework.[29]

Potential support vector machine was employed due to superior performance with unbalanced data.[30] The potential support vector machine model selection was performed twice, once without balancing (to optimise for accuracy) and once with balancing (to optimise for balanced accuracy). Hyperparameter selection was performed using tenfold cross-validation on the respective training sets using all combinations of cost factors $C \in \{8,10,12,\ldots,20\}$ and shrinkage parameters $\varepsilon \in \{0.5,0.7,0.9,\ldots,2.3\}$. In all experiments, potential support vector machine was used in dyadic mode, that is, the model's discriminant function is a linear combination of a subset of features.[30]

RESULTS

An immunological pattern of JIA

JIA is a class of heterogeneous diseases. To identify the potential immunological processes driving JIA subsets, we recruited 85 patients with JIA and 43 age-matched controls. Patients with JIA and healthy controls were assessed for 42 immunological parameters by flow cytometry, using the immune phenotyping platform previously validated.[15] This platform gives a strong representation of key subsets of the adaptive immune system (online supplementary table 2). To determine whether an immune signature could be identified based on disease characteristics, we classified patients with JIA into different subsets. Our primary subtype analysis classified patients with JIA along the ILAR guidelines into oligoarticular persistent JIA (n=15), oligoarticular extended JIA (n=13), RF− polyarticular JIA (n=33), RF+ polyarticular JIA (n=2), enthesitis-related JIA (n=3) and sJIA (n=19). The characteristics of the patient cohort are given in table 1. A first alternative analysis was run with RF− polyarticular JIA, oligoarticular extended JIA and oligoarticular persistent JIA combined and termed 'RF- polygo' under the assumption that RF+ polyarticular JIA, sJIA and enthesitis-related JIA may represent distinct entities, whereas patients in the RF− polygo JIA group may share pathophysiology. This amounted to 61 patients with RF− polygo, 2 patients with RF+ polyarticular, 19 patients with systemic and 3 patients with enthesitis-related JIA (online supplementary table 3). Considering the altered immune profile of patients with oligoarticular JIA may only be observed locally in the inflamed joints, we used a second alternative combined grouping strategy according to the number of joints affected, which divided the patients as persistent oligoarticular (n=15), combined polyarticular and extended oligoarticular (n=48), and sJIA (n=19) (online supplementary table 4).[31] Due to the small number of patients in some ILAR classifications, the reports that ANA positivity distinguishes a relatively homogeneous group irrespective of the number of affected joints (early age at onset, asymmetric arthritis, female predominance, increased incidence of chronic iridocyclitis) and the resultant heightened clinical surveillance in ANA-positive patients,[5 32 33] we finally used a third alternative grouping system, an ANA-based grouping. This

Table 1 Characteristics of patients with JIA classified by the ILAR criteria

	Healthy controls	Oligoarticular persistent	Oligoarticular extended	RF– polyarticular	RF+ polyarticular	Enthesitis-related	Systemic JIA
Total (n)	43	15	13	33	2	3	19
Male, n (%)	16 (37.2)	2 (13.3)	1 (7.7)	8 (24.2)	1 (50)	1 (33.3)	7 (36.8)
Age at time of sampling (years)							
Median (IQR)	9 (4.75–12)	9 (4.5–10.5)	8 (6–11)	8 (6–10)	13 (11.5–14.5)	14 (13–14.5)	12 (6.5–20.5)
Range	2–17	2–16	2–13	2–18	10–16	12–15	0.75–34
Years of disease duration							
Median (IQR)	–	3.45 (1.7–6.9)	3.4 (2.3–7)	2.2 (0.9–5.15)	3.4 (2.1–4.7)	4.4 (2.85–5.95)	6.4 (2–12.05)
Range	–	0.2–13.2	0.1–10.7	0.3–8.3	0.8–6.0	1.3–7.5	0–21.1
Disease properties							
Patients with ANA, n (%)	–	10 (66.7)	12 (92.3)	20 (60.6)	1 (50)	0	1* (5.3)
Patients with sJIA with MAS, n (%)	–	–	–	–	–	–	5 (26.3)
Patients with active disease, n (%)	–	8 (53.3)	10 (76.9)	13 (39.4)	2 (100)	1 (33.3)	8 (42.1)
JIA medication							
No medication or non-steroidal anti-inflammatory drugs, n (%)	–	7 (46.7)	3 (23.1)	9 (27.3)	0	2 (66.7)	1 (5.3)
Methotrexate or leflunomide without biologics, n (%)	–	6 (40)	8 (61.5)	22 (66.7)	1 (50)	0	5 (26.3)
Biologics (Aba, Ada, Can, ETN, Toc,±leflunomide or methotrexate), n (%)	–	2 (13.3)	2 (15.4)	2 (6.1)	1 (50)	1 (33.3)	13 (68.4)
Aba, n (%)	–	0	1 (7.7)	0	0	0	0
Ada and/or ETN, n (%)	–	2 (13.3)	1 (7.7)	2 (6.1)	1 (50)	1 (33.3)	2 (10.5)
Can, n (%)	–	0	0	0	0	0	3 (15.8)
Toc, n (%)	–	0	0	0	0	0	8 (42.1)
Dose of steroids (Medrol) (mg/kg)							
Median (IQR)	–	0 (0–0)	0 (0–0)	0 (0–0.03)	0	0	0.05 (0–0.3)
Range	–	0–0.25	0–0.3	0–0.3	0	0	0–1.4

*4/19 patients transiently developed ANA following Epstein-Barr Virus infection or ETN treatment.
ANA, antinuclear antibody; Aba, abatacept; Ada, adalimumab; Can, canakinumab; ETN, etanercept;ILAR, International League of Associations for Rheumatology; JIA, juvenile idiopathic arthritis; MAS, Macrophage-activation syndrome
; RF, rheumatoid factor; Toc, tocilizumab; sJIA, systemic JIA.

led to a final categorisation of 22 patients with oligoarticular/polyarticular ANA-negative (ANA−), 43 patients with oligoarticular/polyarticular ANA+, and 19 patients with sJIA (online supplementary table 5). As alternative grouping systems exist for JIA, we fully annotated the data set, allowing independent analysis by alternative criteria (online supplementary datasets 1 and 2). MDS analysis of patients with JIA according to the ILAR classification showed complete overlapping of most JIA subsets, with significant separation only of the sJIA group (figure 1A). Likewise, alternative groupings 1, 2 and 3 (online supplementary figure 2A–C) also showed almost complete overlap, with only the sJIA cluster consistently separating. In each case this systemic cluster showed enhanced separation from the healthy controls along the JIA disease axis, consistent with sJIA manifesting as a more extreme polarisation of the immune signature found in patients with non-sJIA. As the primary separation was between patients with JIA and healthy individuals, we reclustered JIA subsets in the absence of healthy individuals (figure 1B, online supplementary Figure 2D–F). Here, while there was a trend for sJIA to separate from the other subtypes (online supplementary dataset 3), few significant differences were observed, demonstrating the overall immune deviation signature of JIA is independent of JIA clinical subtype. Together these results suggest that patients with JIA, while clinically distinct, share common immunological perturbations.

While JIA is a heterogeneous group of conditions, with sJIA in particular shown to be genetically distinct, all JIA subtypes are unified by the presence of chronic childhood arthritis without an identifiable cause.[34] Also, almost half of children with sJIA develop long-standing and often destructive arthritis that appears similar to other forms of JIA. Since our JIA clinical subset analysis demonstrated patients with JIA share pathophysiology distinct from the healthy cohort, we performed a secondary analysis comparing patients with JIA as a whole with disease controls to evaluate specificity of the observed immune pattern. As previously observed,[15] immunological variance within the healthy control population was structured, with strong correlations between variance in different immunological parameters (figure 2A). While much of this interrelatedness was preserved in patients with JIA, for several adaptive immune subsets, such as invariant natural killer T (iNKT) cells, multiple disjunctions were observed (figure 2A). For example, the homeostatic weak relationship between iNKT cells and plasmablasts, observed in healthy controls, was inverted and strengthened in

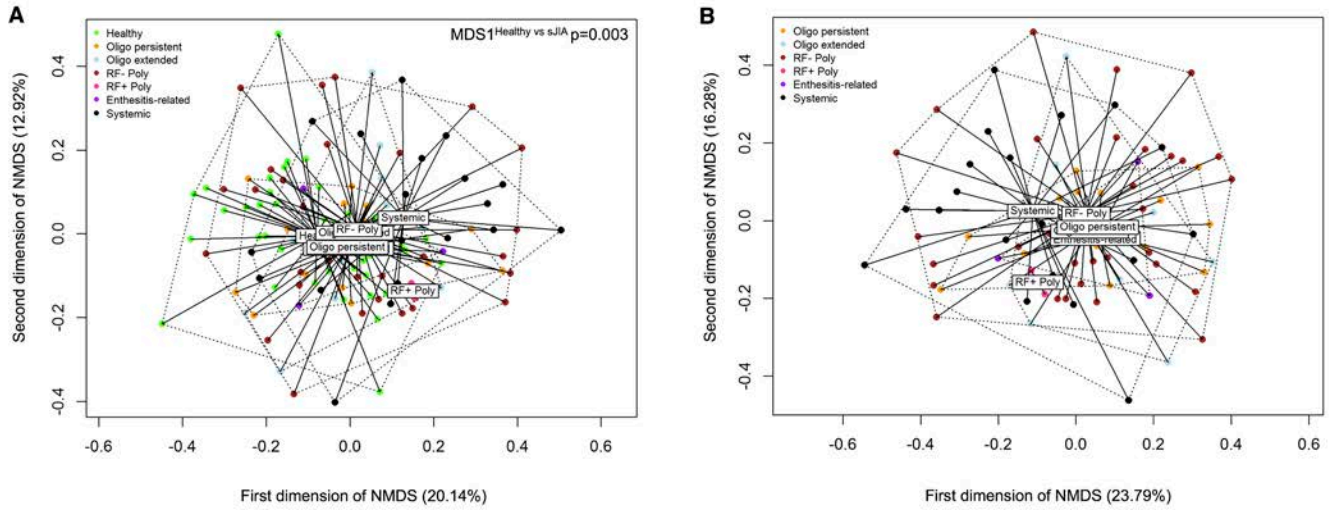

Figure 1 JIA subsets share a common immunological disturbance. Healthy controls (n=43) and patients with JIA (n=85) were assessed for immune phenotype. All individuals were plotted with multidimensional scaling over 42 immunological variables. (A) Healthy individuals clustered with patients with JIA, classified on the ILAR criteria. (B) Patients with JIA, classified on the ILAR criteria, clustered alone. Individuals were plotted with multidimensional scaling showing Bray-Curtis dissimilarity indices over 42 immunological variables. Variation explained by each axis is indicated in parentheses. Differences for MDS1 and MDS2 using Kruskal-Wallis one-way analysis of variance are shown when p<0.05. ILAR, International League of Associations for Rheumatology; JIA, juvenile idiopathic arthritis; MDS, multidimensional scaling; NMDS, non-metric multidimensional scaling; sJIA, systemic JIA; RF–, rheumatoid factor-negative; RF+, rheumatoid factor-positive.

JIA (figure 2A). Taking into account the variance in all assessed parameters, the immunological composition of patients with JIA diverged from healthy controls, with overlapping but distinct separation of the two clusters on an MDS analysis, with significant separation of healthy and JIA on MDS1 (figure 2B). To determine whether this immune signature was a generic inflammatory disease signature, or a JIA-specific signature, we recruited 16 disease controls, juvenile patients with inflammatory disease but no arthritis, including 5 patients with juvenile-onset SLE and 11 patients with juvenile-onset SID. MDS analysis of disease controls showed a little separation from the JIA clusters on an MDS plot (figure 2B). As invariant traits compress MDS clusters, we reclustered individuals with a core immune set of 21 traits, which individually show statistical differences among the three disease groups (figure 2C). In the core trait analysis, the JIA cluster showed separation from the disease control cluster, and both were in turn separated by a significant distance from the healthy cluster, although still with strong overlap among all three groups. These results demonstrate that patients with juvenile inflammatory diseases present with alterations in their immune profile, most of which appear to be driven by a core inflammatory signature shared with other paediatric inflammatory patients.

Patients with JIA present with a disturbed adaptive immune system

To unravel the individual drivers of the global immune profile shifts observed in both sJIA and non-sJIA patients, we assessed each parameter individually. Using a stringent statistical analysis, and taking into account multiple testing, significant changes were observed in 21 distinct immunological parameters in patients with JIA (figure 2D–X, online supplementary table 6). Since patients with sJIA demonstrated the most enhanced separation from healthy controls in the MDS analysis, we show these 21 significant traits for each individual JIA subset according to the ILAR classification in figure 3. Compared with healthy controls, the disease control patients registered 13 significant changes in individual immunological parameters (figure 2D–X), notably with all of these changes overlapping with the JIA-changed parameters. While there was a large overlap in the changed immunological parameters between JIA and disease controls, the degree of change shifted, with some inflammation-associated changes much stronger in JIA than non-JIA patients, and vice versa. Thus we can consider a shared set of immune phenotypes which are sensitive to inflammatory disease in children (a universal inflammatory signature), while the exact constellation of changes can be disease-specific, driving the separation on an MDS plot (figure 2C).

Among the significant changes observed in the immunophenotype of circulating cells in patients with JIA, several were suggestive of important biological changes. First, patients with JIA displayed increased numbers of CD4 T cells (figures 2E and 3B), with particular increases in interleukin (IL)-17 and IL-2 secreting CD4 T cells (figures 2G,H and 3D,E). The increase of Th17 cells, in conjunction with prior evidence,[8–10] indicates that activation of CD4 T cells contributes to the inflammatory manifestations. Changes in the CD8 compartment, by contrast, were more consistent with a decreased, rather than enhanced, CD8 effector response. CD8 T cells were raised in patients with JIA (figures 2I and 3F); however, there was a significant increase in naive and recent thymic emigrant CD8 T cells (figures 2J,K and 3G,H). Notably, these latter increases were not observed in the disease control cohort. Within antigen-experienced CD8 T cells, there was an increase in central memory (figures 2L and 3I) and IL-2-producing CD8 T cells (figures 2O and 3L) at the expense of IFNγ-producing (Tc1) or effector memory CD8 T cells (figures 2M,N and 3J,K). The decrease in IFNγ-producing CD8 T cells is of particular note, with the standard model of progression of sJIA into macrophage activation syndrome driven by excessive production of IFNγ by effector memory CD8 T

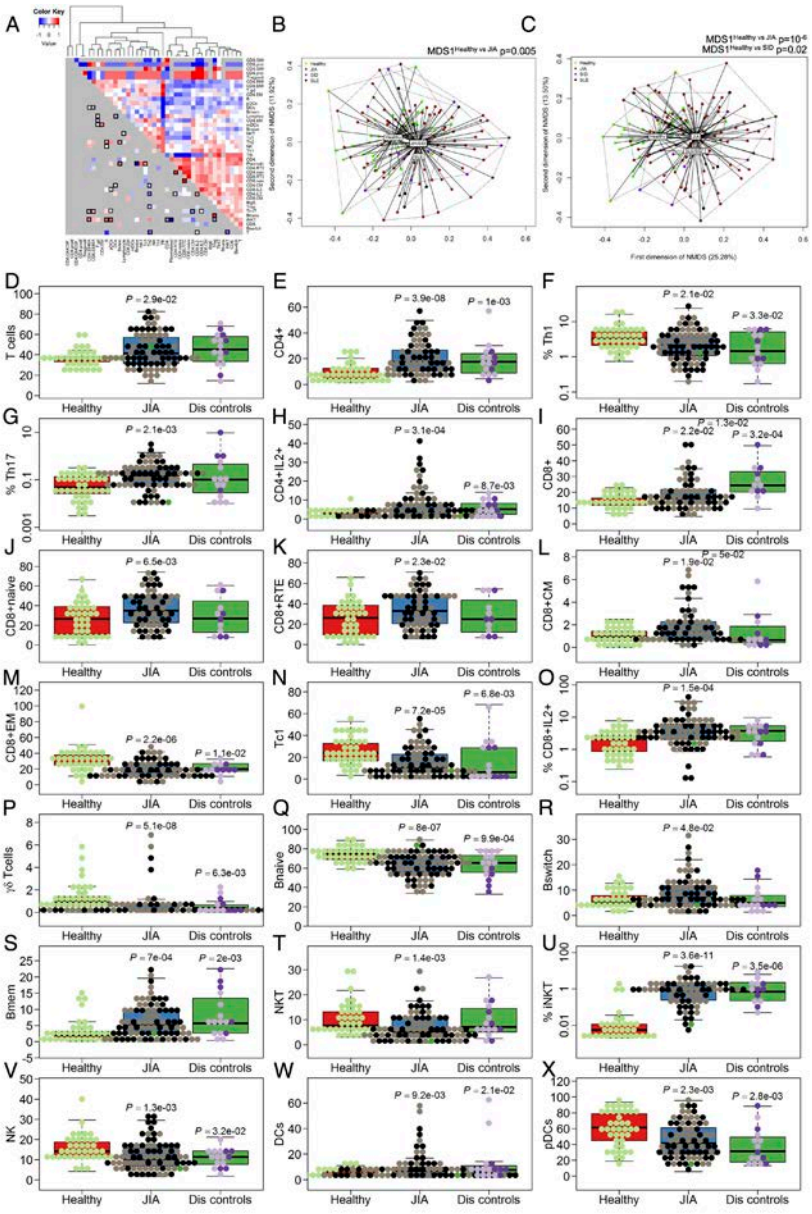

Figure 2 JIA is marked by global immunological shifts and alteration in the relationship between leucocyte subsets; both shared and distinct alterations from paediatric systemic inflammatory disease. Healthy controls, patients with JIA, patients with SLE and patients with SID were assessed for immune phenotype (n=43, 86, 5, 11). JIA active disease status is indicated by the black dots, while quiescent disease is shown by empty dots. Within patients with JIA, no disease status was available for one individual, indicated by green. Within the disease controls, patients with SLE are indicated by dark purple and patients with SID are indicated by light purple. Box plots for each significant immune parameter are shown; non-significant parameters are not shown. (A) Upper right, above the diagonal: coregulation between pairs of cell types in healthy controls (n=43) (red: positive correlation coefficient; blue: negative correlation coefficient; light grey: no data available). Unbiased clustering of coefficients was performed to group coregulated cell types. Lower left, below the diagonal: dark grey indicates coregulation between pairs of cell types in patients with JIA (n=85) that are preserved from healthy controls. Coregulation between pairs of cell types that are significantly altered by disease ($p<0.05$, and boxed if $p<0.01$) is coloured (red: positive correlation coefficient; blue: negative correlation coefficient). (B) All individuals were plotted with multidimensional scaling showing Bray-Curtis dissimilarity indices over 42 immunological variables. Variation explained by each axis is indicated in parentheses. (C) All individuals were plotted with multidimensional scaling showing Bray-Curtis dissimilarity indices over 21 immunological variables, selected for significant variance between groups. Variation explained by each axis is indicated in parentheses. Differences for MDS1 and MDS2 using Kruskal-Wallis one-way ANOVA are shown when $p<0.05$. (D) T cells, (E) CD4+, (F) Th1, (G) Th17, (H) CD4+IL2+, (I) CD8+, (J) CD8+naive, (K) CD8+RTE, (L) CD8+CM, (M) CD8+EM, (N) Tc1, (O) CD8+IL2+, (P) γδ T cells, (Q) Bnaive, (R) Bswitch, (S) Bmem, (T) NKT, (U) iNKT, (V) NK, (W) DCs and (X) pDCs. Boxes and centre lines represent IQR and median, respectively; whiskers, 1.5× IQR. Statistical comparison for D–X was based on Kruskal-Wallis one-way ANOVA, followed by Dunn post-hoc test, adjusted with the false discovery rate method. P values above patient groups indicate significant difference as compared with healthy controls, while p values between patient groups indicate significant differences between JIA and disease controls. ANOVA, analysis of variance; CM, central memory; DCs, dendritis cells; dis controls, disease controls; EM, effector memory; iNKT, invariant natural killer T cells; IL, interleukin; JIA, juvenile idiopathic arthritis; MDS, multidimensional scaling; NK; natural killer cells; NKT, NK T cells; NMDS, non-metric multidimensional scaling; pDCs, plasmacytoid DCs; RTE, recent thymic emigrants; SID, systemic inflammatory diseases; SLE, systemic lupus erythematosus; Tc1, type 1 cytolytic T cells; Th, T helper.

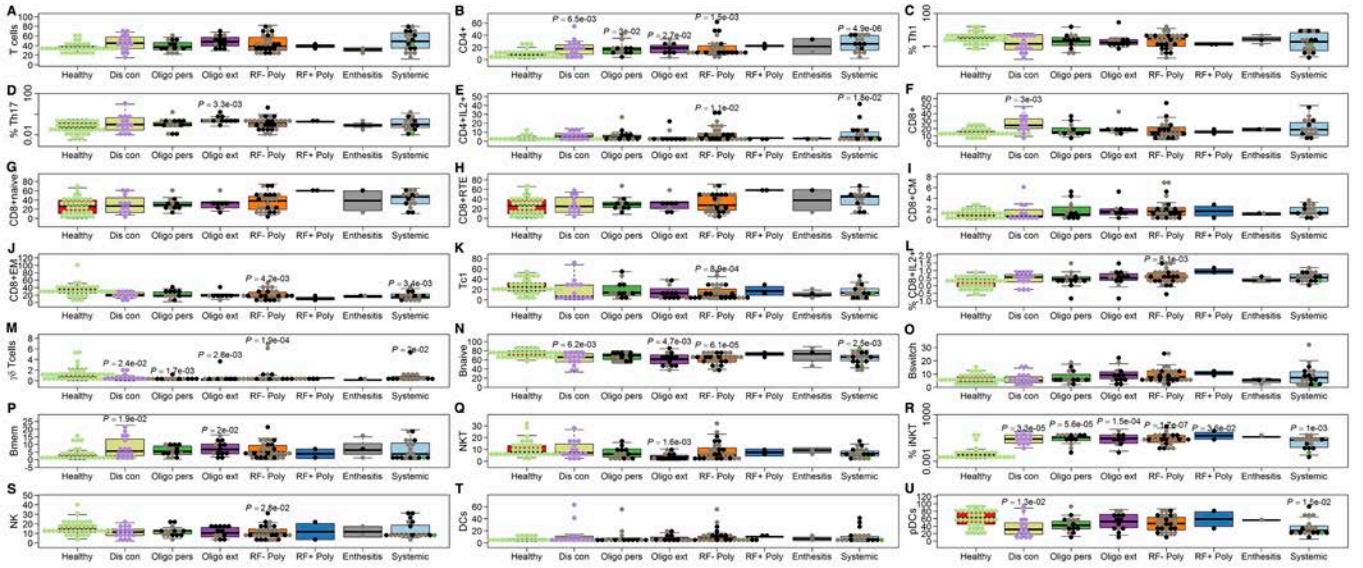

Figure 3 Shared immunological profiles across JIA ILAR subtypes. Healthy controls (n=43), patients with JIA (n=86) and disease controls[16] were assessed for immune phenotype. Patients with JIA were classified as oligoarticular persistent (oligo pers, n=15), oligoarticular extended (oligo ext, n=14), polyarticular RF− (poly RF−, n=33), polyarticular RF+ (poly RF+, n=2), enthesitis-related (enthesitis, n=3) and systemic JIA (sJIA, n=19). JIA active disease status is indicated by the black dots, while quiescent disease is shown by empty dots. Box plots for each significant immune parameter are shown, while non-significant parameters are not shown. (A) T cells, (B) CD4+, (C) Th1, (D) Th17, (E) CD4+IL2+, (F) CD8+, (G) CD8+naive, (H) CD8+RTE, (I) CD8+CM, (J) CD8+EM, (K) Tc1, (L) CD8+IL2+, (M) γδ T cells, (N) Bnaive, (O) Bswitch, (P) Bmem, (Q) NKT, (R) iNKT, (S) NK, (T) DCs and (U) pDCs. Boxes and centre lines represent IQR and median, respectively; whiskers, 1.5× IQR. Statistical comparison was based on Kruskal-Wallis one-way analysis of variance, followed by Dunn post-hoc test, adjusted with the false discovery rate method. P values above patient groups indicate significant difference as compared with healthy controls, while p values between patient groups indicate significant differences between JIA and disease controls. CM, central memory; DCs, dendritic cells; Dis con, disease controls; EM, effector memory; iNKT, invariant natural killer T cells; IL, interleukin; ILAR, International League of Associations for Rheumatology; JIA, juvenile idiopathic arthritis; NK, natural killer; NKT, NK T cells; pDCs, plasmacytoid DCs; RF−, rheumatoid factor-negative; RF+, rheumatoid factor-positive; RTE, recent thymic emigrants; Tc1, type 1 cytolytic T cells; Th, T helper .

cells.[35] Thus, while IFNγ has been proposed as a key proinflammatory cytokine in JIA, our data in the peripheral blood are more consistent with impeded production of IFNγ. Altered activation of T cells in patients with JIA was accompanied by activation of B cells, with a decrease in naive B cells (figures 2Q and 3N), coupled with an increase in switched B cells (figures 2R and 3O) and memory B cells (figure 2S and 3P). Beyond these major adaptive cell types, patients with JIA displayed a relative decrease in γδ T cells, natural killer (NK) cells and plasmacytoid dendritic cells, while iNKT cells were increased (figures 2–3). Together, these results suggest a pathophysiological process in the circulating lymphocytes involving the suppression of IFNγ production by CD8 T cells and excessive CD4 T cell differentiation into the Th17 lineage.

When alternative JIA subset groupings were considered, the changes observed at the individual parameter level reflected the analysis at the global level: the immunological changes occurring in JIA versus healthy children were highly similar among JIA subtypes. In general, immunological changes in each JIA subset mirrored the others in trend, if not statistical significance, regardless of whether the RF− polygo grouping (online supplementary figure 3), grouping based on number of joints affected (online supplementary figure 4) or ANA-based grouping (online supplementary figure 5) was used. Together, these results indicate that, despite the different clinical manifestations, JIA subtypes share a distinct immunophenotype, with only relatively minor immunological changes correlating with the particular JIA clinical subtype. An exception was found with sJIA, which showed a more profound increase in CD4+ T cells and in naive CD8 T cells than the other JIA subsets (online supplementary figures 2–4). While at a global level patients with sJIA presented with an accentuated version of the non-systemic patients (figure 1), several individual parameters broke this general trend, in particular the B cell changes (figure 3, online supplementary figures 2–4). Thus, while sJIA may share a common immune signature with non-sJIA and non-arthritic systemic inflammatory patients, there are potentially unique immunological changes, together with the unique clinical presentation.

Potential confounding factors in the immunological analysis of patients with JIA include disease activity and immunological changes secondary to treatment. As JIA subtypes manifested a mostly similar immune phenotype, or at least phenotypic changes along the same spectrum, we merged JIA subtypes in an attempt to control for disease activity. Separation of patients with JIA into patients who had either active disease or were quiescent revealed a more extreme immune signature in patients with JIA who were sampled at a time point of active disease, although even patients with JIA sampled during disease inactivity showed separation from the healthy controls (figure 4A). The immunological relationships observed in patients with inactive JIA were largely replicated in patients with active JIA (figure 4B); however, patients with active JIA did show altered interrelatedness for several immune parameters. For example, in patients with inactive JIA, no relationship was observed between CD8 T cells and memory B cells; however, a strong positive relationship emerged in patients with active JIA (figure 4B). At the individual parameter level, of the 21 immune parameters that showed a significant difference between JIA and healthy, 20 showed no change between patients sampled at active and inactive disease stages (online supplementary figure 6). Only one parameter, NK

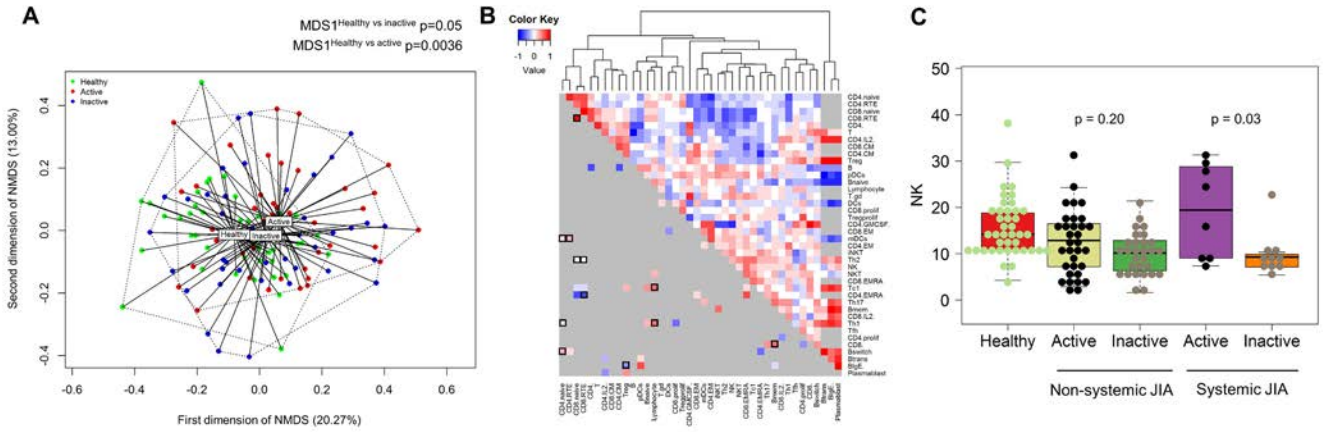

Figure 4 Immunological architecture of JIA is maintained into disease remission. Healthy controls and patients with JIA were assessed for immune phenotype. (A) Healthy controls (n=43), patients with JIA with quiescent disease at sampling (inactive; n=43) and patients with JIA with active disease (JIA active; n=42) plotted with multidimensional scaling over 42 immunological variables. All individuals were plotted with multidimensional scaling showing Bray-Curtis dissimilarity indices over 42 immunological variables. Variation explained by each axis is indicated in parentheses. Differences for MDS1 and MDS2 using Kruskal-Wallis one-way ANOVA are shown when p<0.05. (B) Upper right, above the diagonal: coregulation between pairs of cell types in patients with JIA with inactive disease (n=42) (red: positive correlation coefficient; blue: negative correlation coefficient; light grey: no data available). Unbiased clustering of coefficients was performed to group coregulated cell types. Lower left, below the diagonal: dark grey indicates coregulation between pairs of cell types in patients with JIA with active disease (n=42 that is preserved from patients with inactive JIA). Coregulation between pairs of cell types that are significantly altered by disease activity (p<0.05, and boxed if p<0.01) are coloured (red: positive correlation coefficient; blue: negative correlation coefficient). (C) Box plots NK cells shown. Boxes and centre lines represent IQR and median, respectively; whiskers, 1.5× IQR. Statistical comparison was based on Kruskal-Wallis one-way ANOVA, followed by Dunn post-hoc test, adjusted with the false discovery rate method. ANOVA, analysis of variance; JIA, juvenile idiopathic arthritis; MDS, multidimensional scaling; NK, natural killer cells; NMDS, non-metric multidimensional scaling.

cell percentage, showed a significant difference based on disease state, with the drop in NK frequency only observed in patients with inactive disease (p=0.01). This effect, however, was only significant in the sJIA group (figure 4C). Our findings suggest that disease activity correlates with quantitative rather than qualitative changes in the signature immune dysregulation, with the underlying JIA immune dysfunction remaining present in patients with disease control.

To assess the contribution of medication to the immune phenotype, we grouped patients with JIA into those who were without medication (or on non-steroidal anti-inflammatory drugs only), those who were being treated with methotrexate, leflunomide or oral steroids, and those who were on biologic therapies (including abatacept, tumour necrosis factor blockade, anti-IL-1 and anti-IL-6, with or without additional non-biologic therapy). Using a global analysis, significant separation was observed between healthy controls and patients on either steroidal or biological treatment, while untreated patients showed no significant separation from healthy individuals (figure 5A). This effect was reflected at the individual parameter level, where more pronounced changes were observed in patients undergoing steroidal or biologic treatment (figure 5B). The same result was observed when treatment was considered for each JIA subtype, although with reduced statistical power (online supplementary figure 7). While this effect paralleled that of disease activity, the effects were independent: no segregation of disease active/inactive patients was observed within the treatment categories (figure 5B). The effect could be partially explained, however, by the discrete clustering of patients with sJIA, which, regardless of disease activity, showed a more extreme immune signature and which were heavily enriched for patients on steroidal or biologic treatment. Thus, when only non-sJIA patients were classified by treatment, only patients on steroidal treatment separated from healthy individuals (figure 5C). It remains to be determined whether these treatment effects are driven by medication, or whether the effect reflects underlying diversity within the patient cohort, with strategic therapeutic choice correlating with immune phenotype.

Immune-aided machine learning can discriminate between patients with JIA and healthy children

A key application of immunophenotyping in JIA would be to drive assay-based personalised therapeutic choice of treatment strategy. While such an outcome requires a multiyear longitudinal immunophenotyping data set, we sought to determine in the current data set whether machine learning could identify the primary immunological features of JIA, as a basis for future discovery. Three approaches were trialled: random forests, artificial neural networks and support vector machines. Each of these approaches uses machine learning methods, whereby the algorithm trains on the data set in an unbiased manner; each has different strengths and weaknesses depending on the data set to be analysed, as discussed in ref [36]. Comparing the strength of the models built by the three approaches, based on different measures of ability to segregate the data, found that the random forest approach gave a superior performance in separating healthy individuals from patients with JIA using this data set (online supplementary table 7). A random forest is an algorithm based on an ensemble of deep decision trees, where each tree is constructed using a random subset of samples. The trees are constructed such that every split only considers a random subset of features (ie, immunological parameters) to avoid the selection of only closely correlated features and allow the consideration of alternative features.[21 22] The data were split into 10 random folds (subsets of patients), with hyperparameter training and selection on 9 folds and testing on the 10th, with rotation of the testing fold. This strategy separates the data set into a sample set for building the algorithm and a data set for testing the algorithm; the rotation of the testing data set eliminates algorithms

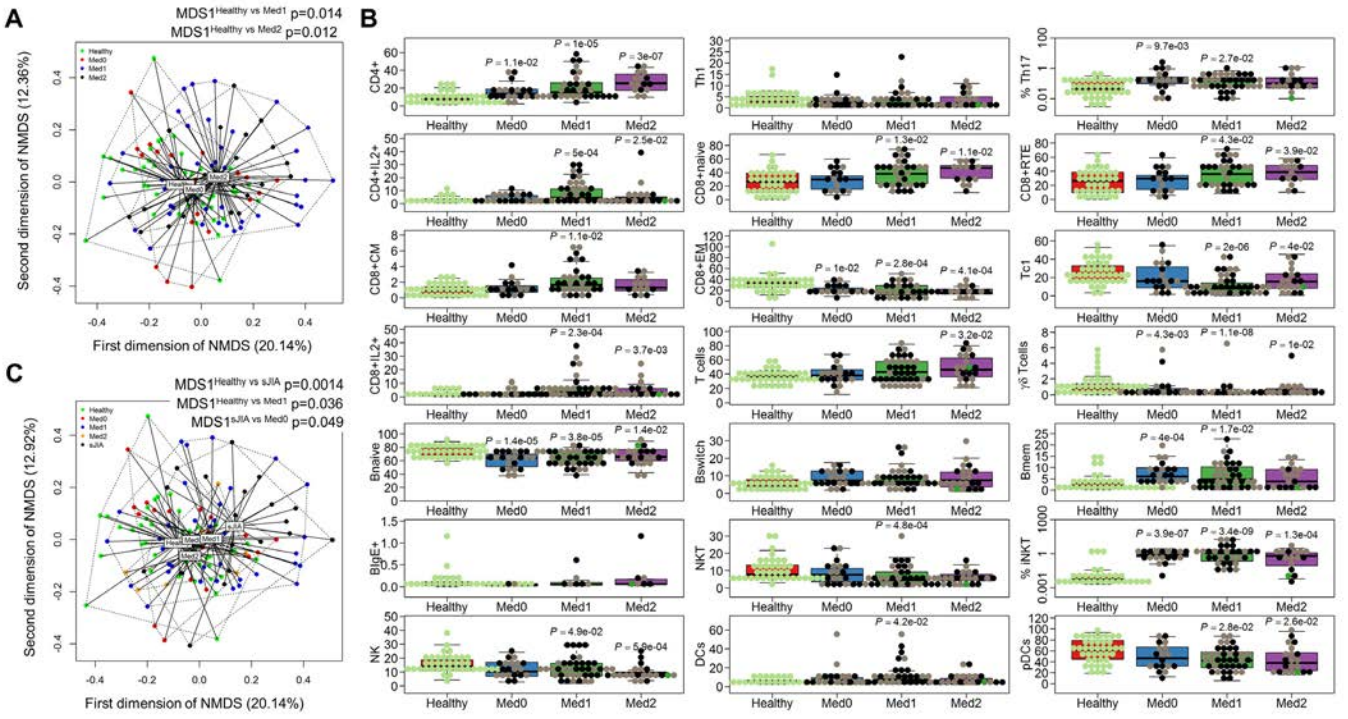

Figure 5 Immunological architecture of JIA is not primarily driven by medication. Healthy controls and patients with JIA were assessed for immune phenotype. (A) Healthy controls (n=43), untreated patients with JIA (Med0, no medication or non-steroidal anti-inflammatory drugs; n=22), steroid-treated patients with JIA (Med1, methotrexate, leflunomide or oral steroids; n=42) or biologic-treated patients with JIA (Med2, canakinumab, tocilizumab, etanercept, adalimumab, abatacept, with or without additional steroid, methotrexate or leflunomide treatment; n=21) were plotted with multidimensional scaling over 42 immunological variables. All individuals were plotted with multidimensional scaling showing Bray-Curtis dissimilarity indices over 42 immunological variables. Variation explained by each axis is indicated in parentheses. (B) Immune phenotypes in which a significant change was observed in one or more JIA subtype were assessed for correlates with treatment status. Active disease status is indicated by the black dots. Boxes and centre lines represent IQR and median, respectively; whiskers, 1.5× IQR. P values show significant difference as compared with the controls. Statistical comparison was based on Kruskal-Wallis one-way ANOVA, followed by Dunn post-hoc test implemented in R, and p values were adjusted with the false discovery rate method. (C) Healthy controls (n=43), untreated patients with non-systemic JIA (Med0, no medication or non-steroidal anti-inflammatory drugs; n=21), steroid-treated patients with non-systemic JIA (Med1, methotrexate, leflunomide or oral steroids; n=37) or biologic-treated patients with non-systemic JIA (Med2, canakinumab, tocilizumab, etanercept, adalimumab, abatacept, with or without additional steroid, methotrexate or leflunomide treatment; n=7) and patients with sJIA (regardless of treatment, n=19) were plotted with multidimensional scaling over 42 immunological variables. All individuals were plotted with multidimensional scaling showing Bray-Curtis dissimilarity indices over 42 immunological variables. Variation explained by each axis is indicated in parentheses. Differences for MDS1 and MDS2 using Kruskal-Wallis one-way ANOVA are shown when p<0.05. ANOVA, analysis of variance; CM, central memory; DCs, dendritic cells; EM, effector memory; iNKT, invariant natural killer T cells; IL, interleukin; JIA, juvenile idiopathic arthritis; NK, natural killer cells; NKT, NK T cells; MDS, multidimensional scaling; NMDS, non-metric multidimensional scaling; pDCs, plasmacytoid DCs; sJIA, systemic JIA; Tc1, type 1 cytolytic T cells; Th, T helper.

built on only a subset of patients. After the construction of 10 001 trees (ie, building 10 001 random forests and identifying the one with best differentiation potential), the optimal random forest selection strategy was capable of discriminating patients with JIA from healthy controls with an area under the curve of 89.6% (figure 6A). The key contributing feature used by the random forest algorithm to discriminate JIA from the healthy was the increased number of iNKT cells (figure 6B). Indeed, the altered frequency of iNKT cells, used as a single parameter, resulted in an area under the curve of 91.2% (figure 6A), equivalent to the random forest in accuracy (p=0.27). Removing iNKT from the data set, a rebuilt optimal random forest selection strategy was capable of discriminating patients with JIA from healthy controls with an area under the curve of 85.5% (figure 6A). This censored machine learning process identified the same contributing parameters as the uncensored analysis (figure 6C). In this regard, the change in iNKT cells is not necessarily functional in JIA, and may instead be a sensitive parameter that responds to other immune parameters that drive the disease. These results demonstrate the utility of machine learning in prioritising identified changes for explanatory power, beyond a priori biological rationale, with potential for use in the design of diagnostic assays.

DISCUSSION

Improved understanding of the immunological architecture of JIA subsets is required for pathophysiology-based diagnosis and treatment. Through extensive immune phenotyping of the adaptive immune system in a large cohort of Belgian children, we found an immunological pattern common to multiple JIA subsets. This signature comprised two components: first, a shared signature of inflammation, common among children with JIA, SLE or diverse inflammatory diseases. Such a shared signature is unlikely to derive from shared pathophysiology, and may instead reflect a signature response of children's immune systems to inflammatory disease. Second, a smaller set of individual immune trait

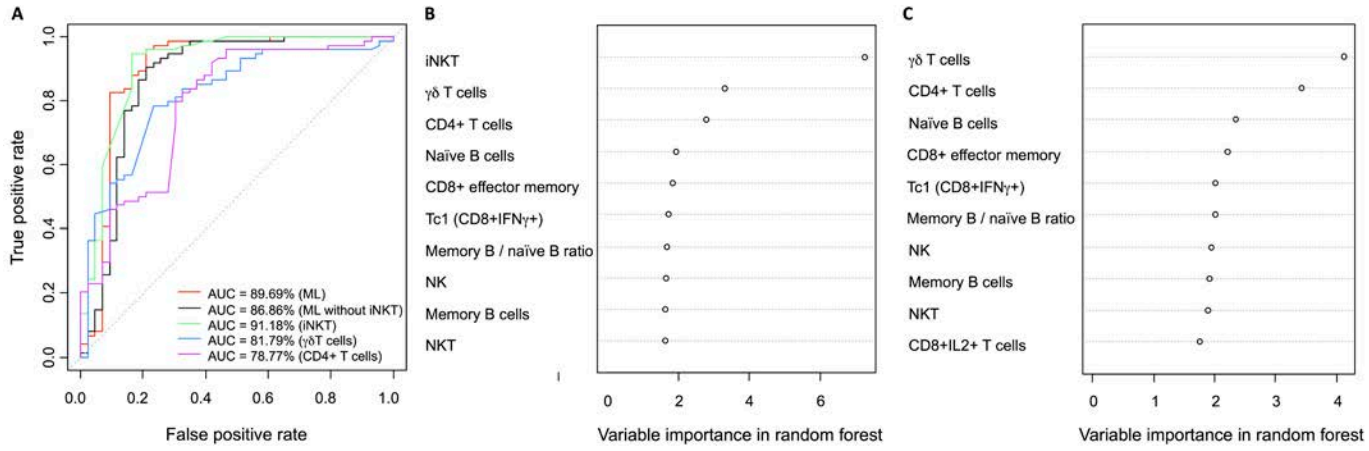

Figure 6 Machine learning identifies iNKT cells as primary predictive immunological change in JIA. Random forests were trained on immunological data from healthy controls (n=43) and patients with JIA (n=72), selecting for capacity to discriminate between the two groups. (A) ROC curves computed from the out-of-bag predictions of a random forests trained on the entire data set (machine learning, 'ML', red curve) and on a data set where iNKT numbers are removed ('ML without iNKT', black curve). For comparison, an ROC curve using only the data of a single parameter is displayed for iNKT, γδ T cells and CD4+ T cells (other curves). (B) The top 10 features in the random forest trained on the entire data set, and contribution to the optimal random forest approach. (C) The top 10 features in the random forest trained on the data set where iNKT numbers are removed, and contribution to the optimal random forest approach. AUC, area under the curve; iNKT, invariant natural killer T cells; IFNγ, interferon gamma; JIA, juvenile idiopathic arthritis; ROC, receiver operating characteristics.

changes were found only in patients with JIA, providing an axis of discrimination between JIA and the non-JIA inflammatory diseases tested here. While all JIA subsets demonstrated overall similar changes, sJIA in particular was a consistent outlier within the JIA diseases with the most pronounced immune deviation. While the distinct clinical presentation of these diseases does not necessitate biologically derived discrimination, such changes may identify the disease-specific pathophysiological processes.

Within the JIA cases here assessed, the immunological signature was broadly shared across disease subtypes, and indeed shared with non-arthritic inflammatory patients. This inflammatory signature was enhanced in two populations: patients with JIA sampled at a time point of active disease and patients with JIA of systemic subtype. sJIA has long been considered an entity distinct from other, more common, JIA subtypes, mediated by abnormalities in the innate immune system with features of an autoinflammatory disease. Indeed, high-depth immunophenotyping of the innate immune system recently identified an sJIA signature response to stimuli.[12] This simple innate versus adaptive division between sJIA and other JIA subtypes is, however, challenged by the genetics, with association of the HLA-DRB*11 class II allele to sJIA susceptibility indicating a strong role for the adaptive immune system.[37] How then to reconcile these data sets? A biphasic clinical course of sJIA was recently proposed where innate immune mechanisms dominate at disease onset, eliciting systemic inflammation through increased levels of IL-6, IL-18, S100A8/A9, S100A12 and IL-1β, followed by a second phase where the adaptive immune system mediates chronic arthritis.[38] Integrating our data with prior studies, the innate drivers at disease onset may be sJIA-specific,[12] while the ongoing adaptive disturbances may reflect shared features with the non-sJIA patients.

The pathophysiological process of JIA disease susceptibility identified through our analysis is one of complex immune network failure, rather than the generation of a single pathology-driving event. A key interaction may be the balance between Th1/Tc1 and Th17 cells. Th17 cells are pathogenic in mouse models, with IL-17- and IL-23 deficiency invoking resistance to arthritis induction.[39–42] Evidence from human studies over the past 20 years provides ample data to support Th17 cells as drivers of autoimmunity in JIA and rheumatoid arthritis (RA). Synovial fluid of both patients with JIA and RA was shown enriched for Th17 cells,[43–46] and elevated plasma levels of IL-17 in oligo-JIA and poly-JIA were shown to correlate with disease activity.[47] Addition of IL-17 to human ex vivo models enhanced IL-6 production and collagen destruction, while inhibiting collagen synthesis by RA synovium explants.[48] In contrast to IL-17, the functional role of IFNγ is decidedly ambivalent. While Th1 and Tc1 cells are considered inflammatory, IFNγ also suppresses the differentiation of Th17 cells, which can drive a net suppressive impact on autoimmunity. Indeed, in mouse models, treatment with IFNγ suppresses arthritis development due to impeded Th17 differentiation.[49] Conversely, deficiency in IFNγ promotes an sJIA-like disease on Freund's complete adjuvant in mice,[11] and IL-12 knockout mice, deficient in Th1 cells, are likewise susceptible to arthritis induction,[39 40 50] both phenotypes attributed to increased production of Th17 cells. A primary defect in IFNγ production could thus drive a pro-Th17 state that is permissive for JIA development. Alternatively, the causality could be reversed; IL-17 production reduces Th1 differentiation in RA,[51] and thus elevated Th17 numbers may be the primary effect. The identification of strong changes in the NKT cell compartment was the most surprising change observed. As these cells can have proarthritic or antiarthritic properties in mice, depending on the model,[52] either a potential mechanistic involvement or a compensatory biomarker function could be envisioned in JIA.

Beyond the mechanistic insights offered into disease pathogenesis, the utility of machine learning to extract JIA discrimination from the immunological data set serves as a proof of principle of immune-directed machine learning in precise diagnosis and personalised therapeutic choice. The data set used here lies at the threshold of manual and automated analyses, while future studies of larger populations and more extensive immune profiling will require automated analysis for full value extraction. Here a machine learning approach was able to build a discrimination algorithm for JIA 'diagnosis', and critically

we were able to extract the informative parameters from the discrimination algorithm. In principle, these limited parameters could be used to design a simplified immunophenotyping assay for JIA diagnosis. In practice, such an assay would be of relatively limited use: JIA diagnosis in secondary and tertiary referral centres is efficient (although diagnosis during primary care may be delayed due to overlapping early symptoms and the lack of specific biomarkers[53]), and the machine learning algorithm was derived from comparison with healthy individuals rather than patients with a relevant confounding disease (such as infectious arthritis). Nonetheless, the ability of machine learning to extract the relevant traits validates this approach for larger longitudinal cohorts, with deeper immunophenotyping. A key area where machine learning and immunophenotyping may be applied in the future is to identify immune signatures that correlate with disease course/outcomes and with successful response to certain treatments. The diversity of mechanisms that is likely to underlie such a heterogeneous group of diseases as JIA, combined with a steady increase in the potential immune-modulating biologics available to the clinician, will increasingly make therapeutic selection challenging. The features of unbiased analysis and identification of parameters with combinatorial diagnostic power would allow an immune-led machine learning process to identify those immune signatures predictive of efficient response to particular treatments. Indeed, machine learning approaches allow for the integration of immunological, clinical, genomic, environmental, biochemical and microbiomic factors, among others, for identification of predictive parameters. Findings could be translated into simple assays that provide added value to the clinician developing a personalised treatment plan for the patient. Such a data-driven personalised medicine approach would greatly improve the appropriate treatment selection for patients.

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Contributors EVN, LVE and JD collected the data. VL, UB, CR, MV and AG analysed the data. SH-B, CW and AL supervised the study.

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Competing interests None declared.

Patient consent for publication Not required.

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Data sharing statement All data are included as an online supplementary data set.

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CLINICAL SCIENCE

Lupus Low Disease Activity State (LLDAS) discriminates responders in the BLISS-52 and BLISS-76 phase III trials of belimumab in systemic lupus erythematosus

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ABSTRACT

Objective We evaluated the discriminant capacity of the Lupus Low Disease Activity State (LLDAS) in post-hoc analysis of data from the BLISS-52 and BLISS-76 trials of belimumab in systemic lupus erythematosus (SLE).

Methods LLDAS attainment, discrimination between belimumab and placebo arms, and the effects in subgroups with high disease activity at recruitment were evaluated at week 52 using appropriate descriptive statistics, χ^2 test and logistic regression.

Results At week 52, for belimumab 10 mg/kg, 17.0% and 19.3% of patients who achieved a Systemic Lupus Erythematosus Responder Index-4 also attained LLDAS in BLISS-52 and BLISS-76, respectively. Significantly more patients attained LLDAS on belimumab 10 mg/kg compared with placebo (12.5% vs 5.8%, OR 2.32, p=0.02 for BLISS-52; 14.4% vs 7.8%, OR 1.98, p=0.04 for BLISS-76). In a subgroup analysis, the difference in week 52 LLDAS attainment between belimumab 10 mg/kg and placebo was greater in patients who had higher disease activity at baseline, compared with the overall group.

Conclusions LLDAS was able to discriminate belimumab 10 mg/kg from placebo in the BLISS-52 and BLISS-76 trials. Our findings support the validity of LLDAS as an outcome measure in SLE clinical trials.

Key messages

What is already known about this subject?

► Lupus Low Disease Activity State (LLDAS) predicts better clinical outcomes and has been found to discriminate responders in a phase II trial of anifrolumab in systemic lupus erythematosus (SLE).

What does this study add?

► This is the first study to demonstrate that LLDAS can discriminate responders in the pivotal phase III trials of belimumab (BLISS-52 and BLISS-76) in SLE, and is a more stringent outcome measure than the Systemic Lupus Erythematosus Responder Index-4 in these trials.

How might this impact on clinical practice or future developments?

► This study lends weight to the potential utility of LLDAS as a novel clinical trial outcome measure for SLE randomised controlled trials.

INTRODUCTION

Measurement of treatment response in systemic lupus erythematosus (SLE) clinical trials has generally been based on measurement of proportions of patients attaining a certain degree of change from baseline; in contrast, a treat-to-target analysis has seldom been applied. The Lupus Low Disease Activity State (LLDAS), a potential response indicator for lupus clinical trials, has been found to correlate with reduced damage accrual in SLE,[1] suggesting that it may be a useful treatment target in the clinic. In a trial setting, LLDAS has been found to correlate with key outcome measures and has discriminated responders from non-responders in phase II trials of anifrolumab and baricitinib.[2 3]

In this study, we sought to evaluate LLDAS utility in discriminating drug from placebo in a post-hoc analysis of data from the pivotal phase III BLISS-52[4] and BLISS-76[5] trials of intravenous belimumab, an anti-BAFF (B-cell activating factor) monoclonal antibody, in patients with moderate to severe SLE.

METHODS

BLISS-52 and BLISS-76 trials

The utility of LLDAS as an outcome measure was assessed in a post-hoc analysis of the data from the phase III, 52-week and 76-week BLISS-52[4] (NCT00424476) and BLISS-76[5] (NCT00410384) trials of intravenous belimumab in patients with SLE. In these large multicentre studies, seropositive (antinuclear or antidouble-stranded DNA [anti-dsDNA] antibody-positive) patients (≥18 years old) with moderate-severe SLE (SELENA-SLEDAI[6] score ≥6), as defined by the revised American College of Rheumatology SLE classification criteria,[7] were randomised in a 1:1:1 ratio to receive belimumab 1 mg/kg or 10 mg/kg, or placebo, by intravenous infusion on days 0, 14 and 28, and then every 28 days until 48 weeks[4] or 72 weeks,[5] in addition to standard of care. The primary efficacy endpoint was improvement in the Systemic Lupus Erythematosus Responder Index-4 (SRI-4) at week 52, defined as a reduction ≥4 points in SELENA-SLEDAI[6] score; no new British Isles Lupus Assessment Group (BILAG)[8] A organ domain score and no more than one new B organ

domain score; and no worsening (<0.3 increase) in Physician Global Assessment (PGA) score, compared with baseline. Patients with active severe or unstable neuropsychiatric SLE or lupus nephritis were excluded. Further details on BLISS-52 and BLISS-76 study design and endpoints have been published.[4 5] Clinical trial data were accessed and analysed via the SAS Data Access System, through a data sharing agreement.

Lupus Low Disease Activity State

As previously published, LLDAS was attained if all of the following were present: (1) SLEDAI-2K (SLE Disease Activity Index 2000) score ≤4, with no activity in major organ systems (renal, central nervous system, cardiopulmonary, vasculitis and fever) and no haemolytic anaemia or gastrointestinal activity; (2) no new features of lupus disease activity compared with the previous assessment; (3) a physician global assessment of activity score (PGA, 0–3) ≤1; (4) current prednisolone-equivalent dosage ≤7.5 mg/day; and (5) standard maintenance dosages of immunosuppressive drugs and approved biologics allowed.[1]

A detailed description of how LLDAS was defined using variables in the BLISS-52 and BLISS-76 data sets is provided in online supplementary table S1.

LLDAS attainment was assessed across all study timepoints. Attainment of LLDAS, association with the primary trial endpoint (SRI-4), and discrimination between belimumab and placebo-treated patients both for the whole study population and subgroups according to the level of disease activity, glucocorticoid dose and presence of damage at recruitment were evaluated using appropriate descriptive statistics, the χ^2 test and logistic regression, where appropriate, using statistical software R (V.3.4.3).

RESULTS

Patient characteristics

BLISS-52 and BLISS-76 patient demographics and baseline characteristics have been published[4 5] and are presented in online supplementary table S2.

BLISS studies efficacy endpoints

Patients receiving belimumab treatment were more likely to achieve an SRI-4 response at week 52 than those treated with standard of care, in both BLISS-52 and BLISS-76 studies (table 1), as previously reported.[4 5]

LLDAS as an outcome measure in BLISS-52 and BLISS-76

LLDAS as a discriminator between placebo and belimumab in BLISS-52 and BLISS-76

Few patients (0% for BLISS-52 and 2.2% for BLISS-76) were in LLDAS at study entry (online supplementary table S3). In contrast to SRI-4, LLDAS was attained by few patients in the placebo arms at week 52 (5.8% in BLISS-52 and 7.8% BLISS-76) (table 1).

Significantly more patients attained LLDAS at week 52 on belimumab 10 mg/kg compared with placebo in both trials (12.5% vs 5.8%, OR 2.32, p=0.02 in BLISS-52; 14.4% vs 7.8%, OR 1.98, p=0.04 in BLISS-76) (table 1). A statistically significant difference was also seen at week 44 in BLISS-52 between the belimumab 1 mg/kg group and placebo (11.52% vs 6.19%, OR 1.97, p=0.05), and at week 72 in BLISS-76 between belimumab 10 mg/kg and placebo (19.4% vs 10.29%, OR 2.09, p=0.02) (online supplementary figure 1).

Of the LLDAS components at week 52, most patients (from 97.1% to 99.0% across treatment groups, in both studies) met criterion 5 (standard dose immunosuppressants allowed), with the fewest patients (22.5%–30.9%) meeting criterion 1 (SLEDAI-2K score ≤4, with no activity in major organ systems and no haemolytic anaemia or gastrointestinal activity) (table 1). Increases in LLDAS attainment across time were largely driven by attainment of criteria 1 and 3 (PGA score ≤1) (online supplementary figures 2-6).

Comparison of LLDAS with SRI-4 as an outcome measure

At week 52, in both studies, fewer patients in the treatment arms attained LLDAS compared with SRI-4 (table 1). LLDAS attainment was more stringent than SRI-4 attainment at week 52, with 13.8% of patients in BLISS-52 and 17.7% of patients in BLISS-76

Table 1 Attainment of LLDAS and SRI-4 at week 52 in BLISS-52 and BLISS-76

	BLISS-52			BLISS-76		
	Placebo (%)	Belimumab 1 mg/kg	Belimumab 10 mg/kg	Placebo (%)	Belimumab 1 mg/kg	Belimumab 10 mg/kg
SRI-4	**43.6**	**51.4% OR 1.37 (0.99 to 1.90), p=0.06**	**57.6% OR 1.76 (1.27 to 2.45), p=0.0007**	**33.5**	**40.7% OR 1.36 (0.95 to 1.93), p=0.08**	**43.2% OR 1.51 (1.07 to 2.14), p=0.02**
LLDAS	**5.8**	**10.9% OR 2.00 (1.02 to 4.11), p=0.05**	**12.5% OR 2.32 (1.20 to 4.71), p=0.02**	**7.8**	**11.6% OR 1.55 (0.79 to 3.11), p=0.21**	**14.4% OR 1.98 (1.04 to 3.91), p=0.04**
LLDAS criterion 1	22.5	28.8%	30.9%	17.7	25.4%	30.6%
LLDAS criterion 2	76.4	73.1%	76.8%	63.0	73.0%	69.3%
LLDAS criterion 3	60.4	64.6%	70.4%	54.7	64.3%	63.6%
LLDAS criterion 4	40.5	43.3%	46.5%	62.4	77.8%	67.0%
LLDAS criterion 5	97.9	99.0%	99.0%	98.6	97.1%	98.2%

LLDAS criterion 1: SLEDAI-2K (SLE Disease Activity Index 2000) score ≤4, with no activity in major organ systems (renal, central nervous system, cardiopulmonary, vasculitis and fever) and no haemolytic anaemia or gastrointestinal activity.
LLDAS criterion 2: no new features of lupus disease activity compared with the previous assessment.
LLDAS criterion 3: Physician Global Assessment of activity score (0–3) ≤1.
LLDAS criterion 4: current prednisolone-equivalent dosage ≤7.5 mg/day.
LLDAS criterion 5: standard maintenance dosages of immunosuppressive drugs and approved biologics allowed.
OR with (95% CI).
LLDAS, Lupus Low Disease Activity State; SRI-4, Systemic Lupus Erythematosus Responder Index-4.

Table 2 Attainment of LLDAS in those achieving an SRI-4 response in BLISS-52 and BLISS-76

	BLISS-52			
	All groups (%)	Placebo (%)	Belimumab 1 mg/kg (%)	Belimumab 10 mg/kg (%)
SRI-4+/LLDAS+	13.8	9.7	13.7	17.0
SRI-4–/LLDAS-	96.7	99.0	93.5	97.4
LLDAS–/SRI-4+	59.1	52.8	59.4	64.9
LLDAS+/SRI-4-	8.7	7.7	23.1	6.7
	BLISS-76			
	All groups (%)	Placebo (%)	Belimumab 1 mg/kg (%)	Belimumab 10 mg/kg (%)
SRI-4+/LLDAS+	17.7	14.8	18.3	19.3
SRI-4–/LLDAS-	95.2	98.1	95.2	91.0
LLDAS–/SRI-4+	47.1	42.1	46.4	53.2
LLDAS+/SRI-4-	20.6	13.3	20.8	24.1

*+Attained outcome measure.
†-Did not attain outcome measure.
LLDAS, Lupus Low Disease Activity State; SRI-4, Systemic Lupus Erythematosus Responder Index-4.

who achieved an SRI-4 also attaining LLDAS across all treatment groups (table 2). However, the majority of patients who did not achieve an SRI-4 at week 52 also failed to attain LLDAS (96.7% in BLISS-52 and 95.2% in BLISS-76). Conversely only 8.7% of LLDAS responders in BLISS-52 and 20.6% of LLDAS responders in BLISS-76 failed to achieve SRI-4.

Subgroup analyses

Subgroup analyses revealed that the difference in LLDAS attainment at week 52 between belimumab 10 mg/kg and placebo was greater in patients with higher disease activity at baseline, relative to all study participants (table 3). The subgroups of patients in which a greater difference was seen were those with a high anti-dsDNA antibody level ≥30 IU/mL (for both studies), low C3 (<90 mg/dL) and/or C4 (<16 mg/dL) (both studies), high anti-dsDNA antibody levels or low complement levels (both studies), SLEDAI-2K score ≥10 (BLISS-52 study only), or prednisolone dose ≥7.5 mg/day (BLISS-52 only) at study entry. Patients in the belimumab 10 mg/kg treatment arms who had baseline high anti-dsDNA levels in BLISS-76, or baseline low C3/C4 and SLEDAI-2K score ≥10 in BLISS-52, were more likely to attain LLDAS at week 52 compared with their lower disease activity counterparts (table 3). In contrast to the greater discrimination in LLDAS attainment between active and placebo seen with higher baseline disease activity, at most timepoints in both studies, patients with SLEDAI-2K score ≤9 at baseline were more likely to attain LLDAS than those with SLEDAI-2K score ≥10 (online supplementary figure 7). Additionally, patients with less organ damage at baseline (SLICC (Systemic Lupus International Collaborating Clinics) Damage Index [SDI]=0) were more likely to attain LLDAS at week 52 than those with an SDI score ≥1 (table 3).

DISCUSSION

We have demonstrated that the LLDAS is able to discriminate active treatment from placebo in the pivotal phase III BLISS studies of intravenous belimumab in moderate-severe SLE, with a greater percentage of patients receiving belimumab 10 mg/kg attaining LLDAS at week 52 compared with placebo in BLISS-52 and BLISS-76. The ability to discriminate treatment arms was evident despite the relatively modest difference measured by the SRI-4 in the original trials, and LLDAS appeared to be a more stringent outcome measure than the SRI-4, with a smaller percentage of patients overall attaining LLDAS compared with the SRI-4 and in particular a very low LLDAS attainment among placebo-treated patients. We would envisage that in a trial where the treatment is more efficacious, a larger proportion of patients in the active treatment arm would achieve LLDAS, with still few achieving this endpoint in the control arm. Of course, this will only be established when LLDAS is used as an endpoint in future trials of novel therapies, although such an outcome is strongly suggested by post-hoc analysis of the phase II anifrolumab trial.[2] Although harder to achieve, a more stringent, and clinically relevant, trial endpoint has potential benefits, for example in permitting the conduct of smaller clinical trials, and distinguishing novel therapies that will effect robust clinical change. This conclusion is supported by the very low rates of LLDAS attainment in the placebo arms of both trials.

These results add to the growing body of evidence that the LLDAS is useful not only as a clinical treatment target,[1 9] but that it has discriminant validity as a clinical trial endpoint in SLE randomised controlled trials.[2] Recently, the LLDAS was found to discriminate responders from non-responders in the phase II clinical trials of anifrolumab[2] and baricitinib in SLE.[3] The frequencies of LLDAS attainment in these studies were higher than in the BLISS studies—17%, 39% and 28% of patients on placebo, anifrolumab 300 mg and anifrolumab 1000 mg at week 52, and 26%, 33% and 38% of patients on placebo, baricitinib 2 mg and baricitinib 4 mg at week 24. This could be due to differences in study patient populations and treatment protocols, and/or reflect differences in efficacy between these treatments. Our study is the first to confirm the discriminant validity of the LLDAS in two large positive phase III clinical trials of SLE treatment.

In pooled post-hoc univariable and multivariable analyses, patients with higher disease activity at baseline (SELENA-SLEDAI score ≥10, low complement, anti-dsDNA positivity and steroid dose ≥7.5 mg/day) have been found to be more likely to achieve an SRI-4 response with belimumab treatment compared with placebo, in the BLISS-52 and BLISS-76 studies.[10] We similarly found that the difference in LLDAS attainment between belimumab 10 mg/kg and placebo was greater in these high disease activity subgroups than for the trial patients overall.

Limitations of this study include its post-hoc nature. However, the study is strengthened by prospectively collected data within the framework of a rigorously conducted, double-blind randomised controlled trial. The LLDAS definition was applied to available data in a stringent manner to ensure minimal or no misclassification. Gastrointestinal activity, part of LLDAS criterion 1, was not captured in the original trials and was here assumed to be captured as part of the PGA (LLDAS criterion 2); this assumption requires further validation. Our application of the LLDAS definition to the BLISS-52 and BLISS-76 data sets required for criteria 1 and 2 to have neither major organ involvement or new activity in either BILAG or SLEDAI measures of disease activity. This is a strength of our post-hoc analysis and may partly explain the relatively low frequency of LLDAS attainment in this study, in comparison with a previous post-hoc analysis of phase II data for anifrolumab in SLE, in which these criteria were assessed based on SLEDAI alone.[2] Despite these stringent criteria, it is important to note that LLDAS was still attained from a relatively high baseline disease activity (average

Table 3 Attainment of LLDAS at week 52 in subgroups of patients with higher disease activity features at study entry, in BLISS-52 and BLISS-76

	BLISS-52			BLISS-76		
	Placebo	Belimumab 1 mg/kg	Belimumab 10 mg/kg	Placebo	Belimumab 1 mg/kg	Belimumab 10 mg/kg
LLDAS (overall)	5.8%	10.9% OR 2.00 (1.02 to 4.11), p=0.05	12.5% OR 2.32 (1.20 to 4.71), p=0.02	7.8%	11.6% OR 1.55 (0.79 to 3.11), p=0.21	14.4% OR 1.98 (1.04 to 3.91), p=0.04
High[a] vs low[b] disease activity subgroups						
n	160	178	182	116	132	131
High anti-dsDNA antibody levels[a]	5.0%	10.1% OR 2.14 (0.93 to 5.34), p=0.08	12.1% OR 2.61 (1.17 to 6.42) p=0.02	5.2%	6.8% OR 1.34 (0.47 to 4.11), p=0.59	15.3% OR 3.30 (1.35 to 9.32), p=0.01
n	65	60	59	76	75	71
Normal anti-dsDNA antibody levels[b]	7.7%	13.3% OR 1.85 (0.58 to 6.44), p=0.31	13.6% OR 1.89 (0.59 to 6.57), p=0.29	11.8%	20.0% OR 1.86 (0.77 to 4.72), p=0.18	12.7% PR 1.08 (0.40 to 2.94), p=0.88
n	139	154	164	108	113	123
Low C3/C4[a]	5.8%	8.4% OR 1.51 (0.61 to 3.92), p=0.37	14.0% OR 2.67 (1.20 to 6.56), p=0.02	4.6%	5.3% OR 1.15 (0.33 to 4.19), p=0.82	13.0% OR 3.08 (1.16 to 9.70), p=0.03
n	86	84	77	84	94	79
Normal C3/C4[b]	5.8%	15.5% OR 2.97 (1.06 to 9.62), p=0.05	9.1% OR 1.62 (0.50 to 5.69), p=0.43	11.9%	19.1% OR 1.75 (0.77 to 4.18), p=0.19	16.46% OR 1.46 (0.60 to 3.63), p=0.41
n	183	204	207	142	149	153
High anti-dsDNA or low C3/C4[a]	5.5%	9.3% OR 1.78 (0.82 to 4.08), p=0.16	13.0% OR 2.60 (1.26 to 5.78), p=0.01	4.9%	6.0% OR 1.24 (0.45 to 3.56), p=0.68	14.4% OR 3.24 (1.40 to 8.43), p=0.01
n	67	90	88	54	60	64
High anti-dsDNA and low C3/C4[a]	4.7%	8.8% OR 2.08 (0.58 to 9.79), p=0.29	11.4% OR 2.74 (0.80 to 12.58), p=0.14	7.4%	6.67% OR 0.89 (0.20 to 3.95), p=0.88	16.7% OR 2.05 (0.62 to 7.93), p=0.25
n	158	148	153	138	147	138
Normal anti-dsDNA and low C3/C4[b]	6.3%	12.2% OR 2.05 (0.93 to 4.76), p=0.08	15.0% OR 2.23 (1.03 to 5.12), p=0.05	8.0%	13.6% OR 1.82 (0.85 to 4.06), p=0.13	16.9% OR 1.96 (0.91 to 4.39), p=0.09
n	119	111	131	90	103	95
SLEDAI-2K score ≥10[a]	4.2%	9.9% OR 2.51 (0.88 to 8.19), p=0.09	13.0% OR 3.40 (1.30 to 10.62), p=0.01	5.6%	6.8% OR 1.24 (0.38 to 4.32), p=0.72	9.5% OR 1.78 (0.59 to 6.00), p=0.32
n	106	127	110	102	104	107
SLEDAI-2K score ≤9[b]	7.5%	11.8% OR 1.64 (0.68 to 4.23), p=0.28	11.8% OR 1.64 (0.66 to 4.31), p=0.29	9.8%	10.8% OR 1.80 (0.79 to 4.28), p=0.17	18.7% OR 2.11 (0.96 to 4.95), p=0.07
n	155	176	168	78	95	89
Prednisolone dose >7.5 mg/d[a]	3.9%	5.7% OR 1.50 (0.54 to 4.49), p=0.44	10.7% OR 2.98 (1.21 to 8.41), p=0.02	7.7%	4.2% OR 0.53 (0.13 to 1.92), p=0.34	6.7% OR 0.86 (0.26 to 2.89), p=0.81
n	70	62	73	114	112	113
Prednisolone ≤7.5 mg/d[b]	10.0%	25.8% OR 3.13 (1.23 to 8.72), p=0.02	16.4% OR 1.77 (0.67 to 5.04), p=0.26	7.9%	17.9% OR 2.54 (1.13 to 6.11), p=0.03	20.4% OR 2.98 (1.35 to 7.11), p=0.009
n	77	75	83	88	106	93
SDI score ≥1[a]	6.5%	8.7% OR 1.25 (0.52 to 3.06), p=0.61	7.2% OR 1.12 (0.47 to 2.73), p=0.80	10.2%	8.4% OR 0.72 (0.40 to 1.27), p=0.26	9.7% OR 0.94 (0.53 to 1.65), p=0.83
n	148	163	158	104	101	109
SDI=0[b]	5.7%	12.3% OR 2.45 (1.36 to 4.59), p=0.003	15.2% OR 3.13 (1.77 to 5.81), p=0.0001	5.8%	15.8% OR 3.07 (1.77 to 5.55), p=0.0001	18.4% OR 3.67 (2.16 to 6.54), $p=3.92\times10^{-6}$

OR with (95% CI).
Shaded boxes denote statistically significant results.
LLDAS, Lupus Low Disease Activity State; SDI, SLICC (Systemic Lupus International Collaborating Clinics) Damage Index; SLEDAI-2K, SLE Disease Activity Index 2000; a, high activity subgroup; anti-dsDNA, antidouble-stranded DNA; b, low activity subgroup.

SLEDAI score at study entry was ~10). There were small differences in LLDAS attainment between the BLISS-52 and BLISS-76 studies, both in the overall group and in subgroup analyses, which could be accounted for by small differences in treatment protocol, in particular the permitted doses of background DMARDs (disease-modifying drugs) and steroids.

In conclusion, we have shown that LLDAS has discriminant validity when applied retrospectively, in two large positive

phase III clinical trials of SLE treatment, and is a more stringent endpoint than that used in the original trials. These findings, and the recent report of applying the LLDAS prospectively in a phase II clinical trial,[3] suggest inclusion of LLDAS as a trial endpoint in studies of novel therapies in SLE.

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EPIDEMIOLOGICAL SCIENCE

Multicriteria decision analysis process to develop new classification criteria for systemic lupus erythematosus

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ABSTRACT

European League Against Rheumatism and are jointly supporting multiphase development of systemic lupus erythematosus (SLE) classification criteria based on weighted criteria and a continuous probability scale. Prior steps included item generation, item reduction and hierarchical organisation of candidate criteria using an evidence-based approach. Our objectives were to determine relative weights using multicriteria decision analysis (MCDA) and to set a provisional threshold score for SLE classification. An SLE Expert Panel (8 European, 9 North American) submitted 164 real, unique cases with a wide range of SLE probability in a standardised format. Using the candidate criteria, experts scored and rank-ordered 20 representative cases. At an in-person meeting, experts reviewed inter-rater reliability of scoring, further refined criteria definitions and participated in an MCDA exercise. Based on expert consensus decisions on pairwise comparisons of criteria, 1000minds software calculated criteria weights and rank-ordered the remaining 144 cases based on their additive scores. The score of the lowest-ranked case for which complete expert consensus was achieved defined the provisional threshold classification score. Inter-rater reliability of scoring cases with the candidate criteria was good. MCDA involved 74 pairwise decisions and was repeated for the arthritis and mucocutaneous domains when the initial ranking of some cases did not match expert opinion. After criteria weights and additive scores were recalculated once, experts reached consensus for SLE classification for all cases scoring>83. Using an iterative process, the candidate criteria definitions were refined, preliminary weights were calculated and a provisional threshold score for SLE classification was determined.

INTRODUCTION

A multinational effort to develop new classification criteria for systemic lupus erythematosus (SLE) for clinical research, jointly supported by the European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR), is underway. The overarching goal is to develop a system that identifies potential participants for clinical research studies, requiring a degree of homogeneity among subjects while simultaneously dealing with the extreme heterogeneity of SLE.[1] The aim was to design a system with the maximum combination of sensitivity and specificity for SLE, retaining face validity. While the classification criteria are not intended for diagnosis or clinical care, it is acknowledged that the only available 'gold standard' for the presence of SLE is expert clinician opinion.

A 12 member Steering Committee was formed with input from EULAR and ACR leadership to oversee a four-phase process.[2] In Phase 1, items were generated using a Delphi exercise,[3] early SLE cohort[4] and SLE patient survey[5]; and antinuclear antibody (ANA) was evaluated as a potential entry criterion.[6 7] During Phase 2, the list of potential criteria was narrowed using nominal group technique.[8 9] Phase 3 began with a literature review for test performance characteristics of candidate criteria and data-driven organisation of criteria into domains.[1] This report outlines the latter part of Phase 3: criteria weighting and threshold score identification through a consensus-based multicriteria decision analysis (MCDA) approach.[10–12] The goal was to develop a criteria system producing a continuous measure of the relative probability that a case (ie, particular combination of clinical features) could be characterised as SLE, and a provisional threshold score above which a case could be definitely classified as SLE for clinical research.[13 14] Phase 4 involves the determination of the final threshold, followed by validation of the classification system.

METHODS

An international panel of SLE experts collected and rank-ordered patient case scenarios, participated in an in-person consensus meeting and held post-meeting email and telephone discussions.

SLE expert panel

The Steering Committee invited 6 additional experts (3 European, 3 North American) to form a 17 person SLE Expert Panel ('SLE experts') to assist with this phase and establish external validity of the criteria development process. SLE experts were senior clinicians focused on SLE, many of whom direct SLE clinics at their institutions, and senior clinical investigators with expertise in SLE.

Development of patient case scenarios

Each of the 17 SLE experts submitted 10 deidentified real cases based on adult patients from his/her own cohort in a standardised online form using REDCap (Research Electronic Data Capture), a secure, web-based application for research studies.[15] Each expert was asked to submit five cases with 'definite' or 'likely' SLE and five cases in which they considered but ultimately did not diagnose SLE and/or diagnosed a condition mimicking SLE such as rheumatoid arthritis, other inflammatory arthritis, Sjögren's syndrome, antiphospholipid antibody syndrome or viral infection. ANA≥1:80 was required of all cases. The REDCap form included three options for each clinical and laboratory criterion: yes (present), no (absent) and unknown.

Rank ordering and scoring of cases

From 164 deidentified cases, three authors of this manuscript (KHC, RPN, SKT) chose a representative sample of 20 reflecting a range of possible SLE cases. Each case was abstracted into standardised paragraph format. Laboratory tests that had not been performed were treated as unknown. SLE experts were asked to rank the cases based on their confidence that the case should be classified as SLE. This exercise introduced SLE experts to the challenge of assessing the relative influence of individual criteria in pointing towards or away from SLE.

SLE experts then scored the 20 cases using a standardised REDCap form reflecting the draft SLE classification criteria as of September 2016, based on the Phase 2 nominal group technique exercise[16] and subsequent work by the Steering Committee.[1] The REDCap form included 10 domains; each domain included 2–6 options. Experts were provided written instructions for scoring and a list of proposed definitions for each criterion. The instructions specified that within each domain, criteria were ordered from least to most supportive of SLE and if multiple criteria were present in one domain only the single criterion furthest down the list (ie, most supportive of SLE) should be scored. The instructions specified that a criterion should not be scored if a cause more likely than SLE existed (eg, other autoimmune disease, malignancy, medication). Criteria did not need to occur simultaneously and could occur before or after the detection of ANA≥1:80 as long as another explanation more likely than SLE did not exist.

In-person consensus meeting, November 2016

During a 1.5-day in-person meeting, RPN and AH moderated discussions among SLE experts leading to consensus decisions. Goals of this meeting included achieving full consensus on criteria definitions, calculating criteria weights via a MCDA exercise and establishing a provisional threshold score for SLE classification.

1. *Review of case scoring and criteria refinement*. Experts reviewed a summary of the REDCap scoring exercise. Discrepancies in scoring individual cases were discussed in depth to understand the underlying reasons. Criteria definitions were discussed in the context of these discrepancies and refined based on consensus agreement.
2. *MCDA to determine weights*. The MCDA exercise is based on the PAPRIKA method (Potentially All Pairwise RanKings of all possible Alternatives),[17] as implemented by 1000minds software (http://www.1000minds.com). This method and software have been used extensively since 2010 for developing classification criteria.[10 11 18] Experts voted on a series of pairwise decisions about hypothetical cases, each defined by two criteria from two domains. For example, hypothetical case A: 'oral ulcers' (mucocutaneous domain) and 'acute pericarditis' (serositis domain) versus hypothetical case B 'alopecia' (mucocutaneous domain) and 'pleural effusion' (serositis domain). Experts were asked to decide whether they would more likely classify hypothetical case A or B as SLE, presuming all else was equal about the cases. Voting was conducted anonymously, but where opinions diverged cases were discussed until full consensus was reached. Consensus opinion was based on the specificity of each manifestation for SLE and how much its presence would increase the likelihood of SLE (although specificity for some manifestations has not been formally evaluated, as discussed in Ref. [1]). Such pairwise-ranking questions were repeated with different pairs of hypothetical cases—always involving trade-offs between different combinations of criteria, two at a time—until enough information about expert preferences had been collected to determine relative criteria weights for all criteria. Each time experts ranked a pair, all other cases that could be pairwise ranked via the logical property of 'transitivity' were identified and eliminated. For example, if experts ranked hypothetical case A over B and B over C, then by transitivity A is also ranked over C (and experts are not asked to choose between A and C). This procedure ensures the number of pairwise-ranking questions posed is minimised, and experts end up having pairwise ranked all possible cases defined on two criteria at a time. Consensus decisions were entered into 1000minds software, which uses linear programming techniques to derive weights for each criterion.[17]
3. *Assessment of the face validity of the weights*. Criteria weights were summed to produce an additive score for each case. Only the highest-weighted criterion in each domain was counted towards the additive score, as specified in the instructions (Box 1). The remainder of the 164 cases were scored and arranged in rank order from highest to lowest score. SLE experts reviewed a spreadsheet listing the criteria present in each case and anonymously voted whether they would classify each as SLE. For cases where expert opinion differed, RPN facilitated discussion to achieve full consensus about case classification. Cases were discussed in descending rank order (confidence that the case should be classified as SLE) until agreement on classification could not be reached.
4. *Determination of an upper threshold score*. The score of the last case for which the group achieved consensus on classification as SLE was the initial threshold.
5. *Review of cases below the threshold*. The cases with scores immediately below the initial threshold were individually reviewed. The threshold thus functioned as a way to focus the discussion on these 'borderline' cases, and the individual criteria present in each of these. SLE experts reached consensus that several of these cases should have been classified as SLE. Experts discussed discrepancies between expert opinion and the initial weights assigned to some of the criteria.
6. *Weighting and upper threshold revision*. The MCDA exercise was repeated once for those criteria whose calculated weights were inconsistent with expert opinion. Weights for all criteria were recalculated using 1000minds and additive scores were recalculated. SLE experts again anonymously voted on classifying each case as SLE, followed by discussion facilitated by RPN to achieve consensus. The score of the last case for which expert consensus was achieved was the provisional full consensus upper threshold score. Phase 4 involves further refinement of the upper threshold score.

Box 1. Provisional SLE classification criteria organisation and definitions

Opening statements:
- A history of a positive ANA by Hep 2 immunofluorescence ≥1:80 is required for consideration of a person for SLE classification.
- For each criterion, do not score if a cause more likely than SLE exists (such as infection, malignancy, medication, rosacea, endocrine disorder, other autoimmune disease).
- Occurrence of a criterion on at least one occasion is sufficient.
- Criteria need not occur simultaneously.
- At least one clinical criterion must be present.
- Within each domain, only the highest weighted criterion is counted towards the total score.

Clinical domains and criteria

Constitutional
- Fever:>38.3°C with no other source identified.

Haematological
- Leucopaenia: WBC<4000/mm^3.
- Thrombocytopaenia: Platelets<100 000/mm^3.
- Autoimmune haemolysis: (1) evidence of haemolysis, such as reticulocytosis, low haptoglobin, elevated indirect bilirubin, elevated LDH and (2) positive Coomb's (direct antiglobulin) test.

Neuropsychiatric
- Delirium: characterised by (1) change in consciousness or level of arousal with reduced ability to focus and (2) symptom development over hours to <2 days and (3) symptom fluctuation throughout the day and (4) either (4a) acute/subacute change in cognition (eg, memory deficit or disorientation) or (4b) change in behaviour, mood or affect (eg, restlessness, reversal of sleep/wake cycle and so on).
- Psychosis: characterised by (1) delusions and/or hallucinations without insight and (2) absence of delirium.
- Seizure: primary generalised seizure or partial/focal seizure, with independent description by a reliable witness. If EEG is performed, abnormalities must be present.

Mucocutaneous
- Non-scarring alopecia, observed by a clinician*
- Oral ulcers, observed by a clinician*
- Subacute cutaneous lupus (SCLE) or discoid lupus (DLE): SCLE is characterised by annular or papulosquamous (psoriasiform) cutaneous eruption observed by a clinician,* usually photodistributed. If skin biopsy is performed, typical changes must be present.[26] DLE is characterised by erythematous-violaceous cutaneous lesions with secondary changes of atrophic scarring, dyspigmentation, often follicular hyperkeratosis/plugging (scalp), observed by a clinician,* leading to scarring alopecia on the scalp. Lesions have a preference for the head and neck, especially the conchal bowl, but may be found in nearly any location. If skin biopsy is performed, typical changes must be present.[26]
- Acute cutaneous lupus: Malar rash (localised) or maculopapular rash (generalised) observed by a clinician,* with or without photosensitivity. If skin biopsy is performed, typical changes must be present.[26]

Serositis
- Pleural or pericardial effusion: imaging evidence (such as ultrasound, X-ray, CT scan, MRI) of pleural or pericardial effusion or both
- Acute pericarditis: ≥2 of: (1) pericardial chest pain (typically sharp, worse with inspiration, improved by leaning forward), (2) pericardial rub, (3) EKG with new widespread ST-elevation or PR depression, (4) new or worsened pericardial effusion on imaging (such as ultrasound, X-ray, CT scan, MRI)

Musculoskeletal
- Synovitis in ≥2 joints: characterised by joint swelling and tenderness, observed by a clinician*

Renal
- Proteinuria>0.5 g/24 hours: on 24 hours urine collection or spot urine protein-to-creatinine ratio representing >0.5 g protein/24 hours
- Renal biopsy with Class II or V lupus nephritis, per International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification[27]
- Renal biopsy with Class III or IV lupus nephritis, per International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification[27]

Immunological domains and criteria

Antiphospholipid antibodies
- Anticardiolipin IgG (>40 GPL units) or anti-β2GP1 IgG (>40 units) or lupus anticoagulant positive

Complement proteins
- Low C3 or low C4
- Low C3 and low C4

SLE-specific antibodies
- Anti-dsDNA antibody
- Anti-Smith antibody

*Direct observation may include physical examination or review of a photograph.[26]

Determining a lower threshold score

SLE experts attempted to set an upper threshold for definite SLE classification and a lower threshold for very low probability for classification. Individuals with scores falling between these two thresholds might be candidates for inclusion in observational studies or SLE prevention trials. Due to insufficient time at the November 2016 meeting, the lower threshold was addressed in emails, secondary exercises and conference calls in the next 2 months. SLE experts were asked to rate the cases that fell below the upper threshold score as 'probable SLE', 'possible SLE' or 'unlikely SLE'. The score of the case for which≥70% indicated 'unlikely SLE' was assigned as the lower threshold.

RESULTS

At the in-person meeting, SLE experts agreed that classification as SLE means a patient is appropriate for inclusion in SLE clinical research—and that classification as SLE should *not* guide clinical decisions about SLE diagnosis or treatment. Experts agreed that the threshold score should have high specificity for SLE, ensuring a high degree of homogeneity among classified patients and facilitating comparisons across clinical studies. SLE experts reached consensus that patients with overlap syndromes could be classified as SLE if they met SLE classification criteria, allowing clinical investigators to decide whether to include or exclude patients with overlap syndromes in specific research studies.

Review of scoring and criteria refinement

There was considerable inconsistency between SLE experts using the REDCap form to score cases. Each expert scored a total of

Case nickname	Expert Rating			
	Definite	Probable	Possible	Unlikely
Lavender	17	0	0	0
Tarragon	17	0	0	0
Pepper	15	2	0	0
Curry	11	4	2	0
Cinnamon	10	5	2	0
Allspice	7	6	4	0
Mint	6	4	3	4
Dill	4	6	5	2
Oregano	3	6	7	1
Garlic	2	5	7	3
Jalapeno	0	5	9	3
Rosemary	0	4	6	7
Mustard	1	3	9	4
Mace	1	3	4	9
Sage	0	3	3	11
Marjoram	0	2	6	9
Fennel	0	2	8	7
Pimento	0	1	3	13
Nutmeg	0	0	6	11
Thyme	0	0	1	16

Figure 1 Rank-ordering exercise results. Seventeen SLE experts ranked each of 20 cases in order of most likely to least likely SLE. More than 85% of experts rated 5 cases as definite or probable SLE and 5 cases as possible or unlikely SLE. Expert opinion varied for the remaining 10 cases. SLE, systemic lupus erythematosus.

200 items (20 cases, 10 domains); all 17 experts scored 127/200 (64%) domains exactly the same. Reasons for discrepant data entry included human error in data entry, not following the instructions, variability in interpreting the candidate criteria based on context and different interpretations of criteria definitions (see online supplement 1 for details).

Review of the rank-ordering exercise

There was agreement on the cases that the majority of SLE experts ranked the highest and lowest, but a spectrum of ranking for cases in between (figure 1). This reflected the different relative weights that individual experts attached to particular criteria.

MCDA exercise to determine consensus weights using 1000minds software. SLE experts anonymously voted on 74 pairs of hypothetical cases. Sometimes it was agreed that hypothetical cases A and B were equally likely to be SLE. For a handful of pairwise comparisons, consensus could not be reached and the decision was to defer that comparison and approach their relativity from other pairwise comparisons. Significant changes to the criteria during this stage included:

- *Mucocutaneous and musculoskeletal domains*. SLE experts decided that observation by a clinician should be required for consistency with other clinical domains. The definition of clinician-observed was broadened to include physical examination or review of a photograph.
- *Neurological domain*. Due to disagreement over whether seizure or cranial neuropathy was more specific for SLE (the SLICC[19] and ACR 1982[20] manuscripts did not present the specificity of these individual items), and because the prevalence of cranial neuropathy is very low in SLE (and none of the 164 patient cases had cranial neuropathy), the group reached consensus to remove cranial neuropathy.
- *Renal domain*. SLE experts decided that Class VI lupus nephritis was not specific for SLE based on clinical experience and lack of published data, and agreed on removing Class VI nephritis. Importantly, since historical manifestations are included in the scoring system, previous evidence of class II, III, IV or V lupus nephritis would be fully accounted for. These steps resulted in the updated definitions depicted in Box 1.

Face validity of the weights and initial upper threshold score

The additive score ranged 0–201 for the 164 cases. SLE experts reviewed the cases in order from highest to lowest score and reached consensus on classifying the 69 highest-scored cases as SLE. The group was unable to reach full consensus for a case with a score of 70; this patient had oral ulcers, leucopaenia, low C3 or C4 and positive anti-dsDNA. The last case for which experts reached consensus (17/17 votes) for classification as SLE had a score of 71, and an initial upper threshold score was set as >70.

Revising criteria weights and provisional upper threshold score

The experts reviewed cases scored 60–70. Many had arthritis and most experts had voted to classify them as SLE. Therefore, the group felt that the weight assigned to arthritis was too low. After reviewing the specific criteria present in these cases, the mucocutaneous domain was reorganised based on expert consensus: acute cutaneous lupus was assigned the most influential position because it is most specific, and subacute cutaneous lupus and discoid lupus were grouped together and less influential than acute cutaneous lupus. Anonymous voting was repeated for pairwise comparisons including arthritis and mucocutaneous criteria. 1000minds software recalculated relative weights for all criteria and rescored all cases using the revised weights.

After this second round of MCDA, arthritis received a greater weight than prior, now identical to the weight of pleural or pericardial effusion. Acute cutaneous lupus was assigned the same weight as acute pericarditis and anti-dsDNA (table 1). The group repeated the anonymous voting exercise and reached consensus about the 82 highest-scored cases. Experts were unable to reach full consensus for the same case that determined the initial threshold. As that case now had a score of 83 using the revised weights, a 100% specific provisional consensus threshold was set as >83. Provisional criteria weights resulting from the MCDA exercise are shown in table 1.

Lower threshold score

SLE experts individually rated the 82 cases below the upper threshold score; the distribution of expert opinion is shown in figure 2. The score of the case for which ≥70% indicated 'unlikely SLE' was 27. Only 7 of 52 unique cases (13.5%) included in this exercise would be classified as 'unlikely SLE' based on this lower threshold, and the remaining 86.5% would potentially be candidates for inclusion into observational or preventive studies. Through a series of telephone calls and emails, it became clear that expert opinion varied considerably concerning the cases below the upper threshold. Additionally, the terms 'probable', 'possible' and 'unlikely' were not being uniformly interpreted. The SLE experts decided against assigning a lower threshold because it would exclude only a few cases from clinical studies.

Table 1 Provisional SLE classification criteria weights determined by a multicriteria decision analysis exercise

Clinical domains and criteria	Weight (points)	Immunological domains and criteria	Weight (points)
Constitutional		Antiphospholipid antibodies	
Fever	13	Anticardiolipin IgG >40 GPL units or anti-β2GP1 IgG >40 units or lupus anticoagulant positive	13
Haematological		Complement proteins	
Leucopaenia	12	Low C3 or low C4	19
Thrombocytopaenia	26	Low C3 and low C4	27
Autoimmune haemolysis	28	SLE-specific antibodies	
Neuropsychiatric		Anti-dsDNA antibody	38
Delirium	12	Anti-Smith antibody	40
Psychosis	20		
Seizure	34		
Mucocutaneous			
Non-scarring alopecia	13		
Oral ulcers	14		
Subacute cutaneous or discoid lupus*	29*		
Acute cutaneous lupus	38		
Serositis			
Pleural or pericardial effusion	34		
Acute pericarditis	38		
Musculoskeletal			
Synovitis in ≥2 joints	34		
Renal			
Proteinuria>0.5 g/24 hours	27		
Renal biopsy with Class II or V lupus nephritis	55		
Renal biopsy with Class III or IV lupus nephritis	74		

*Subacute cutaneous lupus and discoid lupus each received a weight of 29.
SLE, systemic lupus erythematosus.

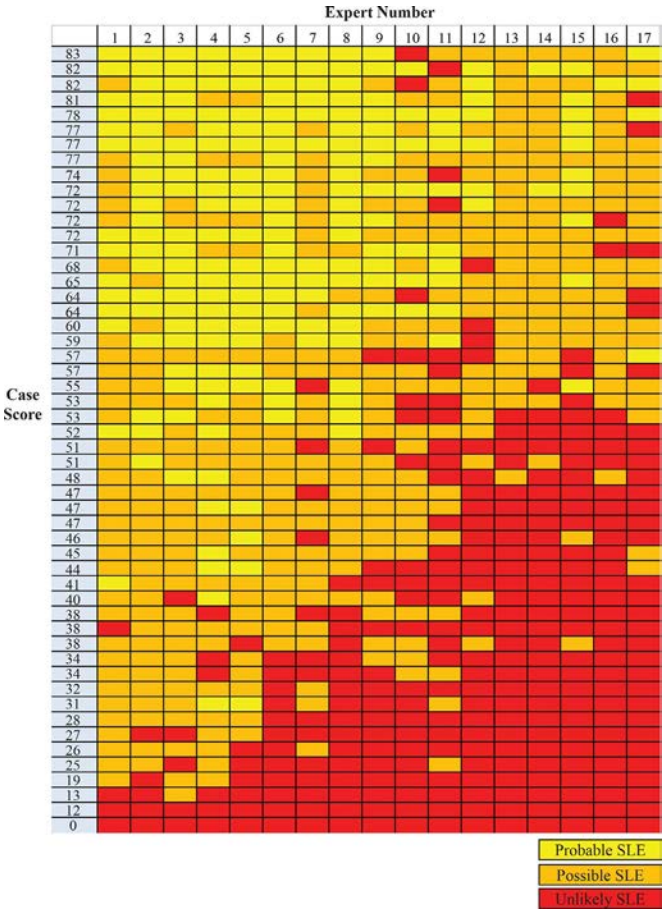

Figure 2 Exercise to consider a lower threshold. SLE experts anonymously labelled unique cases falling below the preliminary upper threshold as probable, possible or unlikely SLE. Among 82 cases below the upper threshold, 52 had unique combinations of criteria. Rows represent unique cases. Columns represent ratings from each SLE expert. SLE, systemic lupus erythematosus.

DISCUSSION

In Phase 3 of this SLE classification criteria development project, we applied a consensus-based, data-driven MCDA approach to assign criteria weights and identify a threshold score for SLE classification among adults for clinical research. This exercise resulted in provisional criteria weights that have face validity and are additive, providing a continuous measure of increasing likelihood for SLE based on combinations of criteria. While full consensus of the 17 SLE experts was reached for cases scoring >83 points, it became evident that expert opinions varied for cases with mid-range or low scores. Many cases with scores just under 83 were still considered SLE by the majority of experts, but in an additional exercise focusing on cases below the threshold for definite SLE, very few were deemed 'unlikely SLE' by ≥70% of experts.

This stage was largely based on the items resulting from the Phase 2 nominal group technique exercise[16] and evidence from our literature review of the sensitivity and specificity of the individual candidate criteria.[1] These efforts followed rigorous data-driven and expert-guided criteria development methodology in order to ensure high face and content validity of the items, and high discriminant validity of the criteria set.[21 22] However, our literature review also revealed knowledge gaps about the sensitivity and specificity of some of the newly proposed criteria, thus expert consensus opinion was critical for decision making.

Consistent with developing other systems of classification criteria,[23 24] there were significant discrepancies in ranking 20 cases regarding likelihood of SLE classification. Discussions centred on two aspects: (1) the precision and thus specificity of clinical and serological manifestations and (2) attribution of manifestations to SLE versus other connective tissue diseases. Some experts expressed concern about misinterpretation of rosacea as acute cutaneous lupus and about false positive anti-dsDNA via ELISA, each of which would reduce the specificity of the proposed classification system. To address these concerns, SLE experts agreed to include detailed definitions for each criterion to mitigate the risk of misinterpreting clinical signs and symptoms. Because particular laboratory assays (eg, Farr method for anti-dsDNA) are not uniformly available in all clinical settings, SLE experts decided that the testing method would not be specified, enabling SLE classification in a wide range of clinics.

The attribution of manifestations to SLE was discussed at length. For some cases, SLE experts were uncertain about how to interpret particular findings when SLE and another disease, such as primary antiphospholipid syndrome or Sjögren's syndrome, seemed equally likely. It became apparent that not all these decisions could be made with certainty and that SLE experts from

different centres could reach opposing conclusions. The criteria system allows for SLE classification in patients with overlap syndromes (eg, SLE with secondary Sjögren's) as long as manifestations are considered to be equally or more likely due to SLE than the other condition.

The decision to exclude Class VI lupus nephritis was unanimous, given the lack of specificity of this end-stage finding. The discussions leading to the consensus elimination of mononeuropathy and cranial neuropathy were of greater interest. It was first mentioned that the specificities of these entities differed and that mononeuropathy is not specific for SLE. The group reached full consensus to eliminate mononeuropathy; cranial neuropathy was initially retained. The group then discussed that cranial neuropathy is a very rare presenting sign in SLE[25] and none of the 164 cases had cranial neuropathy. Experts reached a unanimous decision that the low prevalence of cranial neuropathy in SLE warranted its elimination.

Using a data-driven approach based on literature review[1] combined with an expert-driven MCDA process based on real patient cases, this third phase of the SLE classification project has led to precisely defined criteria with individual weights derived through consensus decisions by 17 international SLE experts. The individual criteria weights have face validity, and taken together they depict current expert understanding of SLE. The provisional threshold sets a high bar for SLE classification (100% specificity), and Phase 4 will consider the appropriate balance between specificity and sensitivity before finalising the threshold. The provisional classification criteria and threshold resulting from Phase 3 are being refined and validated in a large, distinct set of patient cases to finalise the project.

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TRANSLATIONAL SCIENCE

Treatment of primary Sjögren's syndrome with ianalumab (VAY736) targeting B cells by BAFF receptor blockade coupled with enhanced, antibody-dependent cellular cytotoxicity

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ABSTRACT

Objectives To evaluate the efficacy and safety of ianalumab (VAY736), a B cell-depleting, B cell activating factor receptor-blocking, monoclonal antibody, in patients with active primary Sjögren's syndrome (pSS) in a double-blind, placebo-controlled, phase II, single-centre study.

Methods Patients with pSS, EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) ≥6, were randomised to ianalumab single infusion at either 3 mg/kg (n=6), 10 mg/kg (n=12) or placebo (n=9). Outcomes were measured blinded at baseline and weeks 6, 12, 24, and unblinded at end of study (EoS) when B cell numbers had recovered. Clinical outcomes included ESSDAI, EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI), salivary flow rate, ocular staining score, physician global assessment and patient assessments of fatigue and general quality of life. Laboratory-based measures included circulating leucocyte subsets and markers of B cell activity.

Results A similar trend showing positive therapeutic effect by ianalumab was observed across the primary clinical outcome (ESSDAI) and all secondary clinical outcomes (ESSPRI, Multidimensional Fatigue Inventory, Short Form-36, global assessments by physician and patient) versus the placebo-treated group. Rapid and profound B cell depletion of long-lasting duration occurred after a single infusion of ianalumab at either dose. Serum Ig light chains decreased, with return to baseline levels at EoS. Changes in some clinical outcomes persisted through to EoS in the higher dose group. Adverse effects were largely limited to mild to moderate infusion reactions within 24 hours of ianalumab administration.

Conclusions Overall results in this single-dose study suggest potent and sustained B cell depletion by ianalumab could provide therapeutic benefits in patients with pSS without major side effects.

Key messages

What is already known about this subject?

- Patients with primary Sjögren's syndrome (pSS) display numerous signs of B cell activation that appear involved in the pathobiology underlying this autoimmune disease. However, demonstrating the clinical benefits of depleting B cells in these patients have proved challenging in randomised, controlled clinical trials.
- B cell activating factor (BAFF) is a key cytokine for B cells, promoting their maturation, proliferation and survival. Elevated BAFF levels are often present in patients with pSS, supporting autoimmunity and potentially blocking therapeutic elimination of pathogenic B cell clones.

What does this study add?

- This is the first reported use of ianalumab (VAY736), a novel targeted biologic against the BAFF receptor (BAFF-R) on B cells with dual mechanisms of action: direct lysis of B cells and blockade of BAFF:BAFF-R signalling with its receptor.
- This study demonstrates the safety and efficacy of a single dose of ianalumab administered to patients with pSS.

How might this impact on clinical practice or future developments?

- Direct depletion of B cells coupled with blockade of BAFF:BAFF-R signalling may provide more thorough elimination of pathogenic B cells in patients with pSS and improve clinical outcomes.
- Ianalumab is currently under development by Novartis for the treatment of pSS.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease of unknown aetiology characterised primarily by lymphoid infiltration and progressive destruction of exocrine glands.[1] Nearly all patients suffer from mucosal dryness, fatigue and diffuse musculoskeletal pain, with a subset of patients experiencing extraglandular disease manifestations with increased risk for lymphoma development.

Treatment is limited to symptomatic care of mucosal dryness. Steroids and typical

disease-modifying antirheumatic drugs are ineffective, and there is no pharmacological intervention against the fatigue. Early efficacy of B cell depletion therapy in patients with pSS using anti-CD20 monoclonal antibody (mAb) rituximab[2 3] was not replicated in subsequent studies,[4–6] linked to persistence of Ig-producing clonal cells within the salivary glands.[7]

Patients with pSS have elevated levels of B cell activating factor (BAFF) correlating with disease activity, ectopic germinal centre formation and serum autoantibody levels.[8 9] The BAFF receptor (BAFF-R; synonyms BR3, TNFSF13C) is predominantly expressed on B cells. Signalling of BAFF through the BAFF-R is critically involved in B cell maturation, activation and survival, and for isotype class switching in response to T cell-dependent antigens.[10] High baseline BAFF levels in pSS and other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are inversely correlated with extent and duration of B cell depletion by rituximab, two efficacy markers for this treatment.[9 11–13]

Ianalumab (VAY736) is a human IgG1/κ mAb designed to target human BAFF-R and to competitively inhibit binding of BAFF to BAFF-R, thereby blocking BAFF-R-mediated signalling in B cells (online supplementary method 1). In addition, ianalumab was engineered to effectively eliminate B cells from circulation in vivo by antibody-dependent cellular cytotoxicity (ADCC). ADCC activity of ianalumab is greatly enhanced by elimination of fucose residues from the carbohydrate moiety attached to the Fc part of the antibody.[14] Accordingly, ianalumab shows potent ADCC activity in vitro with an EC50 of 2.0 pM (online supplementary method 2). Thus, ianalumab eliminates BAFF-R + mature and immature B cells via dual mechanisms: (1) antibody-dependent cytotoxicity (ADCC) and (2) induction of B cell apoptosis by blocking BAFF:BAFF-R interaction and downstream survival pathway in B cells. BAFF-R expression is limited to immature and mature B cells up to the lymphoblast stage, and thus earlier stage pro-B and pre-B cells are not directly affected by ianalumab. Consequently, ianalumab should represent a more effective therapeutic agent in B cell-driven autoimmune diseases with high BAFF levels such as pSS.[8 9] This clinical study was designed to evaluate safety, tolerability, pharmacokinetics (PK) and therapeutic efficacy of a single ianalumab intravenous infusion in patients with pSS to enable further development of the compound for treating this disease population.

METHODS

This single-centre study (NCT02149420) was conducted between 23 May 2014 and 7 February 2018 (online supplementary method 3). Patients enrolled are 18–75 years fulfilling revised European US consensus criteria for pSS,[15] antinuclear antibody (ANA) ≥1:160 and seropositive for rheumatoid factor (RF) or for anti-Sjögren's-syndrome-related antigen A (anti-SSA), with stimulated whole salivary flow rate of >0 mL/min and active disease (EULAR Sjögren's Syndrome Disease Activity Index [ESSDAI] ≥6). Exclusion criteria included concurrent connective tissue diseases, therapy with prednisone >10 mg/day or azathioprine within 84 days of randomisation, prior use of any B cell depleting therapy, or use of other biologics within 180 days prior randomisation. All patients provided written informed consent before study participation. The protocol and informed consent were approved by local ethics committee before study initiation.

Treatment

Patients meeting eligibility criteria were enrolled into two sequential cohorts (figure 1). Ianalumab (150 mg lyophilisate) was reconstituted with water and diluted in 5% dextrose infusion bag. Placebo was administered as vehicle only. Paracetamol 500 mg was administered 1 hour prior and 5 hours after ianalumab dosing. Patients were allowed use of artificial tears and artificial saliva/salivary stimulants outside of a 48-hour period before or during disease activity assessments.

Assessment time points

Blinded safety and efficacy markers were assessed on day 1 prior to drug administration and again at weeks 6, 12, 24 and at end of study (EoS). Patients returned for safety and PK evaluations at days

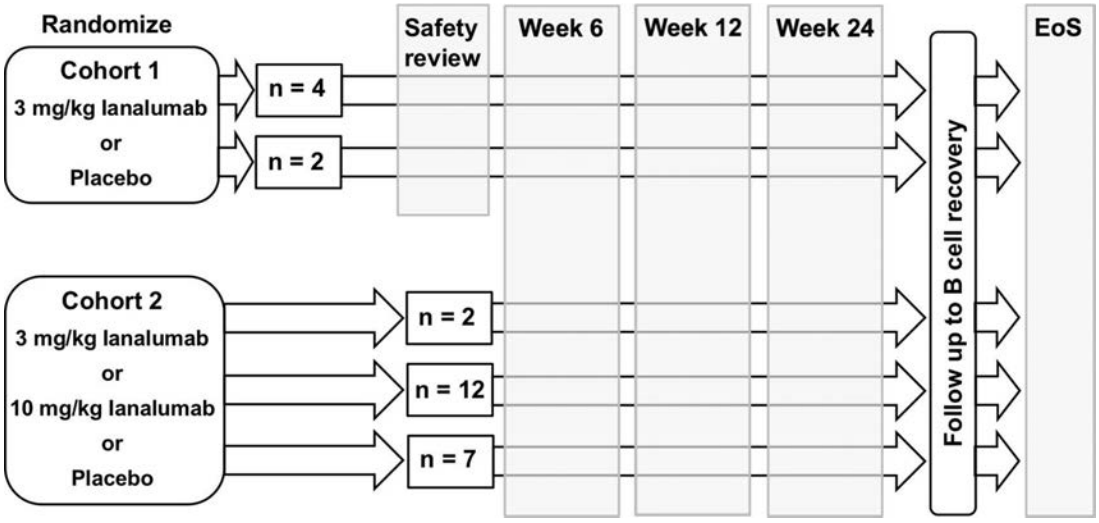

Figure 1 Study design. Cohort 1: six patients randomised to receive single intravenous dose at 3 mg/kg ianalumab or placebo at 2:1 ratio, and cohort 2: 21 patients randomised to receive single intravenous dose ianalumab at 3 or 10 mg/kg, or placebo at a 1:6:3 ratio, respectively. A blinded review of safety data was performed on the first six patients of cohort 1 prior to advancing to cohort 2. Main study visit schedule was as follows: 28-day screening period prior to randomisation, baseline assessments and dosing over domiciled day 1 and day 2, study visits at weeks 1, 2, 3, 6, 9 and 12, then every 4 weeks until week 24. Safety follow-up visit schedule after week 24 was as follows: every 8 weeks until week 40, every 12 weeks to week 52, every 24 weeks to week 100 and every 48 weeks thereafter. Patients achieving B cell recovery criteria proceeded to EoS within 4 weeks. EoS, end of study.

8 and 15, and weeks 3, 6, 9 and 12, and at 4-week intervals thereafter until week 24. Following week 24 assessments, patients were unblinded. Patients achieving B cell recovery criteria, defined as ≥80% of baseline levels or ≥50 cells/μL,[16] proceeded to EoS visit within 6 weeks. Otherwise, patients remained in study for monitoring until meeting recovery criteria.

Clinical outcomes

The primary clinical outcome was the ESSDAI.[17 18] Patient-reported outcomes included the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI),[19] Multidimensional Fatigue Inventory (MFI)[20] and Medical Outcomes Study Short Form-36 (SF-36).[21] Physician's global assessment (PhGA) and patient's global assessment (PaGA) were measured by 100 mm visual analogue scale (VAS). Functional clinical outcomes of disease activity included stimulated and unstimulated salivary flow rate and ocular staining score.[22]

Laboratory-based outcomes

Selective B cell depletion was evaluated by flow cytometry gated on CD45+ leucocytes to measure absolute counts of CD19+ B cells, B cell subsets and other leucocyte classes (online supplementary method 4). Soluble serum biomarkers included BAFF, autoantibodies (RF, ANA, anti-SSA and anti-SSB), β2 microglobulin, immunoglobulins (IgG, IgM) and free Ig light chains (kappa, lambda).

Pharmacokinetics

Serum ianalumab levels were measured on day 1 (before dose and at 2 hours after dose) and at each subsequent visit by validated ELISA (online supplementary method 5) with lower limit of quantification of 0.025 μg/mL.

Statistical analysis

The primary analysis for change from baseline in ESSDAI was conducted via a Bayesian repeated measures model, including data up to week 24. The posterior probabilities were used to evaluate the predefined dual efficacy criteria: more reduction in ESSDAI week 12 in ianalumab-treated patients than placebo with high confidence (90%), and an average magnitude (50%) of effect of 5 points more reduction in ESSDAI week 12 compared with placebo, where the 5 points more reduction is considered clinically meaningful difference. The study was powered for comparison of ianalumab treatment versus placebo regardless of dose levels. However, the data revealed some consistent difference in response between the two ianalumab groups; therefore, the Results section will focus on the individual ianalumab groups for discussion.

RESULTS

Patient characteristics

A total of 27 patients were enrolled and randomised (figure 1) as follows: 3 mg/kg ianalumab (n=6), 10 mg/kg ianalumab (n=12), placebo (n=9). All enrolled patients completed the initial 24-week blinded period and were included for analysis, and all 18 patients from the ianalumab treatment groups completed the study through to time of B cell recovery.

Demographic and other baseline parameters were comparable for the three treatment groups (table 1). The primarily female patients had moderate to severe disease of ESSDAI between 6 and 19, except for one patient in the 3 mg/kg ianalumab group scoring 31. There was also comparable prior use of background corticosteroids, hydroxychloroquine and methotrexate that remained stable throughout the study.

Table 1 Demographic and baseline disease characteristics of enrolled study patients

	Placebo n=9	Ianalumab 3 mg/kg n=6	Ianalumab 10 mg/kg n=12
Age in years, median (range)	50.0 (28, 58)	49.0 (32, 56)	58.5 (25, 70)
Female, n (%)	7 (77.8)	5 (83.3)	11 (91.7)
Caucasian, n (%)	9 (100.0)	6 (100.0)	12 (100.0)
Baseline ESSDAI	10.0 (6, 19)	12.5 (6, 31)	10.0 (6, 18)
Baseline ESSPRI	6.3 (3.0, 9.0)	6.3 (4.7, 7.7)	6.8 (3.0, 8.7)
Intake of oral corticosteroid daily dose in mg, median (range)	5.0 (5.0, 5.0)	5.0 (5.0, 7.5)	2.5 (1.0, 9.0)

ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; ESSPRI, EULAR Sjögren's Syndrome Patient Reported Index.

EULAR Sjögren's Syndrome Disease Activity Index

After one infusion per patient, the difference at week 12 in the change from baseline ESSDAI between placebo and ianalumab-treated patients, including the combined ianalumab group as well as the two individual dose groups, did not meet the predefined criteria. High variability in this endpoint was observed especially in the ianalumab treatment groups (figure 2). Evaluation of individual ESSDAI domains revealed that most improvement was in the articular domain with ianalumab treatment (data not shown). ESSDAI scores at EoS in patients treated with 10 mg/kg ianalumab remained at reduced levels achieved over the 24-week blinded period, while scores in patients receiving 3 mg/kg ianalumab returned towards baseline values.

Patient-reported outcomes

The ESSPRI is the average of scores for the three symptoms of dryness, fatigue and pain. Changes in ESSPRI over the study course are shown in figure 2. At week 12, no significant difference was observed in change from baseline of ESSPRI between ianalumab groups and placebo. In the 3 mg/kg ianalumab group, ESSPRI reduction appeared transient; an early reduction at week 6 returned towards baseline by week 12 and even increased at EoS. In contrast, ESSPRI reduction in the 10 mg/kg ianalumab group was maintained until week 24 and at EoS. A repeated measurement model for this outcome revealed greater reductions in the 10 mg/kg ianalumab group versus placebo at week 12 (1.55 points; 95% CI 0.03 to 3.08) and at week 24 (1.92 points; 95% CI 0.33 to 3.52). Individual evaluation of the three ESSPRI components suggested more improvement occurred in dryness and fatigue for most ianalumab-treated patients compared with patients receiving placebo, especially at week 12 (data not shown).

Severe fatigue affects up to 70% of patients with pSS and is a major contributor to disease-associated disability.[23 24] The MFI individually assesses five different parameters of fatigue, including general fatigue, physical fatigue, mental fatigue, reduced activity and reduced motivation. Early but transient response to treatment with 3 mg/kg ianalumab was observed at week 6 in all the MFI domains (statistically significant for general fatigue and physical fatigue), with scores returning to baseline by week 24 (physical fatigue, figure 2; others in online supplementary figure S1). For the 3 mg/kg ianalumab group at week 6 there was a 5.4-point greater reduction from baseline in general fatigue score (95% CI 0.97 to 9.72) and in physical fatigue score there was a 4.4-point greater reduction from baseline (95% CI 0.87 to 7.96), both with statistical significance. In contrast, in the 10 mg/kg ianalumab group, early reductions were observed at week 6 for all MFI domains,

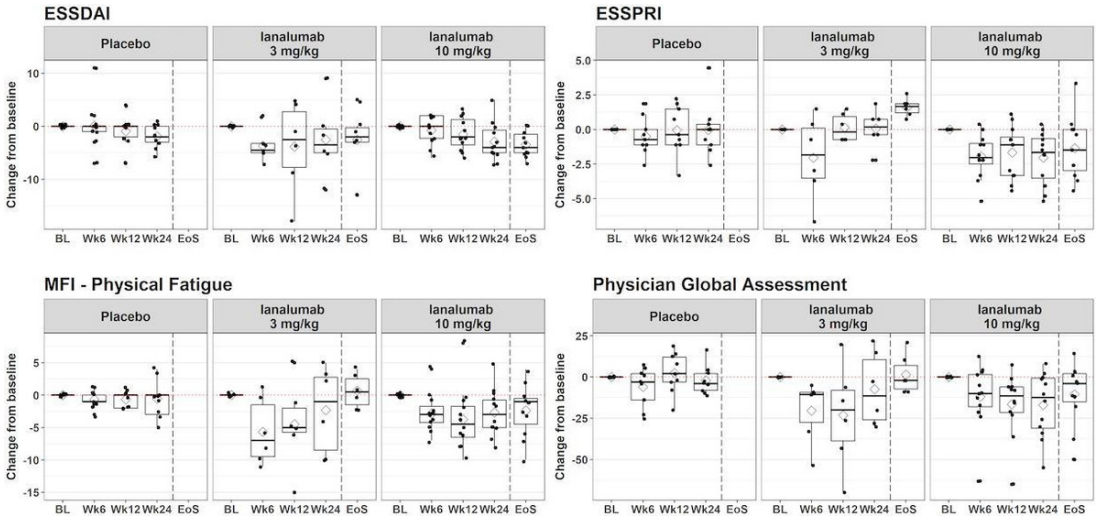

Figure 2 Time course of selected clinical outcomes in the first 24 weeks and EoS. Change from baseline values is shown in box plots. Arithmetic means are shown in diamonds in the box plots. Dotted horizontal line denotes a change from baseline of zero, that is, no difference from baseline. Time of actual EoS visits ranged from 39 to 154 weeks. Comparative EoS data are lacking for placebo arm patients due to transition after week 24 to open-label ianalumab treatment. BL, baseline; EoS, end of study; ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; ESSPRI, EULAR Sjögren's Syndrome Patient Response Index; MFI, Multidimensional Fatigue Inventory.

sustained between week 6 and week 24, and continued to EoS for the MFI domains of general fatigue, physical fatigue and reduced activity.

Changes from baseline over the study for SF-36, PaGA and PhGA were similar to patterns observed for ESSDAI, ESSPRI and MFI (figure 2, online supplementary figures S2 and S3). Early responses in patients receiving 3 mg/kg ianalumab tended to be transient and returned towards baseline levels by week 24 and EoS. Patients in the 10 mg/kg ianalumab group tended towards more sustained clinical responses up to week 24 and, for PaGA and PhGA, responses, extended out to EoS.

Salivary flow rate and ocular staining score

Variability for these two outcomes was high in all groups, making difficult any comparison between ianalumab groups and placebo groups (online supplementary figures S4 and S5). Ianalumab-treated patients had numerically greater increases in unstimulated salivary flow from week 6 to week 24 and in stimulated salivary flow from week 12 to week 24, with both measurements declining back towards baseline at EoS in ianalumab-treated patients. Ocular staining scores for both eyes in patients receiving 10 mg/kg ianalumab remained reduced from baseline through to EoS, but of uncertain clinical relevance.

Laboratory biomarker analysis

B cell and B cell subsets. Minimal changes occurred over time in circulating CD19+ B cell numbers for placebo-treated patients. In contrast, a single infusion of ianalumab resulted in rapid and profound depletion of CD19+ B cells in the two-dose groups (figure 3). Patients in the 10 mg/kg ianalumab group widely varied, with a time to reach minimum, post-treatment B cell numbers ranging from 1 to 83 days. In contrast, patients receiving the 3 mg/kg dose achieved maximum depletion by 2 weeks after treatment.

Time to B cell recovery also varied considerably between individual patients, ranging from week 7 to week 148, both extreme cases occurring in the 3 mg/kg ianalumab group. Recovery time in the 10 mg/kg dose group ranged from 16 to 76 weeks. Median recovery time was 402 days in the 3 mg/kg ianalumab group and 224 days in the 10 mg/kg ianalumab group. Within the CD19+ B cell population, substantial depletion occurred within 24 hours in the peripheral blood mature, naive, memory and transitional B cell subsets (data not shown). By EoS, these B cell subpopulations had returned to baseline levels with the exception of memory B cells which were increased relative to naive B cells. Additionally, at day 2 after ianalumab exposure, transient reductions occurred in non-B cell leucocytes, primarily of effector cells involved in B cell lysis (eg, T cells and natural killer cells). All largely recovered back towards baseline levels by day 7 (data not shown). No impact on leucocyte surface activation markers related to the treatment was observed.

B cell activity markers. There was no consistent difference in percentage change from baseline in autoantibody levels. However, compared with placebo-treated patients, there were numerically greater reductions from baseline in serum levels of free kappa and lambda Ig light chains in ianalumab-treated patients which returned to baseline by EoS (online supplementary figure S6).

Within 24 hours of ianalumab dosing, patients' serum BAFF levels peaked, followed by persistently elevated values until at least week 24, returning towards baseline by EoS (figure 3). Baseline BAFF levels in ianalumab-treated patients did not correlate with the change from baseline in any clinical outcomes at week 12 or at week 24 (online supplementary figure S7).

Pharmacokinetics. Ianalumab exhibited a typical PK profile for a mAb of the IgG1 type (figure 4). A 3.3-fold increase in dose resulted in a 3.1-fold increase in area under the curve and a 3.3-fold increase in Cmax. Elimination of ianalumab was relatively fast for a mAb, with an average half-life around 9–10 days.

Safety analysis

Adverse events (AE). Most AEs were mild to moderate in severity without any severe AEs suspected related to ianalumab (table 2). The most commonly observed AE was mild to moderate infusion-related reaction, characterised by acute onset within hours after ianalumab exposure of one or more of the following: headache, fever, chills, nausea and arthralgias. Fifteen patients receiving ianalumab experienced an infusion reaction (83.3%) compared with one placebo-treated patient (11.1%). Infusion reactions were mild (n=3) to moderate (n=12) in severity and not related to dose

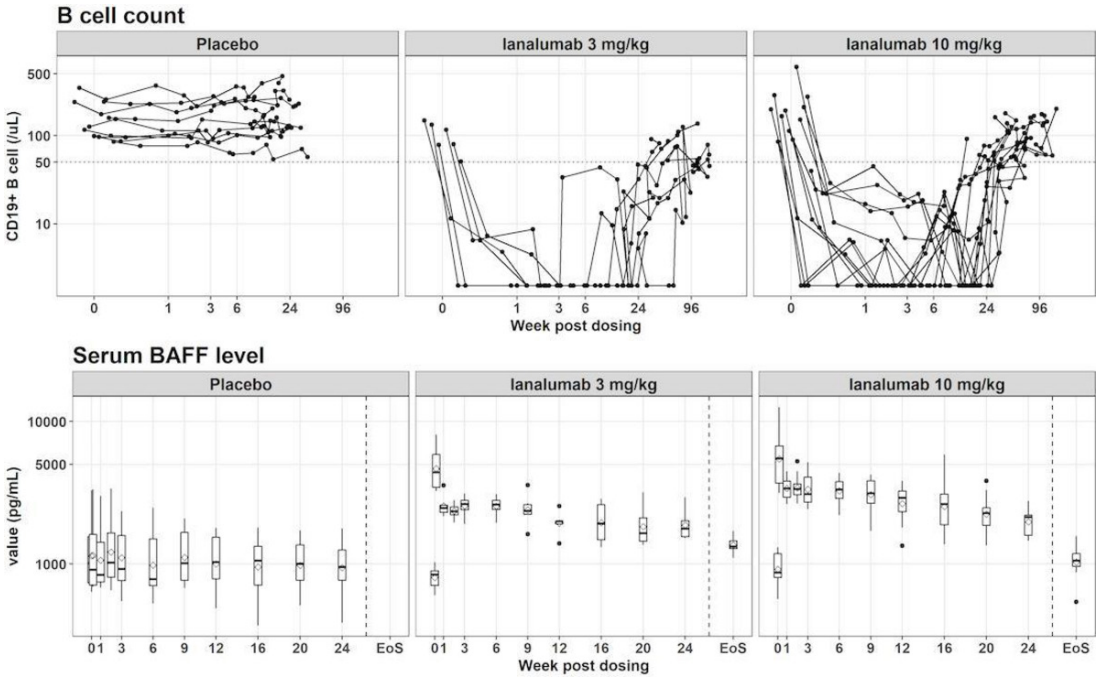

Figure 3 B cell depletion/serum BAFF levels. The top panel shows the individual CD19+ B cell count over time for three treatment groups, with a horizontal line at 50 cells/μL to denote the lower limit of normal. Both x and y axes are presented in log10 scale. The bottom panel shows the BAFF levels in box plots over time, where the arithmetic means are illustrated with diamonds. The y axis is presented in log10 scale. Time of actual EoS visits ranged from 39 to 154 weeks. Absence of EoS data for placebo patients as noted in the Methods section. BAFF, B cell activating factor; EoS, end of study.

but did trend with the number of circulating B cells present at baseline; patients with moderate infusion-related reactions tend to have relatively higher B cell counts at baseline than those with mild reaction or none (online supplementary figure S8). All infusion-related reactions resolved within 24 hours either spontaneously or with additional paracetamol treatment.

The reported incidence of nasopharyngitis was also higher in ianalumab-treated patients (n=6; 33.3%) versus placebo-treated patients (n=1; 11.1%). There was no increase in infections otherwise in the ianalumab group versus placebo group, nor in the incidence of other AEs over the 24-week, blinded study period.

Clinical laboratory findings. Treatment with ianalumab was not associated with significant changes in circulating neutrophils or IgG levels. IgM levels decreased in ianalumab-treated patients but remained within normal limits and had largely returned to baseline levels by EoS (online supplementary figure S9).

DISCUSSION

In this trial, the strongest benefit observed in these patients with pSS after receiving ianalumab (VAY736) was reduction of fatigue, a major pSS disease component typically resistant to therapeutic intervention.[6] Patients treated with ianalumab showed reductions

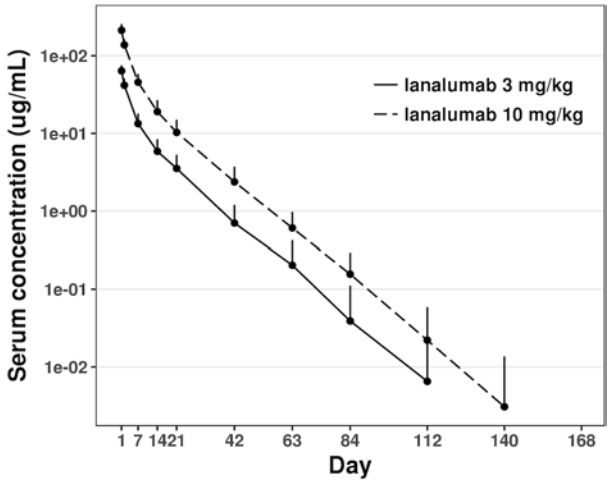

Figure 4 VAY736 concentration-time profiles. Arithmetic mean of VAY736 serum concentration profiles after a single intravenous dose of 3 mg/kg (solid line) and 10 mg/kg (long dash line, only double-blind period), along with SD illustrated by a short vertical interval. The y axis is shown in log10 scale.

Table 2 Most frequent AEs occurring in two or more patients reported during double-blind period

AE preferred terms	Placebo n=9 n (%)	Ianalumab 3 mg/kg n=6 n (%)	Ianalumab 10 mg/kg n=12 n (%)	Total n=27 n (%)
Infusion-related reaction	1 (11.1)	6 (100.0)	9 (75.0)	16 (59.3)
Nasopharyngitis	1 (11.1)	4 (66.7)	2 (16.7)	7 (25.9)
Headache	2 (22.2)	1 (16.7)	2 (16.7)	5 (18.5)
Gastrointestinal infection	1 (11.1)	0 (0.0)	1 (8.3)	2 (7.4)
Influenza	0 (0.0)	1 (16.7)	1 (8.3)	2 (7.4)
Sinusitis	1 (11.1)	0 (0.0)	1 (8.3)	2 (7.4)
Rash	1 (11.1)	0 (0.0)	1 (8.3)	2 (7.4)
Tooth infection	1 (11.1)	1 (16.7)	0 (0.0)	2 (7.4)
Oropharyngeal pain	1 (11.1)	1 (16.7)	0 (0.0)	2 (7.4)
Non-cardiac chest pain	2 (22.2)	0 (0.0)	0 (0.0)	2 (7.4)

All infusion-related reactions occurred within 24 hours after dosing.
AE, adverse event.

in all elements of fatigue measured by the MFI, with particularly strong responses in general fatigue and physical fatigue. This is consistent with reported benefits of B cell-targeted therapy on patient fatigue in pSS and other diseases. Reduction of fatigue in response to rituximab has been observed in patients with RA, SLE and chronic fatigue syndrome.[25–27] Patients with pSS receiving open-label rituximab reported reductions in VAS-measured fatigue.[28] However, rituximab effects on fatigue in larger, placebo-controlled pSS studies showed either only transient reduction of fatigue that did not persist at 24 weeks[4] or no reduction at all.[6] Blockade of soluble BAFF by belimumab in patients with SLE enrolled in the BLISS study had significant improvement of their fatigue,[29] with further improvement in responders over the second half year of exposure. The benefits on fatigue observed in this pSS study with ianalumab will require confirmation in larger trials with longer exposure and observation time.

A single dose of ianalumab did not meet predefined criteria for the primary endpoint ESSDAI. In addition, a post hoc analysis was performed for the more recently developed endpoint, the ClinESSDAI,[30] developed particularly for B cell-targeted therapies. The results in this endpoint (online supplementary figure S10) are similar to findings with ESSDAI (figure 2). However, in addition to the benefits on fatigue (MFI), a trend showing positive therapeutic effect by the compound versus placebo was observed for the ESSDAI and across the other key secondary clinical outcomes, including the primary (ESSDAI) and all secondary clinical outcomes (ESSPRI, SF-36, global assessments by physician and patient).

There was variability between the two ianalumab dose groups in the clinical outcomes of ESSDAI, ESSPRI, MFI and patient and physician global assessments. In some outcomes, the effect of 3 mg/kg ianalumab appeared transient, with early signs of improvement at week 6 returning back towards baseline by week 12 or 24. In contrast, patients receiving 10 mg/kg ianalumab showed sustained effects up to week 24. These observations were in accordance with the observed ianalumab exposure, that is, ianalumab quantifiable levels detected approximately up to 8–12 weeks and to 12–16 weeks for the 3 mg/kg and 10 mg/kg dose groups, respectively. This dose response in duration of clinical outcomes suggests that more sustained exposure to the compound may be more effective against the pSS disease process.

Ianalumab was well tolerated by patients, with AEs largely limited to infusion reactions of mild to moderate intensity occurring within 24 hours after infusion. There was a trend between the baseline B cell numbers and severity of infusion reactions, consistent with the rapid lysis of circulating B cells by ianalumab. Aside from an increase in the incidence of nasopharyngitis, there were no other adverse effects associated with ianalumab that were increased compared with placebo. Importantly, there were no incidences of late-onset neutropenia or hypogammaglobulinaemia in these ianalumab-treated patients over the B cell recovery period.

Rapid, selective and profound B cell depletion occurred in these patients after a single infusion of ianalumab at either 3 mg/kg or 10 mg/kg. Depletion was also long lasting, with only 14 of 18 patients achieving minimal B cell recovery criteria at 1 year. Ianalumab-mediated depletion occurred across B cell subsets, including mature, naive, memory and transitional B cells, with an increase observed in the proportion of mature over naïve B cell populations at EoS; a finding also reported for patients treated with rituximab.[28] Additional evidence of ianalumab effects on the underlying pSS disease process includes reduction of Ig free light chains; a parameter characteristically elevated in B cell-driven autoimmunity and correlated in patients with pSS to disease activity and extraglandular involvement.[31]

Initial, sharp peaks in BAFF levels in the immediate, 24-hour period after ianalumab administration are likely due to a combination of factors, including (1) an acute drop in the number of available receptors for BAFF due to receptor binding by ianalumab and to rapid reduction of B cells expressing BAFF-R, (2) release of stored BAFF by lysed cells, and (3) increased BAFF production in response to lowered B cell numbers. BAFF levels remained elevated in these ianalumab-treated patients, with a gradual return to baseline levels in parallel with B cell recovery, consistent with findings with B cell depletion by rituximab in this patient population.[28]

In this study, no correlations were found between baseline BAFF serum levels and the clinical efficacy endpoints. This is in contrast to B cell depletion by CD20-targeted therapy with rituximab where higher baseline BAFF levels correlated with attenuated efficacy.[9 32] Thus, the absence of such correlations may reflect the ianalumab dual mechanisms of action blocking BAFF:BAFF-R signalling as well as direct, ADCC-mediated B cell depletion. This BAFF-R signalling blockade by ianalumab is important because BAFF levels elevated on B cell depletion are thought to protect B cells from depletion by rituximab and to drive disease relapse in patients with SLE.[33] In patients with immune thrombocytopenic purpura, high BAFF levels following rituximab treatment may cause differentiation of pathogenic, long-lived plasma cells.[34] Thus, the ianalumab dual mechanisms of action may provide more thorough B cell elimination within tissues while also reducing the incidence of BAFF-driven disease flare; a hypothesis pursued by SLE studies combining direct B cell depletion and soluble BAFF blockade by initial treatment with rituximab followed by a maintenance regimen with belimumab[35] and also under consideration for pSS (NCT02631538).

In conclusion, the overall results of this study suggest that potent and sustained B cell depletion by ianalumab could lead to therapeutic benefits in patients with pSS without major safety issues. However, it should be noted that this initial phase II trial involved patients of a heterogeneous phenotype recruited according to ESSDAI score. Also, there was no direct evaluation in these patients of drug effect at the tissue level, for example, through minor salivary gland biopsies. There were also inconsistent effects by ianalumab on objective parameters of pSS disease activity such as salivary flow and ocular staining scores. Nevertheless, patients in this single-dose study, though limited to a small number, appeared to benefit from a single infusion of ianalumab, and further efficacy with greater exposure is suggested by sustained clinical changes in higher dosed patients out to EoS; a time for which minimal pharmacodynamic effects remained of the compound. Although the EoS assessments were by necessity open label, these persisting clinical benefits suggest potential for long-term disease modification with ianalumab treatment. Thus, determination of efficacy for ianalumab in pSS will require further investigation of more sustained treatment in larger numbers of patients, and a larger phase II study in patients with pSS is currently underway (NCT02962895).

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Portions of these reported findings have been presented in poster or podium format at the following conferences: (1) Poster presentation at the European Union League Against Rheumatism 2016 Congress: Dörner T, Posch M, Wagner F, Hüser A, Fischer T, Mooney L, Petricoul O, Maguire P, Pal P, Doucet J, Cabanski M, Kamphausen E, Oliver S. Double-blind, randomized study of VAY736 single dose treatment in patients with primary Sjögren's syndrome (pSS). *Ann Rheum Dis* 2016; 75(suppl 2):S300.

(2) Podium presentation at the American College of Rheumatology 2016 National Meeting: Dörner T, Posch M, Wagner F, Hüser A, Fischer T, Mooney L, Petricoul O, Maguire P, Pal P, Doucet J, Cabanski M, Kamphausen E, Kazma R, Oliver S. Safety and efficacy of single dose VAY736 (anti-BAFFR mAb) in patients with primary Sjögren's syndrome (pSS). *Arthritis Rheum* 2016; 68 (suppl S10):3033.

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Patient consent for publication Not required.

Ethics approval This study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation Guidelines for Good Clinical Practice and local regulations. The protocol, consent form and any other written information provided to patients were approved by the Ethik-Kommission des Landesamt für Gesundheit und Soziales Berlin.

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OPEN ACCESS

CLINICAL SCIENCE

Progressive skin fibrosis is associated with a decline in lung function and worse survival in patients with diffuse cutaneous systemic sclerosis in the European Scleroderma Trials and Research (EUSTAR) cohort

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ABSTRACT

Objectives To determine whether progressive skin fibrosis is associated with visceral organ progression and mortality during follow-up in patients with diffuse cutaneous systemic sclerosis (dcSSc).

Methods We evaluated patients from the European Scleroderma Trials and Research database with dcSSc, baseline modified Rodnan skin score (mRSS) ≥7, valid mRSS at 12±3 months after baseline and ≥1 annual follow-up visit. Progressive skin fibrosis was defined as an increase in mRSS >5 and ≥25% from baseline to 12±3 months. Outcomes were pulmonary, cardiovascular and renal progression, and all-cause death. Associations between skin progression and outcomes were evaluated by Kaplan-Meier survival analysis and multivariable Cox regression.

Results Of 1021 included patients, 78 (7.6%) had progressive skin fibrosis (skin progressors). Median follow-up was 3.4 years. Survival analyses indicated that skin progressors had a significantly higher probability of FVC decline ≥10% (53.6% vs 34.4%; p<0.001) and all-cause death (15.4% vs 7.3%; p=0.003) than non-progressors. These significant associations were also found in subgroup analyses of patients with either low baseline mRSS (≤22/51) or short disease duration (≤15 months). In multivariable analyses, skin progression within 1 year was independently associated with FVC decline ≥10% (HR 1.79, 95% CI 1.20 to 2.65) and all-cause death (HR 2.58, 95% CI 1.31 to 5.09).

Conclusions Progressive skin fibrosis within 1 year is associated with decline in lung function and worse survival in dcSSc during follow-up. These results confirm mRSS as a surrogate marker in dcSSc, which will be helpful for cohort enrichment in future trials and risk stratification in clinical practice.

Key messages

What is already known about this subject?
- Recent evidence-based clinical trial design aimed at including patients with high risk for progression of skin fibrosis.
- However, it is unclear, whether mRSS progression is an appropriate surrogate marker for new onset or deterioration of visceral organ disease and mortality in SSc.

What does this study add?
- Using the large EUSTAR cohort, this study could show that mRSS progression within 1 year is associated with long-term lung deterioration, overall disease progression and all-cause mortality.

How might this impact on clinical practice?
- Patients with short term progressive skin disease should be carefully monitored for other organ progression in the following years.
- The results show that mRSS progression is an excellent surrogate marker for long-term disease progression in SSc, which supports the use of mRSS as an end point in clinical trials.

INTRODUCTION

Systemic sclerosis (SSc) is a highly heterogeneous connective tissue disease with major morbidity and mortality caused by the development of visceral organ complications. These include interstitial lung fibrosis, pulmonary arterial hypertension, scleroderma renal crisis (SRC), and cardiac and gastrointestinal involvement.[1] A major challenge for physicians is to identify patients at high risk of future complications before irreversible visceral involvement occurs. With several new disease-modifying agents in late-stage development,[2] improved identification of at-risk patients will become even more important to inform early treatment intervention. In addition, it will provide important information for cohort enrichment in future clinical trials.[3]

Skin fibrosis is a hallmark of SSc. The modified Rodnan skin score (mRSS) rates skin thickness from 0 (normal) to 3 (severe) at 17 body surface areas in a standardised manner.[4] The mRSS is feasible, reliable and sensitive to change, and is now commonly used in routine practice and clinical trials.[5–7]

Using the European Scleroderma Trials and Research (EUSTAR) database, we previously identified short disease duration (≤15 months) and low baseline mRSS (≤22/51) as independent predictors of progressive skin fibrosis (defined as >5 units and ≥25% increment in mRSS at 1-year follow-up) in patients with diffuse cutaneous SSc (dcSSc).[8 9] While

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this evidence-based strategy of including patients with dcSSc with low baseline mRSS can improve cohort enrichment for progressive skin fibrosis in clinical trials,[10] it might lead to recruitment of patients with overall milder disease. Previous studies have suggested that mRSS may be a potential surrogate marker for disease severity and mortality, but these data were derived from older studies and/or selected patients from clinical trials (D-penicillamine).[11 12] Therefore, new data are required to clarify whether worsening skin fibrosis is an appropriate surrogate marker for new onset or deterioration of visceral organ disease and overall survival in dcSSc.

In a previous single-centre retrospective study of patients with early dcSSc, patients with high baseline mRSS and no subsequent skin improvement within 2 years had significantly higher mortality than those with skin improvement irrespective of baseline mRSS, while the results for internal organ-based endpoints were contradictory.[13] The study thus suggested the prognostic value of the evolution of skin fibrosis, in addition to absolute skin scores, in predicting disease outcome for patients with dcSSc. We herein hypothesise that progression of skin fibrosis within 1 year might be associated with progression of visceral organ disease and mortality in dcSSc during follow-up. The aim of the current study was to test this hypothesis in the large, systematic, longitudinal, real-life EUSTAR registry.

METHODS

More details on methods can be found in the online supplement.

Patients and study design

For this observational study, data from patients' visits from 1 January 2009 to 31 August 2017 were exported from the EUSTAR database. The structure of the EUSTAR database and minimum essential dataset have been described previously.[14 15]

Inclusion criteria for the study were classification of SSc (1980 American College of Rheumatology criteria[16]), diffuse cutaneous involvement as described by LeRoy *et al*,[17] at least one available annual follow-up visit, mRSS ≥7 (the minimal value for subclassification as dcSSc) at the first available visit (baseline) and valid mRSS data at 12±3 months after baseline.

Definition of 'progressor' patients

Patients with progression of skin fibrosis (skin progressors) were defined as those with an increase in mRSS >5 units and by ≥25% from baseline to 12±3 months. This mRSS threshold is considered as the minimally clinical important difference.[18] The 1-year period to define skin progression was chosen as it is considered sufficient to capture significant changes in mRSS and is thus frequently used in clinical trials in skin fibrosis.[19]

Follow-up and outcome measures

Follow-up was defined as the time between the first available visit (baseline) and the last available annual follow-up for each patient. All outcome events were accounted during this period. Outcome measures reflecting visceral organ progression were defined by consensus of an expert group (YA, MM-C, JEP, CPD, DK and OD) using the nominal group technique. Organ progression was defined as occurrence of one of the following events during follow-up: (1) relative decrease in FVC ≥10% from baseline; (2) reduction of left ventricular ejection fraction (LVEF) to <45%, or relative decrease of LVEF >10% for patients with baseline LVEF <45%, assessed by echocardiography; (3) new-onset pulmonary hypertension (PH) as globally judged on echocardiography by the treating physician; (4) new-onset SRC; (5) all-cause death.[20–23] Overall disease progression was defined as the presence of any of the above outcomes. In addition, an exploratory analysis in which lung progression was defined as a relative decrease from baseline to follow-up in FVC ≥10%, or 5%–9% combined with diffusing capacity for carbon monoxide (DLCO) ≥15% (instead of definition 1), was performed based on recently proposed criteria.[24]

Statistical analysis

Baseline characteristics were described as mean (SD) for continuous variables and number (frequency) for categorical variables. Baseline variables were compared between skin progressors and non-progressors by univariate analysis followed by Bonferroni correction. Chi-squared tests or Fisher's exact tests were used for categorical variables, and independent sample t-tests were used for continuous variables.

Kaplan-Meier curves and log-rank tests were performed to compare outcomes between skin progressors and non-progressors for up to 8 years of follow-up. Only the first event was considered. Patients with PH or SRC at baseline were excluded from analyses of PH and SRC outcomes, as these patients could not show any new event of these types. Kaplan-Meier analyses were also conducted in subgroups stratifying patients by either baseline mRSS (≤22/51 vs >22/51 units) or disease duration (≤15 vs >15 months). Multivariable Cox regression analyses were performed to examine independent associations between skin progression and both FVC decline ≥10% and all-cause death. Confounding variables for multivariable Cox regression models were selected using the nominal group technique. Spearman rho analyses were conducted to measure the correlation between variables before multivariable regression. Multiple imputation with 10 imputed datasets was used before regression analysis to handle missing values.

Significance was defined as p value <0.05. Statistical analyses were performed by the biostatistician (NG) using R programming language (V.3.3.3), packages 'survival' and 'mice'.[25–27]

RESULTS

Baseline characteristics

In total, 1021 patients were included for analysis, of whom 78 (7.6%) had progression of skin fibrosis at 1-year follow-up. Demographic and clinical characteristics are summarised in table 1. Mean age was 52.0 years, mean disease duration was 7.7 years and mean±SD mRSS was 16.9±7.7 at baseline. Median follow-up was 3.4 years. By using Bonferroni correction, the modified critical p value (α) was determined as 0.0013. Skin progressors had a significantly shorter disease duration at baseline than non-progressors, confirming previous results.[8 9] All other baseline characteristics were comparable between groups (table 1).

Associations between skin progression and visceral organ progression

Lung progression

In total, 282 of 788 patients (35.8%) met the FVC definition of lung progression (relative decrease in FVC ≥10%) during a median follow-up of 3.7 years (IQR 1.8–6.2 years). In the overall cohort, 403 of 670 patients (60.1%) had lung fibrosis on CT scan at baseline. The mean±SD FVC at baseline was 86.9%±20.5%, with 164 patients (20.8%) having a baseline FVC <70%. There were 30 (53.6%) and 252 (34.4%) events in the skin progressor and non-progressor groups, respectively. The probability of FVC decline was significantly higher for skin progressors than

Table 1 Baseline demographic and clinical characteristics of skin progressors and non-progressors

Characteristics	Missing cases, n (%)	Whole cohort (n=1021)	Progressors (n=78)	Non-progressors (n=943)	P value
Demographic					
Age, years (mean±SD)	0 (0)	52.0±13.7	51.7±12.9	52.0±13.7	0.869
Male sex	0 (0)	248 (24.3)	30 (38.5)	218 (23.1)	0.004
Disease duration* years (mean±SD)	78 (7.6)	7.7±7.5	5.3±6.2	7.9±7.5	0.006
≤15 months	78 (7.6)	126 (13.4)	19 (27.9)	107 (12.2)	<0.001
≤36 months	78 (7.6)	298 (31.6)	36 (52.9)	262 (29.9)	<0.001
Vascular					
Raynaud's phenomenon	2 (0.2)	997 (97.8)	74 (94.9)	923 (98.1)	0.141
Digital ulcers	11 (1.1)	384 (38.0)	30 (38.5)	354 (38.0)	1.000
Active digital ulcers	25 (2.4)	199 (20.0)	16 (21.1)	183 (19.9)	0.925
Skin					
mRSS, unit (mean±SD)	0 (0)	16.9±7.7	14.8±6.2	17.1±7.7	0.010
mRSS ≤22/51	0 (0)	819 (80.2)	67 (85.9)	752 (79.7)	0.245
Musculoskeletal					
Tendon friction rubs	11 (1.1)	156 (15.4)	10 (13.0)	146 (15.6)	0.648
Joint synovitis	6 (0.6)	180 (17.7)	16 (20.5)	164 (17.5)	0.607
Joint contractures	7 (0.7)	505 (49.8)	42 (53.8)	463 (49.5)	0.532
Muscle weakness	6 (0.6)	255 (25.1)	17 (22.1)	238 (25.4)	0.614
Gastrointestinal					
Oesophageal symptoms	1 (0.1)	687 (67.4)	51 (65.4)	636 (67.5)	0.795
Stomach symptoms	2 (0.2)	300 (29.4)	27 (34.6)	273 (29.0)	0.361
Intestinal symptoms	3 (0.3)	281 (27.6)	21 (26.9)	260 (27.7)	0.994
Cardiopulmonary					
Dyspnoea (NYHA)	84 (8.2)				0.186
Stage 1		520 (55.5)	34 (51.5)	486 (55.8)	
Stage 2		315 (33.6)	28 (42.4)	287 (33.0)	
Stage 3/4		102 (10.9)	4 (6.1)	98 (11.2)	
Diastolic dysfunction	150 (14.7)	195 (22.4)	12 (18.5)	183 (22.7)	0.526
Pericardial effusion	215 (21.1)	59 (7.3)	7 (12.1)	52 (7.0)	0.238
Conduction blocks	124 (12.1)	123 (13.7)	6 (8.8)	117 (14.1)	0.300
LVEF <45%	266 (26.1)	16 (2.1)	2 (3.4)	14 (2.0)	0.797
Pulmonary hypertension byechocardiography†	138 (13.5)	120 (13.6)	11 (16.7)	109 (13.3)	0.568
Lung fibrosis on CT scan	351 (34.4)	403 (60.1)	33 (60.0)	370 (60.2)	1.000
FVC, % predicted (mean±SD)	168 (16.5)	87.0±20.7	86.6±17.5	87.0±20.9	0.879
FVC <70% predicted	168 (16.5)	182 (21.3)	13 (21.7)	169 (21.3)	1.000
FEV_1, % predicted (mean±SD)	272 (26.6)	85.7±18.4	87.2±16.5	85.6±18.6	0.547
TLC, % predicted (mean±SD)	427 (41.8)	86.6±20.6	86.5±15.3	86.6±20.9	0.991
DLCO, % predicted (mean±SD)	179 (17.5)	65.6±19.3	65.6±17.2	65.6±19.4	0.995
DLCO <70% predicted	179 (17.5)	479 (56.9)	33 (57.9)	446 (56.8)	0.984
Kidney					
Renal crisis history	4 (0.4)	30 (2.9)	2 (2.6)	28 (3.0)	1.000
Laboratory parameters					
ANA positive	16 (1.6)	961 (95.6)	75 (96.2)	886 (95.6)	1.000
ACA positive	64 (6.3)	88 (9.2)	6 (8.2)	82 (9.3)	0.929
Anti-Scl-70 positive	42 (4.1)	616 (62.9)	49 (66.2)	567 (62.7)	0.628
Anti-U1RNP positive	237 (23.2)	35 (4.5)	1 (1.6)	34 (4.7)	0.514
Anti-RNA polymerase III positive	453 (44.4)	58 (10.2)	5 (9.8)	53 (10.3)	1.000
Creatinine kinase elevation	75 (7.3)	100 (10.6)	8 (10.8)	92 (10.6)	1.000
Proteinuria	78 (7.6)	64 (6.8)	5 (6.9)	59 (6.8)	1.000
Hypocomplementaemia	192 (18.8)	58 (7.0)	3 (4.8)	55 (7.2)	0.613
ESR >25 mm/h	117 (11.5)	371 (41.0)	24 (35.3)	347 (41.5)	0.382
CRP elevation	63 (6.2)	294 (30.7)	31 (41.9)	263 (29.8)	0.041
Active disease (VAI >3)‡	154 (15.1)	340 (39.2)	20 (30.8)	320 (39.9)	0.187
Immunosuppressive therapy§	66 (6.5)	667 (69.8)	54 (73.0)	613 (69.6)	0.632

Definitions of items and organ manifestation are according to EUSTAR.[14]
Data are presented as number (%) unless otherwise stated.
P values of univariate comparisons of baseline characteristics between skin progressors and non-progressors are shown (χ^2 tests or Fisher's exact tests used for categorical variables and independent sample t-tests used for continuous variables, as appropriate).
*Disease duration was calculated as the difference between the date of the baseline visit and the date of the first non-Raynaud's symptom of the disease as reported by the patient.
†Pulmonary hypertension was globally judged on echocardiography by the treating physician.
‡Active disease was defined as a score >3 by calculating European Scleroderma Study Group disease activity indices for systemic sclerosis proposed by Valentini *et al*.[45]
§Immunosuppressive therapy was defined as treatment with corticosteroids (prednisone dose ≥2.5 mg/day or other dosage forms in equal dose) or any immunosuppressant.
ACA, anti-centromere antibody; ANA, antinuclear antibody; Anti-Scl-70, anti-topoisomerase 1 antibody; CRP, C reactive protein;CT, computed tomography; DLCO, diffusing capacity for carbon monoxide; ESR, erythrocyte sedimentation rate; FEV_1, forced expiratory volume in 1 sec; FVC, forced vital capacity; LVEF, left ventricular ejection fraction;mRSS, modified Rodnan skin score.NYHA, New York Heart Association; TLC, total lung capacity; VAI, Valentini activity index;

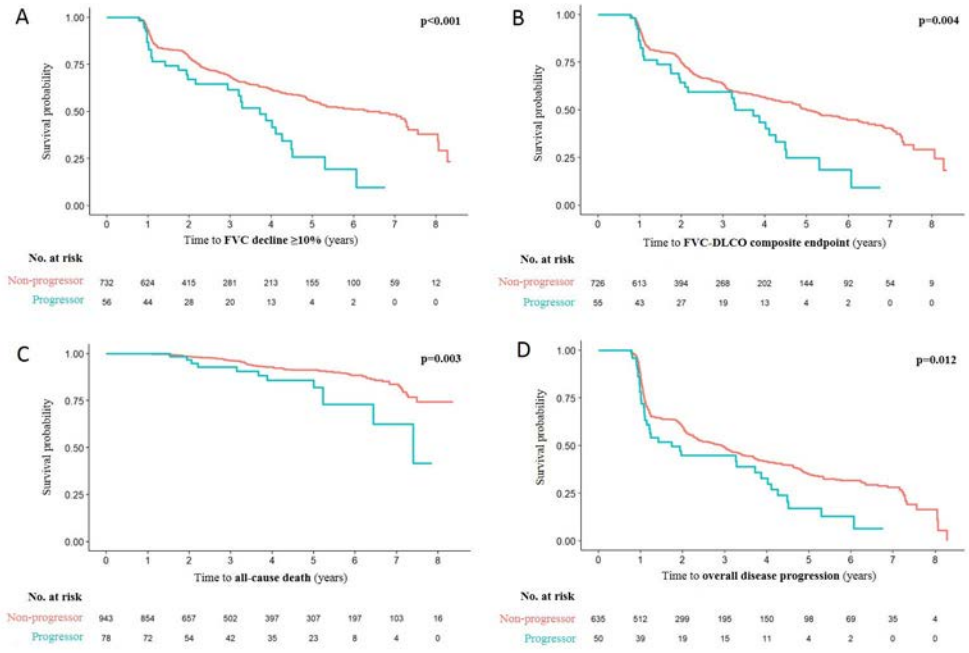

Figure 1 Kaplan-Meier survival plots for (A) time to FVC decline ≥10%, (B) time to FVC-DCLO composite endpoint, (C) time to all-cause death and (D) time to overall disease progression during follow-up depending on the presence or absence of skin progression within 1 year. DLCO, diffusing capacity for carbon monoxide; FVC, forced vital capacity.

non-progressors (log-rank test p<0.001; figure 1A). In the subgroups of patients with low baseline mRSS and short disease duration, which reflect evidence-based recruitment parameters for recent clinical trials in skin fibrosis,[8] skin progressors also had a significantly higher probability of FVC decline than non-progressors (baseline mRSS ≤22/51 units: 27/47 [57.4%] vs 202/596 [33.9%], p<0.001; disease duration ≤15 months: 7/12 [58.3%] vs 26/89 [29.2%], p=0.019, respectively) (figure 2A, C). There was no significant difference in the probability of FVC decline in the subgroups of patients with baseline mRSS >22/51 units and disease duration >15 months (figure 2B, D).

Overall, 320 of 781 patients (41.0%) met the FVC-DLCO composite definition of lung progression (relative decrease in FVC ≥10%, or 5%–9% combined with DLCO ≥15%) during a

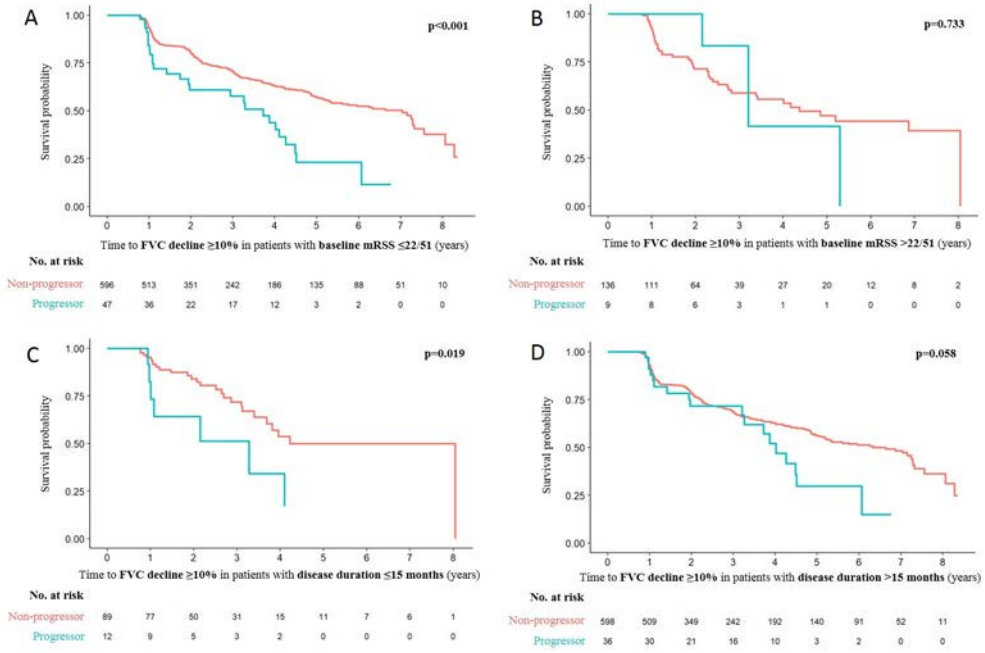

Figure 2 Kaplan-Meier survival plots for FVC decline ≥10% during follow-up depending on the presence or absence of skin progression within 1 year in subgroups of patients with (A) baseline mRSS ≤22/51 units, (B) baseline mRSS >22/51 units, (C) disease duration ≤15 months and (D) disease duration >15 months. FVC, forced vital capacity; mRSS, modified Rodnan skin score.

median follow-up of 3.9 years (IQR 1.9–6.2 years). There were 31 (56.4%) and 289 (39.8%) events in the skin progressor and non-progressor groups, respectively. Again the probability of FVC-DLCO decline was significantly higher for skin progressors than non-progressors (log-rank test p=0.004; figure 1B). In the subgroup of patients with low baseline mRSS, skin progressors also had a significantly higher probability of FVC-DLCO decline than non-progressors (27/47 [57.5%] vs 237/590 [40.2%]; p=0.002). In patients with short disease duration, skin progressors had a trend towards higher probability of FVC-DLCO decline than non-progressors (7/11 [63.6%] vs 29/89 [32.6%]; p=0.050). In the subgroups of patients with baseline mRSS >22/51 units and disease duration >15 months, no significant difference was seen in the probability of FVC-DLCO decline between groups (online supplementary figure S1).

Systolic heart dysfunction and SRC

Despite the large patient cohort, a low number of systolic heart dysfunction and SRC events occurred, limiting interpretation of the data.

During a median follow-up of 3.2 years (IQR 1.3–5.5 years), 15 of 662 patients (2.3%) cumulatively had an LVEF reduction. There were 3 (6.3%) and 12 (2.0%) events in the skin progressor and non-progressor groups, respectively. The probability of LVEF reduction was significantly higher for skin progressors than non-progressors (log-rank test p=0.038; online supplementary figure S2A). However, there was no significant difference in the probability of LVEF reduction between patients with and without skin progression in any subgroup when stratified by either baseline mRSS or disease duration.

During a median follow-up of 3.1 years (IQR 1.6–5.6 years), 21 of 985 patients (2.1%) cumulatively had a new SRC. There were 0 (0.0%) and 21 (2.3%) events in the skin progressor and non-progressor groups, respectively, and no significant difference in the probability of a new SRC between groups (log-rank test p=0.196; online supplementary figure 1). When stratified by either baseline mRSS or disease duration, no significant difference in the probability of a new SRC was observed between patients with and without skin progression in any subgroup.

Pulmonary hypertension

During a median follow-up of 3.8 years (IQR 1.9–5.8 years), 109 of 693 patients (15.7%) developed new PH. There were 5 (10.4%) and 104 (16.1%) events in the skin progressor and non-progressor groups, respectively, with no significant difference in probability of new PH between groups (log-rank test p=0.316; online supplementary figure S2C). When stratified by either baseline mRSS or disease duration, the only significant difference in probability of new PH between groups occurred in patients with disease duration >15 months, in whom skin progressors had a significantly lower probability of new PH compared with non-progressors (0/28 [0.0%] vs 89/528 [16.9%], respectively; p=0.026).

All-cause death

During a median follow-up of 3.4 years (IQR 1.8–5.9 years), 81 of 1021 patients (7.9%) died. There were 12 (15.4%) and 69 (7.3%) deaths in the skin progressor and non-progressor groups, respectively. The probability of all-cause death was significantly higher for skin progressors than non-progressors (log-rank test p=0.003; figure 1C). In the subgroups of patients with low baseline mRSS and short disease duration, skin progressors also had a significantly higher probability of all-cause death than non-progressors (baseline mRSS ≤22/51 units: 9/67 [13.4%] vs 54/752 [7.2%], p=0.017; disease duration ≤15 months: 4/19 [21.1%] vs 3/107 [2.8%], p=0.009, respectively) (figure 3A, C). In the subgroups of patients with baseline mRSS >22/51 units and

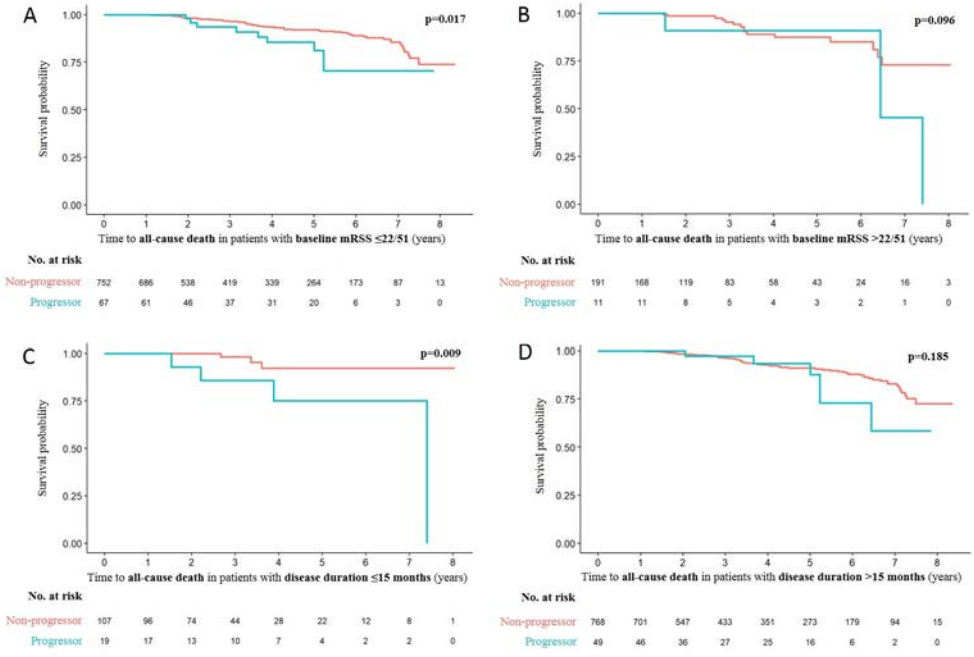

Figure 3 Kaplan-Meier survival plots for all-cause death during follow-up depending on the presence or absence of skin progression within 1 year in subgroups of patients with (A) baseline mRSS ≤22/51 units, (B) baseline mRSS >22/51 units, (C) disease duration ≤15 months and (D) disease duration >15 months. mRSS, modified Rodnan skin score.

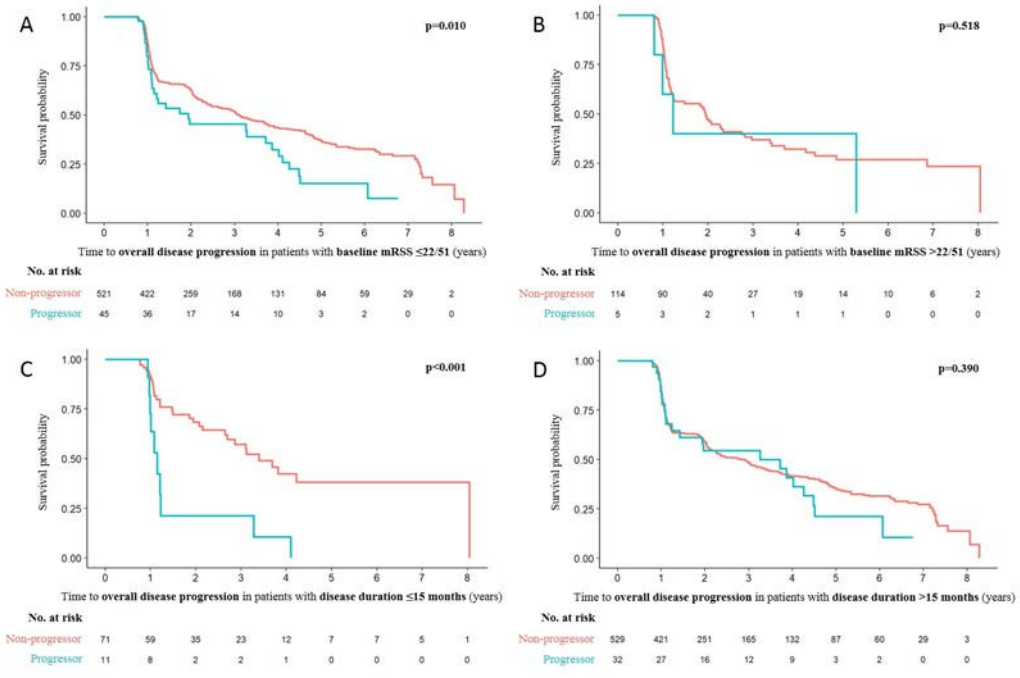

Figure 4 Kaplan-Meier survival plots for overall disease progression during follow-up depending on the presence or absence of skin progression within 1 year in subgroups of patients with (A) baseline mRSS ≤22/51 units, (B) baseline mRSS >22/51 units, (C) disease duration ≤15 months and (D) disease duration >15 months. mRSS, modified Rodnan skin score.

disease duration >15 months, there was no significant difference in probability of all-cause death between groups (figure 3B, D).

Overall disease progression

During a median follow-up of 4.6 years (IQR 2.2–6.6 years), 389 of 685 patients (56.8%) cumulatively had overall disease progression as defined above. There were 37 (74.0%) and 352 (55.4%) events in the skin progressor and non-progressor groups, respectively. The probability of overall disease progression was significantly higher for patients with skin progression than those without (log-rank test p=0.012; figure 1D). In the subgroups of patients with low baseline mRSS and short disease duration, skin progressors also had a significantly higher probability of overall disease progression than non-progressors (baseline mRSS ≤22/51 units: 33/45 [73.3%] vs 283/521 [54.3%], p=0.010; disease duration ≤15 months: 10/11 [90.9%] vs 31/71 [43.7%], p<0.001, respectively) (figure 4A, C). In the subgroups of patients with baseline mRSS >22/51 units and disease duration >15 months, no significant difference was observed in the probability of overall disease progression between groups (figure 4B, D).

Independent associations between skin progression and FVC decline and all-cause death

In the final multivariable Cox regression models, skin progression was independently associated with FVC decline ≥10% (HR 1.79; 95% CI 1.20 to 2.65; p=0.004) and all-cause death (HR 2.58; 95% CI 1.31 to 5.09; p=0.006). History of SRC, LVEF <45%, FVC <70%, DLCO <70% and age at baseline were also independently associated with all-cause death (table 2). Skin progression had a trend-towards association with overall disease progression (HR 1.40; 95% CI 0.98 to 1.99; p=0.063) (online supplementary table S1).

DISCUSSION

We investigated the association between skin progression and subsequent visceral organ progression in the large, prospective, multicentre, real-life EUSTAR cohort. Our findings indicate that patients with dcSSc and skin progression within 1 year have a higher probability of lung progression and worse survival during follow-up. These findings suggest that such patients should be monitored very carefully in clinical practice. The results also support the concept that inclusion of patients with lower mRSS or shorter disease duration can enrich clinical trials for progressive skin fibrosis, and this enrichment leads to study populations with more severe disease at higher risk of organ progression and overall death. Notably, this increased risk of more severe disease occurs at >1 year's follow-up and will thus not be detectable in a classical 1-year randomised controlled trial. Our findings emphasise that mRSS progression within 1 year is an appropriate surrogate marker for more severe disease during follow-up.

This study also provides evidence for cohort enrichment in clinical studies aiming primarily at lung fibrosis. Several parameters, including dcSSc, anti-topoisomerase 1-positive status and decreased baseline FVC have been identified in multiple studies as predictors of lung progression in SSc.[20 28–34] However, few studies have focused specifically on patients with dcSSc. In the current EUSTAR analysis, skin progression was associated with subsequent decline of lung function in patients with dcSSc, even after adjustment for potentially confounding predictors. We examined two definitions of lung progression based on pulmonary function tests. The conventional definition (relative decrease in FVC ≥10%), based on expert group consensus, has been widely used as an endpoint in previous clinical studies, while the exploratory FVC-DLCO composite definition has recently been shown to predict mortality in patients with SSc-related interstitial lung disease.[35] Analyses with both definitions produced similar results, strengthening our findings.

Table 2 Independent factors associated with FVC decline ≥10% and all-cause death as determined by multivariable Cox regression

Baseline characteristics	HR (95% CI)
FVC decline ≥10%	
Skin progression	1.79 (1.20 to 2.65)
Age	1.00 (0.99 to 1.01)
Male sex	0.89 (0.67 to 1.19)
mRSS	1.01 (0.99 to 1.03)
Disease duration	1.00 (0.99 to 1.00)
Lung fibrosis on CT scan	1.25 (0.90 to 1.72)
Pulmonary hypertension by echocardiography	1.31 (0.93 to 1.85)
Dyspnoea NYHA stage ≥2	1.23 (0.94 to 1.62)
Joint synovitis	1.10 (0.81 to 1.49)
FVC <70% predicted	0.89 (0.64 to 1.24)
DLCO <70% predicted	1.28 (0.97 to 1.69)
Anti-Scl-70 positive	0.99 (0.75 to 1.29)
ACA positive	1.07 (0.69 to 1.66)
CRP elevation	1.22 (0.92 to 1.60)
All-cause death	
Skin progression	2.58 (1.31 to 5.09)
Age	1.05 (1.03 to 1.07)
Male sex	1.56 (0.95 to 2.57)
Lung fibrosis on CT scan	1.68 (0.84 to 3.36)
Pulmonary hypertension by echocardiography	0.84 (0.47 to 1.50)
Renal crisis history	3.15 (1.18 to 8.43)
Digital ulcers	1.58 (0.99 to 2.53)
Proteinuria	1.50 (0.74 to 3.04)
LVEF <45%	3.51 (1.22 to 10.12)
FVC <70% predicted	2.60 (1.49 to 4.55)
DLCO <70% predicted	2.00 (1.04 to 3.84)

Factors highlighted in bold are significantly associated with the outcome.
Skin progression is defined as an increase in mRSS >5 and ≥25% from baseline to 12±3 months later.
ACA, anti-centromere antibody; Anti-Scl-70, anti-topoisomerase 1 antibody; CRP, C reactive protein; DLCO, diffusing capacity for carbon monoxide;FVC, forced vital capacity; LVEF, left ventricular ejection fraction;NYHA, New York Heart Association; mRSS, modified Rodnan skin score.

We also found that skin progression within 1 year was independently associated with higher all-cause mortality. Previously, several prognostic studies have tried to predict mortality in patients with SSc. The most common baseline characteristics independently associated with worse survival reported in different cohorts include older age, male sex, dcSSc, lung fibrosis, PH, systolic heart dysfunction, restrictive lung function defect, defective diffusing capacity of the lung, proteinuria, history of SRC and digital ulcers, all of which have been confirmed in studies derived from the EUSTAR database.[21 22 36–44] We included these potentially significant and clinically relevant predictors in our multivariable Cox regression analysis, and found that skin progression, along with several other factors, was still an independent prognostic factor for all-cause death.

In our cohort, average disease duration at baseline was >7 years, indicating that most cases were not early disease. In subgroup analyses, we confirmed that disease course is worse in patients with dcSSc with early disease, although there were also patients with later-stage disease who showed organ progression. This underlines the heterogeneity of the disease course and clinicians should therefore pay attention to all patients with progression of skin fibrosis, even those with longer disease duration. Our findings are supported by the results of a study that focused on early dcSSc using a different definition of skin progression.[23]

One limitation of our analysis is the problem of missing values and loss to follow-up, which was inevitable in such a huge multicentre registry database. This partly explains the low number of patients during long-term follow-up. However, we tried to overcome this by multiple imputation before regression analysis and for most variables there were relatively few missing values. Second, we were unable to determine specific causes of death at all participating centres, and therefore only all-cause mortality, regardless of attribution to SSc, could be assessed. However, all-cause mortality is considered a more robust measure of disease outcome than SSc-associated mortality, as cause of death is often difficult to assign. Third, there was a relatively high proportion of new PH cases during follow-up in our cohort. This was the result of basing the definition on assessment of PH on echocardiography by the treating physician rather than on right heart catheterisation, which is required for formal diagnosis of PH. Unfortunately, right heart catheterisation data are not reliably available in the EUSTAR database, and echocardiography was the best available approximation of PH for the present analysis. Finally, as a result of the observational design, we did not evaluate the effect of treatment on outcomes. However, treatment of SSc, especially with immunosuppressive therapy, is always individualised and organ specific, and it is therefore difficult to accurately exclude the influence of treatment in an unselected heterogeneous cohort. In addition, there is a meaningful treatment-by-indication error in observational studies, making interpretation of results difficult. In our cohort, the proportions of patients receiving immunosuppressive treatment between groups at baseline were equal.

In conclusion, progressive skin fibrosis is associated with decline in lung function and worse survival in dcSSc during follow-up. The evidence-based findings obtained from the large prospective EUSTAR cohort allow optimisation of cohort enrichment in future clinical trials aimed at skin and lung fibrosis, and also help clinicians to identify patients at risk of lung progression in clinical practice.

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CLINICAL SCIENCE

[18F]Florbetapir positron emission tomography: identification of muscle amyloid in inclusion body myositis and differentiation from polymyositis

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ABSTRACT

Objectives With the tools available currently, confirming the diagnosis of inclusion body myositis (IBM) can be difficult. Many patients are initially misdiagnosed with polymyositis (PM). In this observational study at a UK adult neuromuscular centre, we investigated whether amyloid positron emission tomography could differentiate between IBM and PM.

Methods Ten patients with IBM and six with PM underwent clinical review, [18F]florbetapir positron emission tomography and MRI of skeletal musculature. Differences in [18F]florbetapir standardised uptake value ratios in skeletal muscle regions of interest were evaluated. Relationships between [18F]florbetapir standardised uptake value ratios and measures of disease severity (clinical and by MRI of skeletal muscle) were assessed.

Results [18F]florbetapir standardised uptake value ratios were significantly higher in those with IBM compared with PM for all assessed regions (total-[18F]florbetapir standardised uptake value ratio 1.45 (1.28 to 2.05) vs 1.01 (0.80 to 1.22), p=0.005). For total-[18F]florbetapir standardised uptake value ratios≥1.28, sensitivity and specificity for IBM was 80% and 100%, respectively.

Conclusions [18F]florbetapir amyloid positron emission tomography differentiates IBM from PM. Successful development could facilitate accurate diagnosis, inclusion in clinical trials and help avoid unnecessary exposure to potentially harmful treatments.

Key messages

What is already known about this subject?

- Positron emission tomography can detect tissue deposits of amyloid, potentially allowing non-invasive differentiation of inclusion body myositis (IBM) from polymyositis (PM).

What does this study add?

- Significantly increased intramuscular amyloid levels were found in IBM.
- Amyloid levels generally correlated poorly with disease severity, muscle inflammation and fatty infiltration levels.

How might this impact on clinical practice or future developments?

- Muscle amyloid imaging can differentiate between IBM and PM and could prove a useful future diagnostic modality.

INTRODUCTION

Inclusion body myositis (IBM) is an acquired muscle disease with a slowly progressive course, culminating in severe disability.[1] IBM is categorised as an inflammatory myopathy and shares histopathological features with polymyositis (PM), but immunosuppression does not modify progression.[2] IBM is often diagnosed late and is commonly misdiagnosed initially as PM, due in part because differentiation on histopathological grounds can be difficult. In one study, five of nine patients with a diagnosis of 'PM' developed clinical features of IBM during follow-up, with such patients receiving unnecessary and potentially harmful immunosuppressive treatments.[3]

The presence of intramuscular beta-amyloid forms part of several IBM diagnostic criteria and is a key difference from PM.[4] While this feature has a high diagnostic specificity, a relatively low sensitivity has been demonstrated, particularly in early IBM.[5] Recent diagnostic criteria for IBM have shifted towards identification of the characteristic pattern of muscle weakness, with less strict histopathological requirements.[4] While this has improved sensitivity, clinically detectable weakness implies that significant and irreversible muscle damage has occurred, reducing the likelihood that novel treatments will be effective.

We hypothesise that using amyloid positron emission tomography (amyloid-PET) to detect beta-amyloid within muscle can distinguish IBM from other inflammatory myopathies. Unlike muscle biopsy, imaging is non-invasive and large volumes of muscle can be studied, potentially improving sensitivity and facilitating earlier diagnosis. In this imaging study we compared the intramuscular amyloid burden, as determined using amyloid-PET, between IBM and PM. (E)-4-(2-(6-(2-(2-(2-18F-fluoroethoxy)ethoxy)ethoxy)pyridin-3-yl)vinyl)-N-methyl benzenamine, here referred to as [18F]florbetapir, was used as the amyloid imaging agent.[6 7]

METHODS

Participants

Between October 2015 and October 2016, written informed consent was provided by 10 cases with IBM and 6 with PM selected from the database of patients attending the adult neuromuscular service

at Salford Royal NHS Foundation Trust, UK. For the PM cohort, we restricted recruitment to those aged >45 years (online supplementary appendix section 3). IBM cases met European Neuromuscular Centre 2011 diagnostic criteria ('clinicopathologically defined' (n=8) or 'clinically defined' (n=2)).[8] Those with PM met Bohan and Peter diagnostic criteria (probable or definite) and had a minimum classification probability of 75% using the International Myositis Classification Criteria Project criteria.[9–11]

Study procedures

Clinical outcomes

For those with IBM the Functional Rating Scale (IBM-FRS) was performed.[12] In PM, the International Myositis Assessment & Clinical Studies Group disease activity core set measures were completed.[13] Both groups had muscle strength assessed using the manual muscle testing 260 (MMT26) score and completed the Health Assessment Questionnaire disability index (HAQ-DI).[14]

PET

A target dose of 370 MBq (18F)florbetapir was administered by intravenous bolus. A CT scan from shoulders to ankles was performed using a Siemens Biograph TruePoint PET/CT camera for attenuation correction and definition of regions of interest (ROI).[15] A PET emission scan of the same area commenced 45 min after radiotracer injection. Five minutes for each of the eight or nine bed positions was used, depending on subject height. PET images were reconstructed using 3D Ordered Subset Expectation Maximisation with three iterations and 21 subsets producing whole body images with almost isotropic voxels (2.6728 mm×2.6728 mm×2.027 mm) and a matrix size of 256×256 voxels per transaxial plane. A 3D Gaussian filter (full width at half maximum 3 mm) was applied postreconstruction to regularise images.

MRI

On the same day, whole body MRI was performed on a Philips Achieva 1.5 T scanner. A T1-weighted (TR 500 ms, TE 20 ms, bandwidth 220 Hz) sequence (to assess fatty infiltration of muscle) and a short tau inversion recovery (TR 5320 ms, TE 50 ms, TI 150 ms, bandwidth 170 Hz) sequence (to assess myoedema, a surrogate for muscle inflammation) were performed.

Image processing

PET

Seven muscle ROIs were defined for each subject, consisting of all muscle within a 10 cm vertical stack of consecutive images from the anatomical CT scan. The placement of this section was centred on a slice 1/3 of the distance from the superior border of the patella to the anterior superior iliac spine for the thigh, 1/3 of the distance from the inferior border of the patella to the summit of the medial malleolus for the calf, 1/2 of the distance from the greater tuberosity of the humerus to the medial epicondyle for the left arm and 1/2 of the distance from the tip of the olecranon to the ulnar styloid process for the forearm. Each ROI was constructed using semiautomated threshold active contour segmentation tools within ITK-SNAP (online supplementary appendix section 1).[16] Intensities of fat and muscle were specified (muscle: −10 to +100 HU; fat: −150 to −50 HU) and seed 'bubbles' placed within all visible musculature. Contour evolution could iterate until no further expansion of the ROI occurred.

For correction of non-specific radiotracer binding, a reference region was defined within the lumbar fat pad using the same centre landmark as the forearm ROI. Standardised [18F]florbetapir uptake values (SUVs) were calculated for each ROI by dividing the decay-corrected tissue mean concentration of radioactivity by the total injected radioactivity per body weight. Sum intensity means for all regions, upper limb regions and lower limb regions were calculated. SUV ratios (SUVRs) were calculated using the lumbar fat pad reference. This region was chosen as large volumes were available for selection and the location was easily matched between participants. Cerebral amyloid imaging studies have also shown increased statistical power when using lipid-rich reference regions.[17] Given the lipophilic nature of florbetapir, it was assumed that tracer binding in the subcutaneous adipose was predominantly of the non-specific type.

MRI

Images were scored by a blinded musculoskeletal radiologist (JH) using semiquantitative scoring tools based on those in the literature.[18–20] Severity of fatty infiltration (0: normal, 5: end-stage appearance) and extent of inflammatory change (0: normal, 5: entire muscle) were scored (online supplementary appendix section 2). For comparison with the amyloid-PET, mean fatty infiltration and inflammation scores for corresponding muscle regions were calculated.

Statistical analysis

[18F]florbetapir SUVs and SUVRs for IBM were compared with PM using the Mann-Whitney Ranksum test in STATA for Windows V.13.0 (College Station, Texas, USA). For the IBM group, correlations between [18F]florbetapir SUVRs and clinical and MRI parameters of disease severity were examined using Spearman's ranked correlation. Two-sided students t-test or Fisher's exact test were used where appropriate. Receiver operating characteristic analysis was performed regarding the sensitivity and specificity of the total-[18F]florbetapir SUVRs for IBM. $P<0.05$ was considered as significant. Disease duration refers to the interval between diagnosis and the date of participation in the study.

Ethical and regulatory approvals

The study was sponsored by the University of Manchester and authorised by the UK National Research Ethics Service (Greater Manchester West, 15/NW/0547) and the Administration of Radioactive Substances Advisory Committee (RPC number: 595/3586/33509).

RESULTS

Thirteen male and three female participants were studied (table 1). Three of the IBM group had previously received immunosuppressant medication, compared with all in the PM group. Visible differences were evident when comparing [18F]florbetapir PET/CT images between those with IBM and those with PM (figure 1). [18F]Florbetapir SUVRs were significantly higher in those with IBM for all ROIs (p value range 0.002–0.030) (table 1 and figure 2). For [18F]florbetapir SUVs (ie, without adjustment for non-specific radiotracer binding), only trends towards higher values in the IBM group were observed, except for the total-SUV region, where significantly higher values were also seen (table 1). For a total-[18F]florbetapir SUVR≥1.28 the diagnostic sensitivity for IBM was 80% and specificity 100% (area under curve 0.93).

Table 1 Clinical characteristics of subjects and muscle [18F]florbetapir uptake values

		IBM (n=10)	PM (n=6)	P value
Mean age in years at diagnosis (SD)		64.3 (8.4)	58.2 (10.7)	0.222*
Mean age in years at scan (SD)		68.3 (8.0)	59.7 (11.1)	0.092*
Mean disease duration at scan in years (SD)		4.0 (3.0)	1.5 (1.4)	0.079*
Gender (Male | Female)		9 | 1	4 | 2	**0.036**†
Mean manual muscle testing score (0–260) (SD)		236 (22.9)	256 (2.3)	0.052*
Mean Health Assessment Questionnaire disability index (SD)		1.3 (0.7)	0.8 (0.8)	0.192*
Mean IBM-Functional Rating Scale (0–40) (SD)		28.9 (5.3)	–	–
Mean physician global disease activity VAS (0–10) (SD)		–	1.8 (1.5)	–
Mean serum total creatine kinase level (IU/L) (SD)		579 (408)‡	308 (220)	–
Current immunosuppressive treatments (n)		Nil	Prednisolone (5/6) Methotrexate (2/6) Azathioprine (2/6) Cyclophosphamide (1/6)	–
Previous immunosuppressive treatments (n)		Prednisolone (3/10) Azathioprine (1/10) Mycophenolate (1/10)	Cyclophosphamide (2/6) Prednisolone (1/6) Mycophenolate (1/6) Azathioprine (1/6) Ciclosporin (1/6) IVIG (1/6)	–
Median [18F]florbetapir SUV (IQR)	Left arm	0.47 (0.41–0.55)	0.40 (0.36–0.48)	0.104§
	Right forearm	0.39 (0.35–0.42)	0.32 (0.27–0.40)	0.104§
	Left forearm	0.45 (0.32–0.55)	0.33 (0.30–0.36)	0.129§
	Right thigh¶	0.44 (0.43–0.52)	0.41 (0.37–0.45)	0.288§
	Left thigh¶	0.48 (0.43–0.51)	0.41 (0.36–0.45)	0.059§
	Right calf	0.51 (0.45–0.61)	0.46 (0.44–0.50)	0.233§
	Left calf	0.51 (0.40–0.58)	0.43 (0.39–0.45)	0.233§
	Overall (total-SUV)	0.48 (0.44–0.51)	0.42 (0.39–0.45)	**0.039§**
Median [18F]florbetapir SUVR (IQR)	Left arm¶	1.61 (1.43–1.81)	0.96 (0.82–1.08)	**0.002§**
	Right forearm	1.26 (1.05–1.60)	0.79 (0.67–0.91)	**0.005§**
	Left forearm	1.26 (1.12–1.52)	0.83 (0.58–0.96)	**0.005§**
	Right thigh**	1.34 (1.31–1.77)	1.04 (0.79–1.21)	**0.013§**
	Left thigh**	1.40 (1.40–1.87)	0.99 (0.79–1.18)	**0.005§**
	Right calf	1.59 (1.36–2.29)	1.09 (0.94–1.35)	**0.013§**
	Left calf	1.56 (1.29–2.40)	1.00 (0.75–1.31)	**0.030§**
	Overall (total-SUVR)	1.45 (1.28–2.05)	1.01 (0.80–1.22)	**0.005§**

Bold values indicate statistically significant differences.
*P values derive from two-sided students t-test.
†Fisher's exact test.
‡For the IBM group, this refers to the peak serum creatine kinase level (it was not rechecked at the time of the scan).
§The Mann-Whitney Ranksum test.
¶The right arm was not used because radiotracer administration was via a venous cannula in the right antecubital fossa, except in two subjects (one with PM, one with IBM) where the reverse was true due to difficulties with cannula placement.
**n=9 for IBM group. Measurement in one subject could not be obtained due to very high levels of muscle atrophy and fatty replacement.
IBM, inclusion body myositis; IVIG, intravenous immunoglobulin; PM, polymyositis; SUV, standardised uptake value; SUVR, standardised uptake value ratio with reference region in lumbar fat pad; VAS, visual analogue scale.

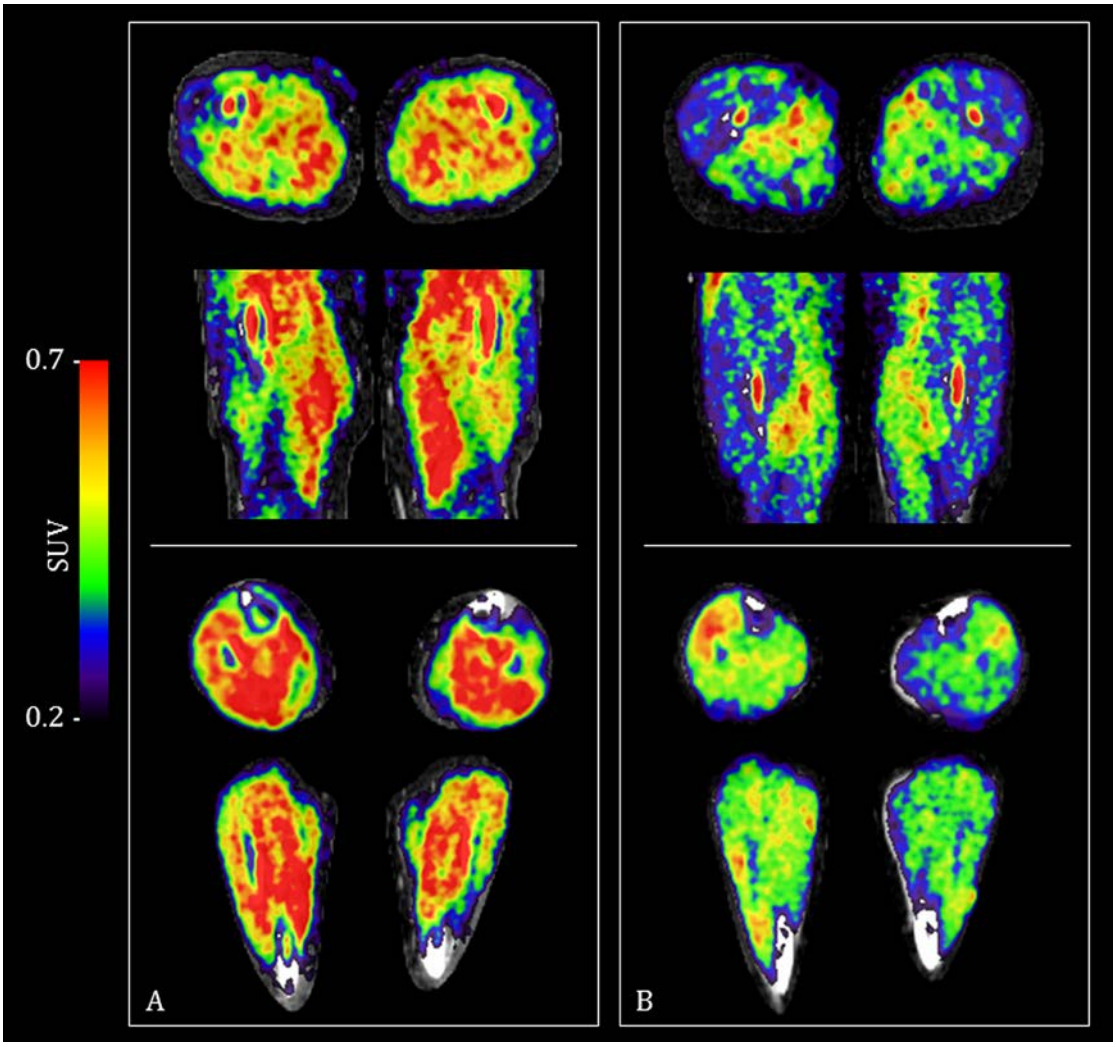

Figure 1 (18F)florbetapir PET/CT images showing differences in uptake between a participant with inclusion body myositis (panel A) and one with polymyositis (panel B). Increasing SUVs (red) indicate increased tracer uptake. [18F]Florbetapir PET images overlay spatially aligned CT images. Top of each panel depicts axial and coronal slices though the thigh. Bottom of each panel depicts axial and coronal slices though the calf. Each image is centred on the middle of the defined region of interest. PET, positron emission tomography; SUVs, standardised uptake values.

In those with IBM, only in the calves were strong negative correlations between [18F]florbetapir SUVRs and muscle inflammation levels (by MRI) found (right calf Rho −0.73, p=0.02; left calf Rho −0.68, p=0.03). No significant correlation between [18F]florbetapir SUVRs and levels of fatty infiltration were identified. Furthermore, no significant relationships between the total-[18F]florbetapir SUVR and the age at scan, disease duration, MMT26, HAQ-DI or IBM-FRS were identified. This included subsets of the MMT26 and IBM-FRS restricted to upper limb and lower limb components compared with corresponding upper limb and lower limb [18F]florbetapir SUVRs (online supplementary appendix section 1 table 1) . Amyloid deposits (by congo red staining) were only found in the diagnostic muscle biopsy of one IBM participant. No differences in the total-[18F]florbetapir SUVR were found according to the presence of degenerative biopsy features, including rimmed vacuoles (online supplementary appendix section 1 table 2).

DISCUSSION

In all assessed muscle groups, significantly increased [18F]florbetapir SUVRs were evident in IBM compared with PM. Sensitivity and specificity of the total-[18F]florbetapir SUVR for IBM was high, highlighting the potential diagnostic usefulness of muscle amyloid-PET. Further development of this technique could facilitate accurate diagnosis of IBM in those with early and otherwise undifferentiated disease, avoiding the use of potentially harmful treatments and facilitating inclusion in clinical trials.

To our knowledge, only one other published study used PET to detect intramuscular amyloid in IBM.[21] Maetzler *et al* used the Pittsburgh-B (PiB) compound; a carbon-11 based radionucleotide with a half-life of approximately 20 min (compared with 110 min for fluorine-18), limiting its clinical use. Uniquely, we also performed same day muscle MRI and collected standardised clinical disease severity measures.

We used a semiautomated contour evolution method to select large sections of muscle for ROIs.[16] It is likely that our method, rather than selecting small ellipsoid regions, produces more reliable results due to lower susceptibility to noise and bias from manual ROI placement. Borderline lower [18F]florbetapir SUVRs were found in the forearm when compared with other regions in both groups, potentially due to increased noise at the edge of the field of view. As we performed sequential exposures, comparison between different regions is susceptible to error, even after correction for radioactivity decay.

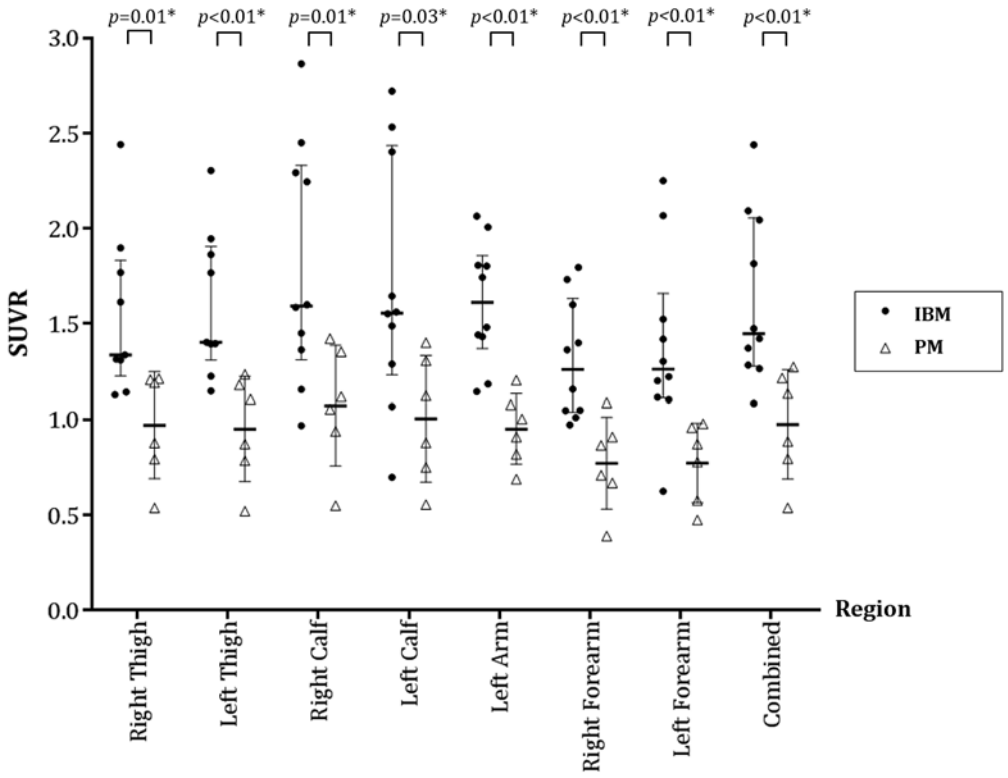

Figure 2 Comparison of SUVRs of [18F]florbetapir between participants with IBM (filled circles) and those with PM (open triangles) across seven different muscle regions and a combined region. Thick horizontal lines represent median SUVR and thin horizontal lines indicate the IQR. P values derived from Mann-Whitney Ranksum test. *Statistically significant difference (p<0.05). IBM, inclusion body myositis; PM, polymyositis; SUVR, standardised uptake value ratio.

Our study is small and it is possible that factors other than diagnosis are confounding the results. A trend towards increased age at the time of scan is evident in the IBM group, but no significant correlations between age and the total-[18F]florbetapir SUVRs were evident (Rho=0.33, p=0.22), indicating that age alone is unlikely to explain the differences in intramuscular amyloid content between the groups. The IBM group also had borderline lower MMT26 scores. However, total-[18F]florbetapir SUVRs did not correlate significantly with measures of disease severity in this group, including the MMT26. Gender ratios are also different between the groups, but we are not aware of a clear rationale as to why this would independently influence the [18F] florbetapir SUVR.

This study has demonstrated the usefulness of muscle amyloid imaging using [18F]florbetapir PET in differentiating IBM from PM. By potentially improving the ability to accurately diagnose IBM, further development and validation of this technique could help to avoid the use of unnecessary medication and enhance involvement in clinical trials.

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TRANSLATIONAL SCIENCE

mTOR inhibition by metformin impacts monosodium urate crystal–induced inflammation and cell death in gout: a prelude to a new add-on therapy?

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ABSTRACT

Objective Gout is the most common inflammatory arthritis worldwide, and patients experience a heavy burden of cardiovascular and metabolic diseases. The inflammation is caused by the deposition of monosodium urate (MSU) crystals in tissues, especially in the joints, triggering immune cells to mount an inflammatory reaction. Recently, it was shown that MSU crystals can induce mechanistic target of rapamycin (mTOR) signalling in monocytes encountering these crystals in vitro. The mTOR pathway is strongly implicated in cardiovascular and metabolic disease. We hypothesised that inhibiting this pathway in gout might be a novel avenue of treatment in these patients, targeting both inflammation and comorbidities.

Methods We used a translational approach starting from ex vivo to in vitro and back to in vivo.

Results We show that ex vivo immune cells from patients with gout exhibit higher expression of the mTOR pathway, which we can mimic in vitro by stimulating healthy immune cells (B lymphocytes, monocytes, T lymphocytes) with MSU crystals. Monocytes are the most prominent *mTOR* expressers. By using live imaging, we demonstrate that monocytes, on encountering MSU crystals, initiate cell death and release a wide array of proinflammatory cytokines. By inhibiting *mTOR* signalling with metformin or rapamycin, a reduction of cell death and release of inflammatory mediators was observed. Consistent with this, we show that patients with gout who are treated with the mTOR inhibitor metformin have a lower frequency of gout attacks.

Conclusions We propose mTOR inhibition as a novel therapeutic target of interest in gout treatment.

Key messages

What is already known about this subject?
- Increased mechanistic target of rapamycin (mTOR) signalling has recently been observed in monocytes after encountering uric acid–containing medium.
- Gout is mediated by monosodium urate crystals consisting of precipitated uric acid in joints.

What does this study add?
- We show that monocytes encountering MSU crystals go into pyroptosis and provoke an mTOR-mediated proinflammatory environment.

How might this impact on clinical practice or future developments?
- Both pyroptosis and inflammation are reduced with mTOR inhibitors metformin and rapamycin, which leads to a lower gout flare rate in clinical practice.

INTRODUCTION

Gout is the most common inflammatory arthritis affecting approximately 4% of the population in Europe and the USA. The inflammation is caused by the deposition of monosodium urate (MSU) crystals in the joints, which predominantly occurs in hyperuricemia (0.42 mmol/L serum urate). The level of comorbidity in gout patients is high; 74% have hypertension, 71% have chronic kidney disease and more than 10% suffer from either a myocardial infarction, heart failure or a major stroke.[1–3] Gout is associated with senescence and with increased mortality due to cardiovascular and infectious diseases and cancer.[4–7] Recently, it has become apparent that an important driver of inflammation in gout is interleukin-1 beta (IL-1β)–mediated NLRP3-inflammasome activation.[8–10] This process is initiated by autophagy of MSU crystals in macrophages, and the same effect is observed when stimulating peripheral blood mononuclear cells (PBMCs) or monocytes in vitro with MSU crystals.[10–12] In addition, interleukin 8 (IL-8) levels seem to be constitutionally increased in the circulation of patients with gout with concomitant cardiovascular disease and diabetes.[13]

A recent study showed that stimulating monocytes with MSU crystals in vitro leads to a higher expression of mechanistic target of rapamycin (mammalian target of Rapamycin) (mTOR) and increased IL-1β.[10] The mTOR signalling pathway partially regulates IL-8 production and IL-1β and therefore might be of interest as a target in inhibiting the chronic inflammation in patients with gout.[14 15] The mTOR pathway is well conserved in eukaryotes, and its signalling is tightly entwined with regulation of lymphocyte proliferation, immune-cell activation, autophagy, and lipid and glucose metabolism. As a consequence of its central role in

Table 1 Baseline characteristics of patients with gout and healthy participants

	Gout (n=89)	Healthy participants (n=89)
Male N (%)	72 (81.5)	77 (85.60)
Age	62.66±13.44	47.84±17.87
Colchicine (yes) N (%)	44 (48.9)	–
NSAID (yes) N (%)	14 (16.20)	–
Allopurinol (yes) N (%) (mean 200 mg/day)	76 (84.40)	–
Corticosteroids (yes) N (%)	40 (44.40)	–
Metformin (yes) N (%)	23 (25.84)	–
Diabetes (type 2) (yes) N (%)	19 (21.35)	–
Stroke (yes/no) N (%)	5 (5.6)	–
Myocardial infarction (non-fatal) (yes) N (%)	14 (15.6)	–
Heart failure (yes) N (%)	12 (13.30)	–
Angina pectoris (yes) N (%)	12 (13.30)	–
Creatinine level (µmol/L)	95.59 (±31.32)	–
Body mass index (kg/m^2) (mean±SD)	29.95 (±6.12)	25.79 (±4.18)
Smoking (yes) N (%)	12 (13.30)	1 (1.24)
Serum urate (mmol/L)	0.50 (±0.12)	–
Total no of flares per year (mean±SD)	4.41 (±5.17)	–
Presence of tophi (yes) N (%)	40 (45)	–
Systolic blood pressure mean (mm Hg) (SD)	142.65 (±17.37)	–
Diastolic blood pressure mean (mm Hg) (SD)	85.72 (±10.19)	–

NSAID, non-steroidal anti-inflammatory drug.

cellular signalling, increased mTOR signalling has been implicated in multiple diseases and is a common causative pathway in vascular disease, inflammation, obesity, progressive renal disease and diabetes.[16–18] These comorbidities are a heavy concomitant disease burden in gout, for which contemporary urate-lowering treatments have not been effective. The most potent clinically approved drug that inhibits mTOR is rapamycin, which is used as an immunosuppressant agent in transplant patients and as a coating for coronary stents.[19] In addition, a number of reports have been published on using rapamycin as an add-on therapy in rheumatoid arthritis, systemic lupus erythematosus and Sjögren's disease.[20–22] A less well-known, weak inhibitor of mTOR but more widely used is metformin. Metformin inhibits mTOR signalling indirectly through AMPK activation and has been shown to reduce IL-8 production and might be able to reduce inflammasome activation.[23 24] In addition, metformin has been shown to reduce the risk for cardiovascular disease and diabetes development in clinical trials and might have a beneficiary effect on these concomitant diseases in gout.[25 26]

In the current translational study, we were interested whether we could find evidence for increased mTOR signalling in patients with gout, to pinpoint the immune cells mostly involved and to test whether mTOR inhibition might be an approach to reduce MSU crystal–induced inflammation in vitro and in vivo in patients with gout.

PATIENTS AND METHODS

Demographics of patients and healthy participants

We included 89 Dutch patients with intercritical gout and 89 healthy participants (table 1 and online supplementary table 1).

The significance of the association between the two classified subgroups of patients with gout and healthy participants was tested using the Mann-Whitney U test (non-parametrical continuous values) and Fisher's exact test (categorical values) and ($p<0.05$). The data are presented as mean±SD.

Cell isolation and culture

Using lithium heparin tubes, peripheral blood of patients and healthy participants was collected. Total PBMCs were isolated using Ficoll (Ficoll-Paque Plus; GE Healthcare).

Monocytes ($CD14^+/CD16^-$) were isolated from total PBMCs of healthy participants (online supplementary table 1) through a monocyte isolation microbead kit (lot no. 5170817557) by AutoMACS apparatus (Miltenyi) according to the manufacturer guidelines. After 30 min of resting in RPMI-1640 (Gibco RPMI 1640 Glutamax medium enriched with 10% fetal bovine serum [FBS] and 1% penicillin/streptavidin; Sigma-Aldrich), 0.5×10^6 monocytes per condition were either kept unstimulated or stimulated with 0.1 mg/mL of MSU crystals (5 mg, catalogue no. tlrl-msu; InvivoGen) suspended in sterile phosphate buffered saline buffer (lot no. RNBG2264; Sigma-Aldrich Life Science), MSU in combination with 10 nM rapamycin (catalogue no. S1039, batch no. S103911 [sirolimus]) and MSU in combination with sterile metformin (1 g, catalogue no. tlrl-metf; InvivoGen) suspended in RPMI medium (as described above) at a final concentration of 38.71 µM (1 g, catalogue no. tlrl-metf; InvivoGen). The study design was optimised and the incubation times were applied according to the readout of the experiment. To exclude bacterial endotoxin contamination within the MSU crystal preparation that might cause activation of the cells during incubations, a Limulus Amebocyte Lysate (LAL) assay (LAL Chromogenic Endotoxin Quantitation Kit, catalogue no. 88282; ThermoFisher Scientific) was performed following the manufacturers' procedure. The quantified endotoxin level (EU/mL) was below the detection limit which excludes any endotoxin contamination in MSU crystals.

Gene expression analysis

RNA was isolated from total PBMCs of patients with gout and healthy individuals (catalogue no./ID: 80204, Qiagen All-prep RNA purification) according to the manufacturer guidelines. Subsequently, cDNA was created using the Biorad iScript kit. Quantitative PCR (qPCR) was performed on a Quantstudio QPCR apparatus, with Taqman Beadchip technology (Applied Biosystems) under conditions as specified by the manufacturer. As housekeeping genes, GUSB and GAPDH were included to normalise expression. The following genes were included in the analyses: protein kinase B (*AkT1*), DEP domain-containing mTOR-interacting protein (*DEPTOR*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-glucuronidase (*GUSB*), interleukin 10 (*IL-10*), interleukin 6 (*IL-6*), mammalian target of rapamycin (*mTOR*), nuclear factor-kappa-B p105 subunit (*NFκB1*), phosphatase and tensin homolog (*PTEN*), rapamycin-insensitive companion of mammalian target of rapamycin (*RICTOR*) and regulatory-associated protein of mTOR (*RAPTOR*). These specific genes were chosen due to their involvement in mTOR complex.

The expression level of *mTOR* genes was determined using synthesised cDNA (Biorad iScript kit) from RNA that was extracted from 0.5×10^6 of total PBMCs, T lymphocytes ($CD3^+/CD56^-$), B lymphocytes ($CD19^+$), monocytes ($CD14^+/CD16^-$) and classical, intermediate and non-classical monocytes. The cells were lysed after 6 and 24 hours and consecutively cDNA was generated. Taqman single-gene qPCR assays were performed on a Quantstudio apparatus (Applied Biosystems). The Housekeeping

GUSB and *GAPDH* Genes (HKG) were included to normalise the gene expression.

Fluorescence-activated cell sorting (FACS) quantification and analysis

Healthy participants' PBMCs were assessed by FACS (FACSAria_III; BD Biosciences) (online supplementary table 2). Cellular markers that were included in FACS quantifications were $CD3^+$ (AF700, mouse anti-human, Clone UCHT1 [isotype IgG2a], 1:50 dilution, catalogue no. 300424; Biolegend)/$CD56^-$ (PE-CF594, mouse anti-human, Clone B159 [isotype IgG1], 1:25 dilution, catalogue no. 562328; BD) for T lymphocytes, $CD19^+$ (PECy7, mouse anti-human, Clone LT19 [isotype IgG1], 1:40 dilution, catalogue no. 130-091-247; Miltenyi) for B lymphocytes, $CD14^+$ (BV785, mouse anti-human, Clone M5E2 [isotype IgG2a], 1:100 dilution, catalogue no. 301840; Biolegend)/$CD16^+$(FCγRII) (APC, mouse anti-human, Clone ebio-CB16 [isotype IgG1], 1:20 dilution, catalogue no. 17-0168-42; eBioscience) monocytes and $CD3^-$/$CD56^+$ NK cells. The three cell subsets within the main group of monocytes were differentiated by gating the cells from the $CD14^+$/$CD16^+$ gate according to the brightness of $CD14^{++}$ (classical), $CD14^+CD16^+$ (intermediate) and $CD16^{++}$ (non-classical).

The percentage of activation markers of classical, intermediate and non-classical monocytes' subsets were quantified after gating the $CD14^+$/$CD16^+$ monocytes, by measuring the expressed $CD163^+$ (APC, mouse anti-human, eBioGHI/61 [isotype IgG1], 1:20 dilution, catalogue no. 17-1639-42; eBioscience) and $CD86^+$ (BV605, mouse anti-human IT2.2 [isotype IgG2b], 1:70 dilution, catalogue no. 2127150; Sony Biotechnology) percentage on the surface of the cells. Isolation and stimulation (6 and 24 hours) of the cell subsets were performed as described above. The cells were subsequently acquired using flow cytometry (FACSAria_III; BD Biosciences).

Intracellular FACS was applied to assess the activation level of intracellular mTOR pathway at the protein level after stimulating monocytes for 15 min according to the abovementioned protocol. Monocytes were first stained extracellularly for the abovementioned cell marker panel to distinguish classical, non-classical and intermediate monocytes. After being fixed and permeabilised, monocytes were stained for phosphorylated S6 (pS6) with human anti-pS6 antibody (anti-S6 pS240-FITC human, monoclonal recombinant IgG1, 1:5 dilution; Miltenyi biotec). The pS6 level was quantified and represented as the mean fluorescence intensity in monocytes.

Live imaging technique

The microscopic live imaging technique was used to visualise the monocytes over time. Medium rested monocytes (2×10^5/condition) were administered to the medium (RPMI 1640 [10% FBS, 1% penicillin–streptavidin]) containing Hoechst 33342 (20 µM) for 30 min at 37°C. The cells were then washed and stimulated according to the previously described stimuli/inhibitors in RPMI 1640 (without phenol red) (10% FBS, 1% penicillin–streptavidin) containing 4 nM Sytox Green (Life Technologies) and plated in precoated wells of a 96-well plate (clear bottom) (Ibidi). Monocytes were recorded on the Pathway 855 bioimaging system (BD Biosciences) with a ×20 objective during a period of 5 hours at 5% CO_2 at 37°C. Using an Orca high-resolution CCD camera and four fields of view, every 13 min, a set of two images (Exc/Em: 350/461 nm [Hoechst] and 504/523 nm [Sytox Green]) was captured. AttoVision software (V.1.7/855) controlled the system.

Monocyte markers

Cytokine measurements by Luminex

Cytokines were quantified using a multiplex Luminex assay. Quantification of the cytokines was done using an in-house developed and further validated (ISO9001 certified) multiplex immunoassay (Laboratory of Translational Immunology, University Medical Center Utrecht) based on Luminex technology (xMAP; Luminex, Austin, Texas, USA). Each sample was a supernatant of 0.5×10^6 monocytes per condition that were left either untreated, incubated with MSU crystals, MSU crystals and rapamycin and MSU crystals and metformin during 6 and 24 hours. The monocytes were centrifuged (300*g*, 8 min) and the supernatant was collected and kept in −80°C until measured. The cytokine panel included interleukin 1 receptor alpha (IL-1Rα), interleukin 1 (IL-1α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 18 (IL-18), tumour necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein (MIP-1) and interferon gamma-induced protein 10 (IP-10). A Biorad FlexMAP3D (Biorad Laboratories, Hercules, California, USA) in combination with xPONENT software V.4.2 (Luminex) was included to perform the acquisition. To analyse the data, five-parametric curve fitting using Bio-Plex Manager software V.6.1.1 (biorad) was assessed.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics V.23 and GraphPad Prism V.6 (GraphPad Software, San Diego, California, USA). Microscopic live imaging captures were analysed using ImageJ 1.51 hour program (Java V.1.8.0_111; National Institutes of Health, USA). Where appropriate, testing for significant differences in categorical groups was performed using Student's t-test ($p<0.05$).

RESULTS

Genes of mTOR pathway have a higher relative expression in patients with gout compared with healthy controls

Exploiting a custom Taqman gene expression array, we investigated the expression of genes involved in the mTOR pathway (*mTOR*, *Rictor*, *Raptor*, *Deptor*, *AKT1* and *PTEN*) in ex vivo PBMCs from 89 crystal-proven patients with gout and 89 healthy controls (table 1). A higher expression of the genes involved in the mTOR complex was observed in patients with gout ($p<0.0001$). The expression of *PTEN*, an mTOR inhibitor, was lower in patients ($p<0.0001$). Taken together, these results demonstrate an upregulation of various genes involved in mTOR signalling in gout (figure 1A).

Stimulation of PBMCs from healthy subjects with MSU crystals leads to increased mTOR gene expression in vitro

To investigate if the increased expression of mTOR genes in patients with gout could be caused by contact with MSU crystals in these patients, we cultured PBMCs from healthy subjects with MSU crystals in vitro for 24 hours and quantified mTOR expression. We observed an increase of *mTOR* expression in the PBMCs challenged with MSU crystals ($p=0.0007$) (figure 1B).

MSU crystal stimulation induces mTOR gene expression in immune-cell subsets in vitro

The gene expression level of *mTOR* on (in vitro) MSU crystal stimulation was measured in T and B lymphocytes and total monocytes of 10 healthy participants immediately after isolation

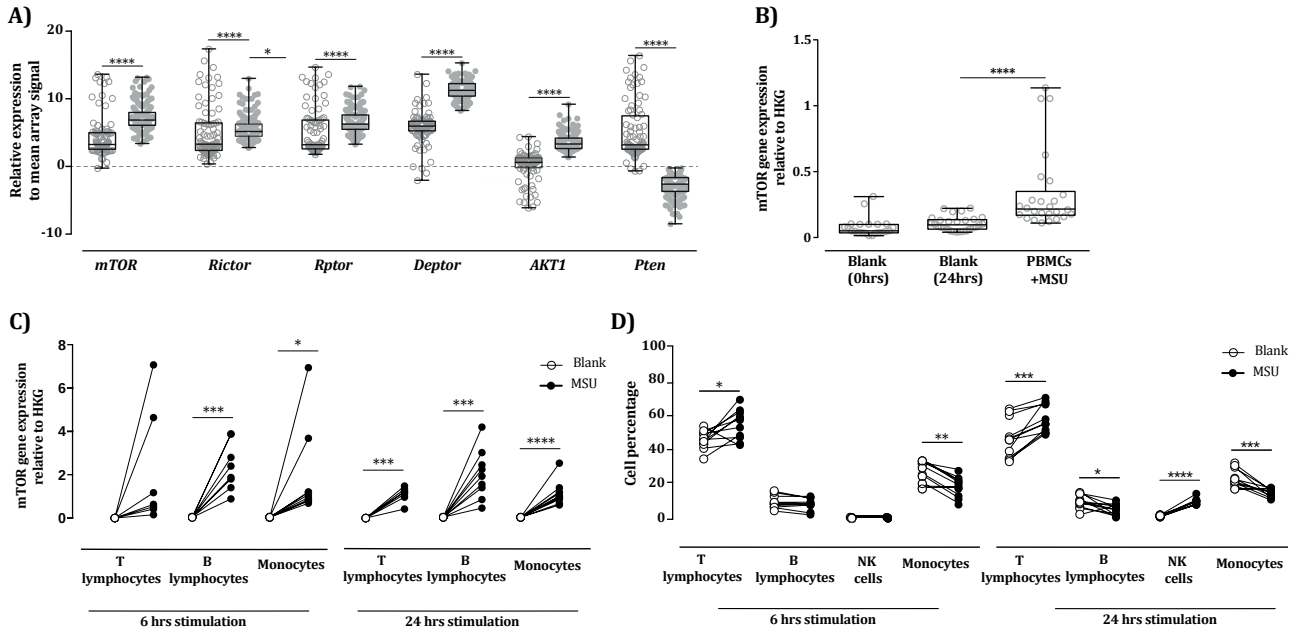

Figure 1 (A) Gene expression of mTOR pathway–related genes in patients with gout (N=89) and healthy participants (N=89) (filled dots and empty dots, respectively). (B) Expression of *mTOR* after stimulating peripheral blood mononuclear cells (PBMCs) of healthy participants with monosodium urate (MSU) crystals in vitro (N=28). (C) Gene expression level of *mTOR* on MSU stimulation after 6 and 24 hours of stimulation as compared with basal level of the gene at T=0 in immune cell subsets. The gene expression level of *mTOR* is increased in B lymphocytes (p=0.0006) and monocytes (p=0.024) after 6 hours of stimulation. After 24 hours of stimulation, there was a significant induction of *mTOR* gene expression in T lymphocytes (p=0.0001), B lymphocytes (p=0.0008) and monocytes (p<0.0001) as compared with the T=0 conditions. (D) After 6 hours of stimulation with MSU crystals, there was a reduction in the proportion of monocytes within the total PBMCs cultured (p=0.0002) within the MSU-challenged condition compared with the control. Reciprocally, there was an increase in the proportion of T lymphocytes (p=0.012). In line with this, the proportion of monocytes in the PBMCs that had been incubated for 24 hours showed a further decrease in the proportion of monocytes (p=0.001). Accordingly, an increment of the proportion of T lymphocytes (p=0.0004) and NK cells (p<0.0001) was observed.

of the cells (T=0) and after stimulating the cells for 6 and 24 hours. After 6 hours of stimulation, MSU crystals induced *mTOR* gene expression in B lymphocytes (p=0.0006) and monocytes (p=0.024) but not in T lymphocytes (p=0.085). After 24 hours, there was an induction of *mTOR* gene expression in T lymphocytes (p=0.0001), B lymphocytes (p=0.0008) and monocytes (p<0.0001) as compared with the T=0 conditions (figure 1C).

Encountering MSU crystals in vitro substantiates a reduction of monocytes in PBMCs

In order to study the effect of MSU crystal stimulation on immune-cell subsets in more detail, PBMCs were challenged with MSU crystals for 6 and 24 hours (figure 1D). After 6 hours of stimulation with MSU crystals, there was a significant reduction in the proportion of monocytes within the total PBMCs cultured (p=0.0002). Reciprocally, there was an increase in the proportion of T lymphocytes (p=0.012). Consistent with this, the proportion of monocytes in the PBMCs that had been incubated for 24 hours showed a further decrease in the proportion of monocytes (p=0.001). Accordingly, an increase of the proportion (as ratio of the total) of T lymphocytes (p=0.0004) and NK cells (p<0.0001) was observed (figure 1D). In order to investigate the immune-cell subsets that might be responsible for mTOR activation and subsequently the inflammatory reaction in patients with gout, we evaluated the ratio of the subsets. In PBMCs of patients with gout and healthy participants, the ratio of the T (p=0.22) and B (p=0.01) lymphocytes and NK (p=0.01) cells was higher in patients with gout as compared with healthy participants. The total monocytes, however, were lower in patients with gout as compared with healthy participants (p=0.007). The percentage of classical (p=0.01) and intermediate (p=0.03) monocytes were lower in patients with gout. There was a similar trend in non-classical monocytes (p=0.05) (online supplementary figure 2). The mean percentages (±SD) of the immune-cell subset of patients and healthy participants are presented in online supplementary table 2.

Monocytes actively engage MSU crystals and undergo cell death after contact

To better gauge the reaction of monocytes towards MSU crystals, we performed live imaging of $CD14^+$ monocytes encountering MSU crystals. We used two dyes, namely, Sytox Green (green colour that visualises dead cells) and Hoechst (blue colour that visualises live cells), to quantify the number of monocytes dying on encountering MSU crystals. During 7 hours of imaging, we observed an active movement of monocytes towards MSU crystals. A large proportion of these monocytes undergo cell death on encountering these crystals. The full movies are made available on the website of the journal (online supplementary movies S1). In figure 2A, we show representative snapshots made every hour. After 7 hours, 61% of the monocytes cultured in medium only were still alive, whereas only 35% of the monocytes stimulated with MSU crystals survived (p<0.0001) (figure 2B). The imaging experiment was repeated eight times with analogous outcomes. The results of the similar assessments and analysis on classical, non-classical and intermediate monocytes are presented in online supplementary figure 1.

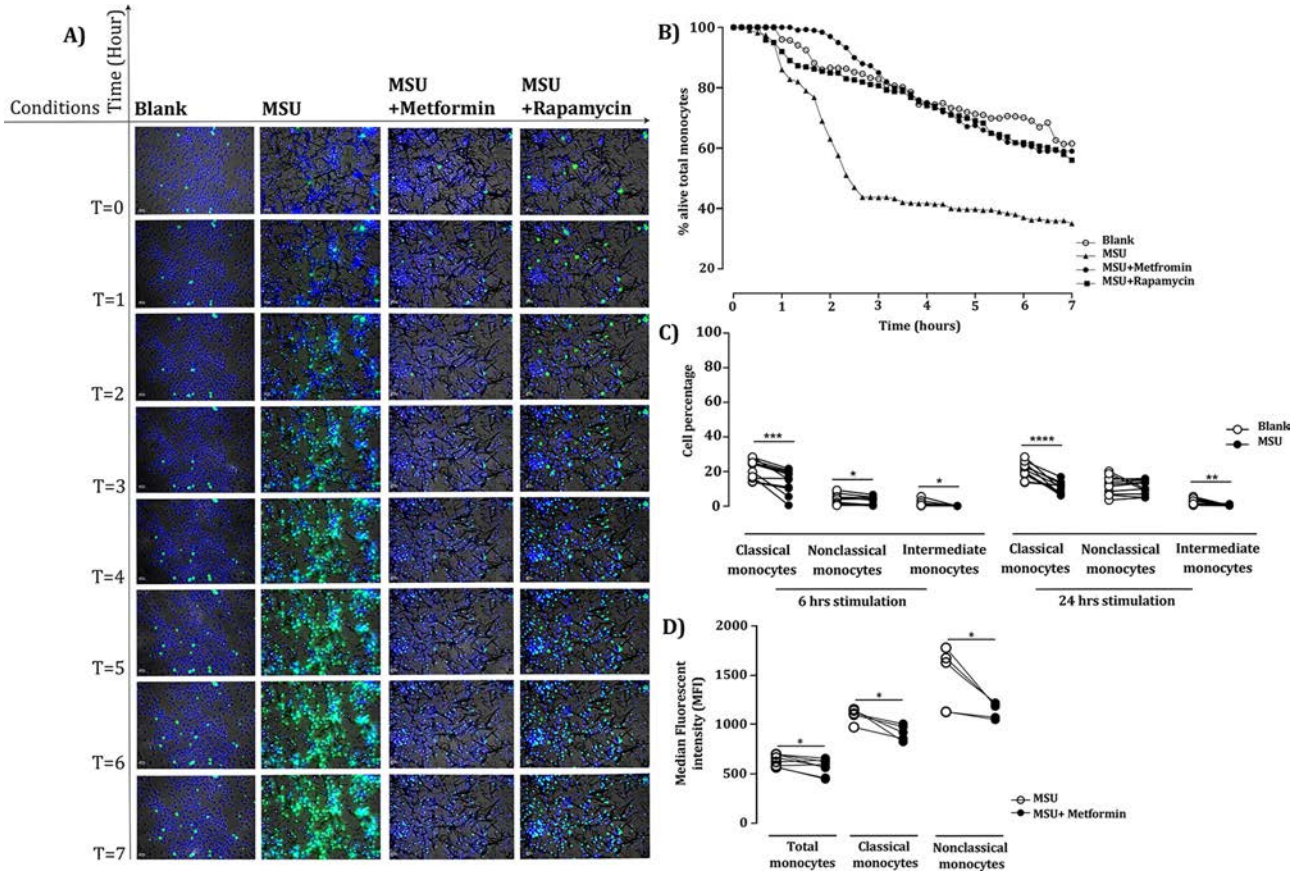

Figure 2 (A) Captures made from monocytes, collected from one healthy participant that were kept unstimulated, stimulated with monosodium urate (MSU) crystals, MSU crystals with rapamycin and MSU crystals with metformin at the time 0 to 7 hours are demonstrated (blue is live cell, green is dead cell). (B) Captures made every 13 min from the same cells were analysed and plotted against the time represented in hours. Treating the monocytes with rapamycin ($p<0.0001$) and metformin ($p<0.0001$) on MSU stimulation induces cell survival as compared with MSU crystal stimulation alone. (C) In vitro, 6 hours of MSU stimulation of peripheral blood mononuclear cells showed a reduction in percentage of classical ($p=0.0008$), non-classical ($p=0.02$) and intermediate ($p=0.04$) cells. After 24 hours of stimulation, classical ($p<0.0001$) and intermediate ($p=0.001$) monocytes were significantly reduced. After crystal stimulation, while non-classical are unchanged ($p=0.34$) (D), in monocytes from healthy participants (N=10), metformin gave a significant reduction in phosphorylation of S6 protein after 15 min of stimulation with MSU crystals.

Proportions of $CD14^{++}$ (classical), $CD14^{+}CD16^{+}$ (intermediate) and $CD16^{++}$ (non-classical) monocytes within the total PBMCs are all decreased after encountering MSU crystals

After 6 hours of stimulating PBMCs with MSU crystals, we quantified the number of monocytes by flow cytometry and further differentiated the monocytes from the $CD14^{+}/CD16^{+}$ gate according to the brightness of $CD14^{++}$ (classical), $CD14^{+}CD16^{+}$ (intermediate) and $CD16^{++}$ (non-classical) monocytes.

After culturing PBMCs for 6 hours with MSU crystals, we observed a significant reduction in proportion of classical monocytes ($p=0.0008$), non-classical monocytes ($p=0.02$) and intermediate monocytes ($p=0.04$) within the total PBMC number. The PBMCs that were incubated for 24 hours showed a significant reduction in classical ($p<0.0001$) and intermediate ($p=0.001$) monocytes, while the reduction of non-classical monocytes ($p=0.34$) was not significant (figure 2C). The mean percentages (±SD) of the immune-cell subsets of patients and healthy participants are presented in online supplementary table 2.

MTOR inhibition by rapamycin or metformin reduces MSU crystal–induced monocyte death

Since we observed an increased rate of cell death and an increased expression of mTOR in monocytes encountering MSU crystals, we investigated whether mTOR inhibition, which promotes autophagy and decreases inflammatory responses and response to apoptotic cells, would have a dampening effect on monocyte death and MSU crystal–induced inflammation. First, we evaluated whether the observed increased mTOR gene expression was reflected in the protein level. We measured the phosphorylation of S6 ribosomal protein (S6) at serine 240/244, which is downstream from mTOR activation and therefore commonly used as readout of mTOR activation. After resting, monocytes were stimulated for 15 min with MSU crystals and MSU crystals with metformin. As presented in figure 2D, metformin caused a decrease of the pS6 mean fluorescence intensity in total ($p=0.013$), classical ($p=0.015$) and non-classical ($p=0.040$) monocytes within 15 min.

To investigate temporal stability of the inhibitory effect of metformin in monocytes, we performed titration assays where we quantified the expression level of *mTOR* gene in monocytes (N=5) after 3, 6, 9 and 12 hours of incubation in the presence of MSU crystals. After 3 hours of metformin stimulation in MSU crystal–challenged monocytes, we observed a significant decrease in *mTOR* gene expression as compared with MSU crystal–challenged monocytes ($p=0.0007$). This inhibitory effect of metformin was stable after 6 hours ($p=0.008$) (figure 3D). The inhibitory effect of metformin in MSU crystal–challenged

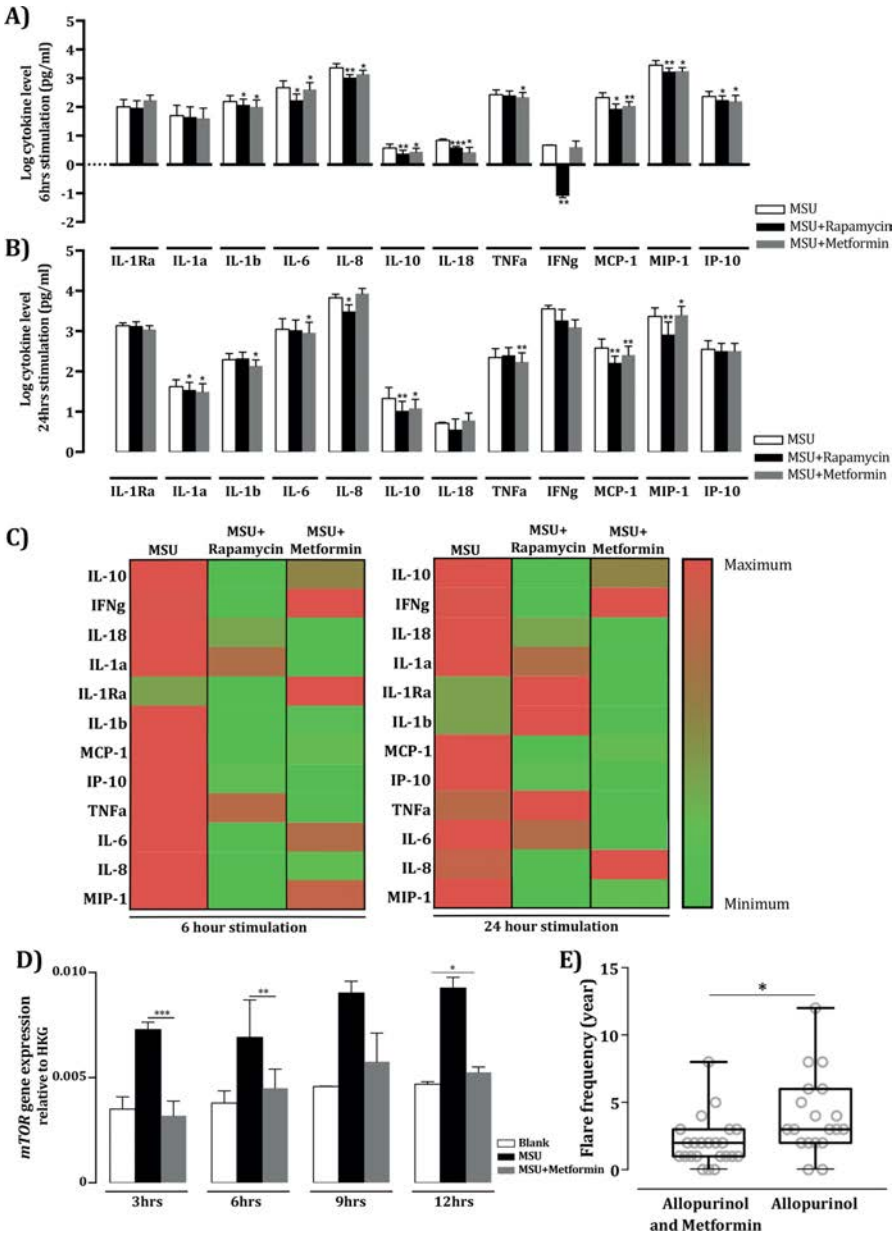

Figure 3 (A, B) Differential cytokine expression in monocytes stimulated with monosodium urate (MSU) crystals with and without mTOR inhibition by metformin or rapamycin. The cytokines that were significantly differently secreted from monocytes treated with MSU crystals and rapamycin as compared with MSU only after respectively 6 and 24 hours. (C) Heatmap representing the changes in monocytes' cytokine expression on stimulation with MSU crystals only, MSU crystals with rapamycin, and MSU crystals with metformin. (D) Inhibitory effect over time of metformin on *mTOR* gene expression (normalised for housekeeping gene (HKG) expression) in MSU-challenged monocytes. In monocytes of healthy participants (N=5), blank condition contains unstimulated monocytes that have the same incubation time as the stimulated conditions. Only the significant differences are indicated. (E) Patients with gout treated with a combination of allopurinol and metformin have significantly less recurrent flares as compared with patients treated only with allopurinol (p=0.010).

monocytes was until 9 hours of stimulation (p=0.19) and reached its minimum after 12 hours of stimulation (p=0.27). In the same monocytes and the same setting, *NFkB* gene expression was quantified. After 3 (p=0.027) and 9 (p=0.026) hours of metformin stimulation, there was a significant inhibition of *NFkB* (online supplementary figure 3A). Interestingly, metformin had an inhibitory effect on *IL-1β* in monocytes after 3 (p=0.023), 9 (p=0.024) and 12 (p=0.041) hours. Similarly, after 6 hours (p=0.063), there was a trend of inhibitory effect of metformin on monocytes (online supplementary figure 3B).

Since we were now able to inhibit mTOR in vitro with metformin and rapamycin, we co-cultured monocytes with medium only, with MSU crystals, with MSU crystals and metformin, or with MSU crystals and rapamycin. In addition, we cultured monocytes with rapamycin and metformin without MSU crystals. These conditions were all evaluated alongside live imaging, at the same time, in which monocytes from a healthy participant were cultured in every condition mentioned and analysed. This experiment was performed six times. When we quantified the proportion of cell death by ImageJ, comparing 'live cell' dye within each snapshot (time between each snapshot T=13 min), we observed that monocytes co-cultured with MSU crystals and rapamycin (cells alive 56%) or metformin (cells alive 59%) had a significantly lower death rate as compared

with monocytes stimulated with MSU crystals only after 7 hours (cells alive 35%) (both p<0.0001). The rate of cell death in the monocytes treated with mTOR inhibitors and MSU crystals was similar to that of monocytes cultured without MSU crystals (61% alive). In figure 2A, we show representative snapshots made every hour of monocyte cell culture at T=0 to 7 hours, and figure 2B shows the number of alive cells per condition over time. We did not observe any differences when cells were stimulated with metformin and rapamycin only mTOR inhibition by metformin or rapamycin reduces proinflammatory cytokine release by monocytes on encountering MSU crystals in vitro

To assess whether *mTOR* inhibition leads to less cytokine production on monocyte exposure to MSU crystals in vitro, we quantified the release of IL-1Rα, IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-18, TNF-α, IFN-γ, MCP-1, MIP-1 and IP-10 by Luminex in monocytes from 11 donors. Monocytes were cultured with MSU crystals. We compared cytokine levels between MSU-cultured monocytes with MSU crystals alone or co-cultured with metformin or rapamycin, which are both mTOR inhibitors. The monocytes co-cultured with MSU crystals and rapamycin showed a reduction in levels of IL-1β (p=0.02), IL-6 (p=0.02), IL-8 (p=0.0017), IL-10 (p=0.024), IL-18 (p=0.0009), IFN-γ (p=0.001), MCP-1 (p=0.01), MIP-1 (p=0.0026) and IP-10 (p=0.029). In the presence of metformin, a reduction in levels of IL-1β (p=0.02), IL-6 (p=0.31), IL-8 (p=0.01), IL-10 (p=0.0051), IL-18 (p=0.045), TNF-α (p=0.042), MCP-1 (p=0.006), MIP-1 (p=0.04) and IP-10 (p=0.046) was observed when compared with the monocytes cultured with MSU crystals alone. The reduced cytokine level after 24 hours of incubation with MSU crystals and rapamycin was IL-1α (p=0.027), IL-8 (p=0.02), IL-10 (p=0.005), MCP-1 (p=0.0032) and MIP-1 (p=0.005). Reduced cytokine levels in monocytes incubated with metformin and crystal stimulation after 24 hours was IL-1α (p=0.032), IL-1β (p=0.04), IL-6 (p=0.034), IL-10 (p=0.015), TNF-α (p=0.0024), MCP-1 (p=0.008) and MIP-1 (p=0.04). The quantified values are represented on a logarithmic scale (figure 3A). Colour heatmaps represent the effect of stimuli and inhibitors on the cells (figure 3B). There was no difference in cytokine secretion by the cells when stimulated with metformin and rapamycin only.

Metformin treatment associates with low flare frequency in patients with gout

To scrutinise whether mTOR inhibition through metformin in patients with gout leads to a lower frequency of gout flares, we performed a retrospective cohort analyses in 23 Caucasian patients with gout and metformin use in comparison with 19 patients with gout and diabetes without using metformin. As diabetic comedication, insulin use was allowed. Patients were selected from the Dutch cohort (table 1) and Caucasian patients with gout from New Zealand (online supplementary table 3). Our analysis demonstrates that patients with gout who were treated with a combination of metformin and allopurinol have a significantly lower attack frequency as compared with patients who were treated with allopurinol alone (p=0.010) (figure 3E). In our small retrospective cohort, we recorded a mean flare frequency of 2.04 (95% CI 1.29 to 2.38) flares per year in the allopurinol with metformin group versus the 4.00 (95% CI 2.57 to 5.43) flares in the allopurinol-only group. Finally, our results demonstrate that patients with gout have a significantly higher mTOR gene expression level. We observed that patients with gout who received colchicine treatment do have a significantly lower level of *mTOR* gene expression level as compared with patients who did not receive any colchicine treatment (p=0.0412). An additional analysis demonstrated no significant difference in mTOR expression level in patients who were treated/not treated with non-steroidal anti-inflammatory drugs (p=0.8139). Assessing the association between mTOR gene expression level and uric acid level (by Pearson correlation) showed a significant positive correlation (p=0.014).

DISCUSSION

The main conclusion of this study is that PBMCs from patients with gout have a signature of increased mTOR signalling as compared with healthy participants. By performing in vitro experiments, we showed that MSU crystals provoke upregulation of mTOR pathways gene expression, IL-1β, IL-6, IL-8, IL-18 release and cell death in monocytes. We were able to inhibit these phenomena by adding mTOR inhibitors rapamycin and metformin. When we analysed the effect of metformin on gout flares in a retrospective analysis of patients with gout with diabetes stratified according to metformin treatment, we observed a significantly lower gout attack frequency as compared with patients not treated with metformin.

An interesting finding of our study is the active engagement of monocytes towards MSU crystals, which induces a form of acute cell death. It is well known that there is an overlap in apoptosis and necrosis in vivo when immune cells encounter strong danger signals.[27] It is established that necrosis leads to NACHT, LRR and PYD domains–containing protein 3 (NLRP3) activation and increased IL-1β production, an important feature of gout and also observed in our study. Interestingly, mTOR activation enhances the process of necrosis.[28] To apply this to gout and our study, it is conceivable that necrosis of monocytes when encountering MSU crystals leads to activation of the inflammasome pathway and release of proinflammatory cytokines, as we demonstrate. The high expression of mTOR within monocytes further facilitates the pro-necrotic state within patients with gout. When mTOR is inhibited, there is a lower tendency towards cell death and consequently less inflammasome activity and inflammation, as we display in our study as well. Hence, the very start of the gout attack might lie in the encounter of monocytes with MSU crystals and seems to be modulated by mTOR.

Our findings are in line with a recent study also showing that stimulation of monocytes with MSU crystals enhances mTOR activation.[10] Very little research has been performed within the field of mTOR inhibition and gout; however, most available data concern the effects of metformin treatment in gout disease activity. A large retrospective case–control study (N=7536) in patients with diabetes showed that the use of metformin decreases the ORs for developing gout compared with patients not using metformin.[29] The authors, however, mainly focused on the finding that poorly controlled diabetes as defined by HbA1c levels is correlated with a decreased incidence of gout. Two small-scale studies conducted in Russia (N=30 and N=26) in patients without diabetes with gout showed that metformin reduces the frequency of gout attacks, lowers uric acid and led to normo-uricemia in 11 patients.[30 31] Of interest is the observation that metformin is able to interfere directly with the purine pathway, which might be the mode of action for the lowering of uric acid levels; the latter, however, has not yet been clearly proven.[32 33]

The evidence for an anti-inflammatory effect of metformin has been mounting over the past years. It is known that metformin activates AMPK (5′ adenosine monophosphate-activated protein kinase) to inhibit NF-κB via the PI3K (phosphoinositide

3-kinase)–Akt1 pathway and reduces the production of NO (nitric oxide), prostaglandin E2 and proinflammatory cytokines (IL-1β, IL8, IL-6 and TNF-α) in monocytes and macrophages.[34 35] One study that included over 4000 patients with pre-diabetes showed a significant reduction of CRP levels when treated with metformin as compared with placebo after 12 months.[36] Moreover, in monocyte-derived macrophages, metformin seemed to interfere directly with the inflammasome, orchestrating an inhibition of IL-1β maturation in patients with type 2 diabetes treated with metformin.[37] Patients with gout are typified by inflammasome induction and high circulating IL-8 levels and metformin is likely to be a suitable treatment for these patients since it is an effective inflammasome and IL-8 suppressor.

Metformin is the first-choice drug for treating type 2 diabetes; it is effective in reducing the hyperglycaemic state and decreases insulin resistance. Less obvious but well proven is the fact that metformin reduces the cardiovascular risk in patients with diabetes. The UK Prospective Diabetes Study (N=5500) demonstrated a substantial beneficial effect of metformin therapy on cardiovascular disease outcomes, with a 36% relative risk reduction in all-cause mortality and a 39% relative risk reduction in myocardial infarction.[38] The exact mechanism of action by which metformin protects the vasculature is not known, but it is thought to be a combination of improving lipid metabolism, AMPK induction and reduction of reactive oxygen species. Of interest for the gout population, which is at high risk to have or develop diabetes, metformin reduced the incidence of diabetes in high-risk groups.[39]

Our study has strengths and weaknesses; the strength of our study lies in the fact that we started from ex vivo patient material and observations, which we translated in an in vitro model and validated retrospectively in an in vivo observation. This chain of experimental settings makes our findings more robust to translation to the clinical setting. Our experiments were performed in parallel on the same apparatus and analysed by the same algorithms to avoid mistakes or bias by measurement or observer. Another strength is that the observations were made on both gene expression and protein level with various techniques. All patients included in the ex vivo study had crystal-proven gout, which is the gold standard of diagnosis. Moreover, the concentrations of metformin and rapamycin used in our experimental settings were derived from real-life plasma concentrations of these drugs in patients being treated with these drugs in clinical practice. This makes the results more relevant to clinical use. A weakness of our study is the small cohort in which we performed a retrospective analysis on the effects of metformin on the frequency of gout attacks. Although highly informative in the light of our study, these results need to be confirmed in a larger prospective study to make way for use of metformin in gout clinical practice. In our retrospective study, we did not have longitudinal data on glucose status, kidney function, treatment adherence and dose escalation; therefore, these results should be regarded with caution for direct extrapolation to clinical practice without further prospective and preferably randomised clinical trials.

Our data add to the understanding of the inflammatory reaction that occurs when monocytes encounter MSU crystals. Previous studies have strongly implicated inflammasome activation in the pathogenesis of gout as well; the role of inflammasome activation in gout is well described and witnessed by the effect that inflammasome-modulating drugs such as canakinumab and anakinra have in gout.[40–42] It is therefore very interesting to discuss how these pathways might entwine. Previous studies have shown that IL-1β secretion is partially regulated by mTOR signalling; a study by Harris *et al* showed that mTOR inhibition with rapamycin in macrophages leads to degradation of pro-IL-1β, subsequently reducing NLRP3 inflammasome activation.[43] A similar observation was made in an in vitro model for sepsis.[44] Another important study showed that MSU priming in monocytes leads to mTOR activation in concert with IL-1β expression.[12] We believe that therapeutic targeting of the inflammasome directly and indirectly by inhibiting mTOR in patients with gout might have a symbiotic effect in reducing the inflammatory response mediated through IL-1β.

Somewhat counterintuitive, we observed an increase in IL-10 levels when the monocytes were stimulated with MSU crystals. Although the role of IL-10 in the biology of monocytes has been under debate, this might be attributed to the high apoptosis rate in these cells.[45]

As described above, metformin has many potential beneficial effects on the disease course in gout. It has properties that inhibit inflammation through the mTOR and NLRP3 pathways, it decreases cardiovascular risk and it potentially might be able to decrease gout flares (online supplementary figure 4). The currently available drugs are well able to target one of these domains, (eg, allopurinol/colchicine in uric acid lowering, canakinumab for inflammasome targeting); however, none of them are able to target all three domains. Up until now, it is not clear if any of the currently used drugs reduce cardiovascular and metabolic risk.

A large body of evidence shows that metformin reduces cardiovascular risk and increases insulin sensitivity, reducing the burden of diabetes. Hence, taking also into account the favourable drug profile and our observations, we advocate to investigate metformin as an add-on therapy for patients with gout in a prospective study to clarify whether metformin is able to reduce the burden of gout flares and comorbidities.

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Contributors All authors approved the final version after being involved in drafting and revising the article for important intellectual content. As the corresponding author, NV had full access to the data and takes responsibility for the accuracy of the performed analysis and the integrity of the data. NV, TRDJR and JCAB were involved in design of the study. Execution, analysis and writing of the manuscript was performed by NV. AO and MvdL respectively contributed in FACS and Live Imaging of this study. CGKW was involved in performing gene arrays. MS and MZ thought along on rapamycin and metformin stimulations. EvL and MJ were involved in inclusion of Dutch patients with gout and TM participated by including patients with gout from New Zealand.

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Competing interests None declared.

Patient consent for publication Obtained.

Ethics approval This study was performed according to the guidelines of the Declaration of Helsinki and study meets the approval of ethical and review committees of the the Rijnstate hospital (Nijmegen, the Netherlands), University Medical Center of Utrecht in the Netherlands, VieCuri Hospital of Venlo in the Netherlands and University of Otago in Dunedin, New Zealand.

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TRANSLATIONAL SCIENCE

Active immunisation targeting nerve growth factor attenuates chronic pain behaviour in murine osteoarthritis

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ABSTRACT

Objectives Nerve growth factor (NGF) has emerged as a key driver of pain in osteoarthritis (OA) and antibodies to NGF are potent analgesics in human disease. Here, we validate a novel vaccine strategy to generate anti-NGF antibodies for reversal of pain behaviour in a surgical model of OA.

Methods Virus-like particles were derived from the cucumber mosaic virus (CuMV) and coupled to expressed recombinant NGF to create the vaccine. 10-week-old male mice underwent partial meniscectomy to induce OA or sham-surgery. Spontaneous pain behaviour was measured by Linton incapacitance and OA severity was quantified using OARSI histological scoring. Mice (experimental and a sentinel cohort) were inoculated with CuMVtt^{NGF} (Vax) or CuMVtt^{ctrl} (Mock) either before surgery or once pain was established. Efficacy of anti-NGF from the plasma of sentinel vaccinated mice was measured in vitro using a neurite outgrowth assay in PC12 cells.

Results Anti-NGF titres were readily detectable in the vaccinated but not mock vaccinated mice. Regular boosting with fresh vaccine was required to maintain anti-NGF titres as measured in the sentinel cohort. Both prophylactic and therapeutic vaccination demonstrated a reversal of pain behaviour by incapacitance testing, and a meta-analysis of the two studies showing analgesia at peak anti-NGF titres was highly statistically significant. Serum anti-NGF was able to inhibit neurite outgrowth equivalent to around 150 ug/mL of recombinant monoclonal antibody.

Conclusions This study demonstrates therapeutic efficacy of a novel NGF vaccine strategy that reversibly alleviates spontaneous pain behaviour in surgically induced murine OA.

Key messages

What is already known about this subject?
- Nerve growth factor (NGF) is a validated target for pain in human and mouse OA.
- Neutralising antibodies to NGF show therapeutic efficacy in Phase III clinical studies.

What does this study add?
- Here, we demonstrate efficacy of an NGF vaccine that reversibly induces neutralising anti-NGF antibodies and suppresses pain behaviour in murine OA.

How might this impact on clinical practice or future developments?
- Vaccination potentially offers a tuneable, cheaper and easier to use alternative to biological therapy in patients.

OA is the most prevalent joint disease costing approximately 1%–2.5% of the gross domestic product of developed countries.[1] Greater than 75% of patients experience pain on a daily basis.[2] Current standard therapies for pain relief, such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioids are limited by their modest efficacy and long-term safety.[3] In the last decade, nerve growth factor (NGF), a key pain sensitiser, has emerged as a promising target for OA pain. In humans, neutralising antibodies to NGF significantly suppress pain associated with late-stage OA.[4] However, biological therapy in OA is likely to be limited by cost[5] and by treatment failure due to anti-drug antibodies.[6] Active immunisation targeting NGF represents an attractive alternative to deliver effective analgesia, while potentially providing a more economically sustainable substitute for patients. The latter is particularly the case as biosimilars replace proprietary products.[7]

Chronic pain in late OA can be modelled using surgical models of joint destabilisation in mice. Spontaneous pain behaviour is assessed by differential weight distribution of the hind limbs using incapacitance testing. Following joint destabilisation, mice display two phases of pain behaviour: one immediately following surgery (postoperative pain) and a second late phase that starts between weeks 7 and 11 after surgery and which is progressive (online supplementary figure 1a) with worsening joint destruction (online supplementary figure 1b, c).[8 9] Both phases of pain behaviour correspond to an increase in NGF expression in the joint (online supplementary figure 1d, e)[10 11] and can be neutralised by delivery of NGF's soluble receptor (TrkAd5).[10]

Immunisation against self-proteins can be achieved by displaying the antigen of interest on virus-like particles (VLPs). Due to their optimal size and geometry, VLPs can effectively transit to

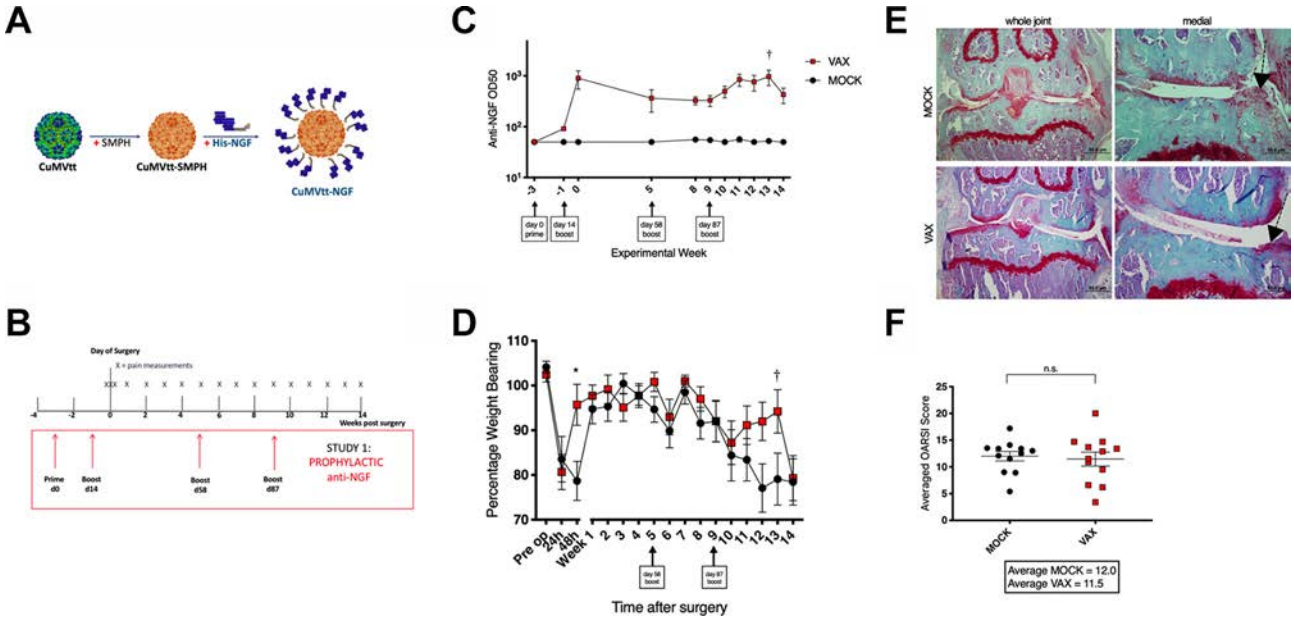

Figure 1 Prophylactic NGF vaccination blocks murine OA pain. (A) VLP is chemically cross-linked (SMPH) to enable conjugation with His-NGF. (B) Prophylactic vaccination protocol. (C) Anti-NGF titres in sentinel cohort (n=10). (D) Painful behaviour following surgical induction of OA (n=40) measured by Linton incapacitance where 100% represents equal weight distributed across R and L limbs. Repeated-measures two-way ANOVA with Bonferroni multiple comparisons test applied, *adjusted p<0.05. SEM shown. Differences between treatment groups during late OA pain phase were not significant after correcting for multiple testing. † identifies time point of highest anti-NGF titre (see figure 2D). (E) Representative histological sections for (F) cartilage degradation (OARSI) scores 18 weeks after PMX surgery in mice treated with mock or NGF vaccine. Statistical significance is shown by two-tailed t-test. Bars represent mean±SEM, n.s.—non-significant., **p<0.01 by t-test. CuMVtt adapted from EMD: 3855.[14] ANOVA, analysis of variance; NGF, nerve growth factor; PMX, partial meniscectomy; VLP, virus-like particle.

draining lymph nodes to drive antigen-dependent immunogenicity.[11] Antigens are arranged as a repetitive array on the particles' surfaces via genetic fusion or chemical conjugation to generate good polyclonal antibody responses without breaking T cell tolerance. This means that the antibody response will only occur when the antigen is presented on the VLP.[12 13]

Here, we describe a novel plant virus derived VLP based on the cucumber mosaic virus,[14] that incorporates a tetanus toxoid epitope for T cell help (herein referred to as CuMVtt, figure 1A).[15 16] Addition of a non-coding, 3' untranslated region in the VLP expression construct, leads to increased retention of encapsulated RNA suggesting greater particle stability (online supplementary figure 2a). Purified His-tagged NGF was covalently conjugated to CuMVtt (online supplementary figure 2b) as previously described for RNA-phage based VLPs.[17] Native conformation of recombinant NGF was tested by its ability to bind a neutralising monoclonal antibody and the interacting domain of the high-affinity receptor (TrkA-d5) (online supplementary figure 2c, d).

To test the therapeutic efficacy of NGF vaccination, mice were immunised with either CuMVtt^{NGF} (Vax) or CuMVtt^{ctrl} (Mock) 2 weeks prior to joint destabilisation (figure 1B). Non-operated sentinel control mice also underwent vaccination to enable regular blood sampling over the experimental course. Immunisation led to seroconversion by week 3, followed by a decline in antibody titres. Additional boosts were necessary to maintain antibody levels (figure 1C). No difference in pain behaviour was detected in NGF immunised animals 24 hours postoperatively (postop), but CuMVtt^{NGF} vaccinated animals recovered from pain behaviour faster than mock-vaccinated animals (within 48 hours) (figure 1D). As expected mice were pain free for several weeks, but pain behaviour started to develop from 8 weeks postsurgery. Following a boost at 10 weeks postop, and in keeping with a concomitant rise in the serum levels of anti-NGF antibody, a reversal of pain behaviour was observed. This was maintained for 3 weeks until anti-NGF titres fell and pain behaviour resumed. At termination of the experiment, joints were harvested and scored for OA severity. No difference in disease severity between mock and vaccinated groups was observed (figure 1E,F). Sera were also collected from experimental mice at the end of the study (week 18) to measure general antibody responses. Anti-CuMV IgG levels were elevated in both vaccinated and mock-vaccinated groups compared with non-vaccinated control animals. Total IgG and IgM levels were largely consistent across all groups. There was no evidence of induction of autoantibodies such as rheumatoid factor in any of the groups (online supplementary figure 3).

A second experiment was carried out to establish whether analgesia could be induced by immunisation after induction of pain behaviour i.e. therapeutic vaccination (figure 2A). When pain behaviour was established (10 weeks postop) mice were randomised into two groups: vaccinated and mock-vaccinated. Vaccine boosts were delivered at weeks 12 and 15 postop to maintain titres. Higher titre anti-NGF levels at the end of the experiment (around OD50 10^3) appeared to be associated with an analgesic response between weeks 14 and 18 postop (figure 2B,C). A subsequent spontaneous reduction in titres was associated with resumption of pain behaviour. Direct correlation between antibody levels and pain behaviour during the experiment was not possible as titres were only measured in the sentinel and not the experimental group. A meta-analysis comparing the analgesic effects across both studies at the point of highest titre in the sentinel group (week 13 for the prophylactic study and week 17 for the therapeutic study, marked by †) yielded a significant difference (p=8.93e-05) between mock and vaccinated cohorts (figure 2D). No heterogeneity of effect was detected between

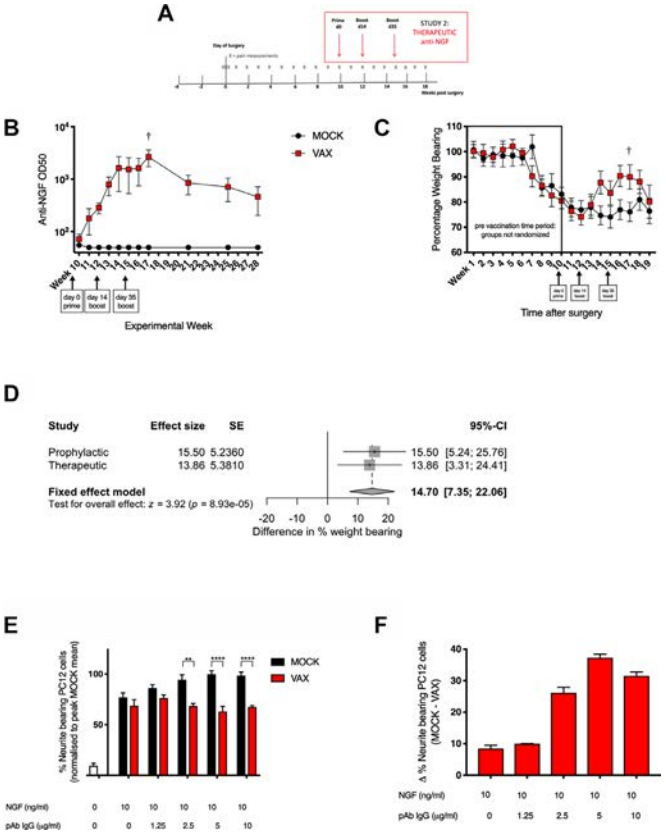

Figure 2 Therapeutic NGF vaccination reduces murine OA pain. (A) Therapeutic vaccination protocol. (B) Anti-NGF titres in sentinel cohort (n=10). (C) Painful behaviour measured by incapacitance testing where 100% represents equal weight distributed across R and L Limbs (n=40). Mice were randomised to receive mock or NGF vaccine at 10 weeks postsurgery. Repeated-measures two-way ANOVA with Bonferroni multiple comparisons test applied. SEM shown. Differences between treatment groups during late OA pain phase were not significant after correcting for multiple testing (D) Forest plot of meta-analysis comparing the effect size of analgesic response between mock and vaccinated cohorts at points of highest titre in the sentinel groups (week 13 for the prophylactic study, week 17 for the therapeutic study, marked by †). (E) Neurite outgrowth inhibition with increasing concentrations of IgG isolated from serum of vaccinated animals and (F) their normalised difference compared with mock-vaccinated animals. Bars represent mean±SEM, *p<0.05, ***p<0.001, ****p<0.0001 by t-test. ANOVA, analysis of variance; NGF, nerve growth factor.

the two studies ($I^2=0$, p=0.827). The sentinel cohort was maintained to follow the fall in antibody titres over the following 10 weeks, which was similar to that observed in previous studies.[17] IgG purified from the serum of CuMVtt^{NGF} vaccinated, but not control mice was able to dose-dependently inhibit NGF induced neurite outgrowth in PC-12 cells (figure 2E,F), to a level similar to that seen with 150 ug/mL monoclonal anti-NGF antibody (online supplementary figure 4). The effect appeared to plateau after 5 ug/mL.

Vaccines to self-antigens have been developed for other non-communicable diseases over the years. Early studies showed preclinical success but with limited clinical efficacy, which may have been due to poor immunogenicity of the vaccine platform, requiring the use of codelivery of adjuvant in preclinical models. Recent studies using refined vaccine platforms have demonstrated translatable efficacy from mouse to large animals including humans.[18–20] Our results show that CuMVtt^{NGF} vaccination produces analgesia in mice when delivered both before and after pain behaviour has become established. A unique aspect of this study is to combine a novel VLP-based therapeutic vaccine with measures of spontaneous pain behaviour in murine OA; its success confirming NGF as a valid target for OA related pain.[21]

Implementation of this type of strategy to treat OA pain has additional benefits. It induces a polyclonal response that might be more effective than a recombinant monoclonal antibody as it will stimulate antigen removal mediated by Fc-dependent clearance mechanisms.[7] It should also prevent a reduction in efficacy over time by anti-idiotypic antibodies. However, safety is also a concern. Accelerated arthropathy (rapidly progressive OA, RPOA) has been described in a small proportion of patients receiving high dose anti-NGF therapy, especially in combination with NSAIDs.[3] The mechanism for this is unclear and may be related to loss of joint protection when pain is abrogated or due to, as yet, undefined disease modifying actions of NGF.[3] It is therefore reassuring that this vaccination strategy does not induce long-lived antibody responses and requires regular boosting to maintain titres. While we did not observe accelerated disease in our NGF-vaccinated cohort, we recognise that safety remains a significant issue, and this would need to be monitored carefully in any future clinical development. This proof of concept study has significant translational potential; in the first instance within veterinary practice where activity measures are validated pain outcomes.[22] Ultimately, this has the potential to reduce the burden of disease in humans (online supplementary files 5–7).

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Contributors AE-T, ISvL and TLV designed the studies. MFB originated the concept of the vaccine. JM-Z and ISvL conducted the mouse surgery and ISvL conducted the behavioural studies. AZ provided VLP constructs and developed purification strategies. AE-T produced and characterised the CuMVttNGF vaccine. AET performed the vaccinations and immunological assays. IP performed the histological preparation and in conjunction with ISvL conducted the histological analysis. LJ conducted and approved of the statistical analysis. JM-A conducted the ELISA experiment.

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Competing interests MFB is founder of SAIBA GmbH and Hypopet AG, that own the VLP-platform IP and is involved in the development of therapeutic VLP-based vaccines for commercial purposes.

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TRANSLATIONAL SCIENCE

Exploration of CRISPR/Cas9-based gene editing as therapy for osteoarthritis

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ABSTRACT

Objectives Osteoarthritis (OA) is a painful and debilitating disease and it is associated with aberrant upregulation of multiple factors, including matrix metalloproteinase 13 (MMP13), interleukin-1β (IL-1β) and nerve growth factor (NGF). In this study, we aimed to use the CRISPR/Cas9 technology, a highly efficient gene-editing tool, to study whether the ablation of OA-associated genes has OA-modifying effects.

Methods We performed intra-articular injection of adeno-associated virus, which expressed CRISPR/Cas9 components to target each of the genes encoding MMP13, IL-1β and NGF, in a surgically induced OA mouse model. We also tested triple ablations of NGF, MMP13 and IL-1β.

Results Loss-of-function of NGF palliates pain but worsens joint damage in the surgically induced OA model. Ablation of MMP13 or IL-1β reduces the expression of cartilage-degrading enzymes and attenuates structural deterioration. Targeting both MMP13 and IL-1β significantly mitigates the adverse effects of NGF blockade on the joints.

Conclusions CRISPR-mediated ablation of NGF alleviates OA pain, and deletion of MMP13-1β or IL-1β attenuates structural damage in a post-traumatic OA model. Multiplex ablations of NGF, MMP13 and IL-1β provide benefits on both pain management and joint structure maintenance. Our results suggest that CRISPR-based gene editing is useful for the identification of promising drug targets and the development of feasible therapeutic strategies for OA treatment.

Key messages

What is already known about this subject?

- Nerve growth factor (NGF), interleukin-1β (IL-1β) and matrix metalloproteinase 13 (MMP13) are upregulated and play pivotal roles in the pathogenesis of osteoarthritis (OA). Thus, these factors could be promising drug targets for OA treatment.
- Positive results from clinical trials of NGF inhibition for the treatment of OA pain have been announced recently.

What does this study add?

- Intra-articular delivery of CRISPR/Cas9, a highly efficient gene editing tool, causes gene-nullifying mutations of NGF, IL-1β and MMP13 in osteoarthritic murine knee joints.
- CRISPR-mediated ablation of NGF palliates OA pain but worsens articular cartilage (AC) destruction and osteophyte outgrowth in the mouse OA model.
- Loss-of-function of IL-1β or MMP13 attenuates AC degradation and osteophyte formation.
- Multiplex targeting against NGF, IL-1β and MMP13 mitigates both OA pain and structural damage in the mouse model.

How might this impact on clinical practice or future developments?

- CRISPR-based gene editing is useful for the identification of promising drug targets and the development of feasible therapeutic strategies for OA treatment.

INTRODUCTION

Osteoarthritis (OA) is a painful joint disease affecting more than 10% of the adult population.[1 2] Pathological changes of OA are complicated and involve multiple tissues, as manifested by articular cartilage (AC) destruction, joint space narrowing, synovial hyperplasia, osteophyte formation and subchondral bone sclerosis.[3 4] It is recognised that OA is a highly heterogeneous disease, as patients with OA often show varied degrees of pathological features including pain, inflammation, cartilage degradation and bone spurs. Currently, OA is not curable, and clinical trials targeting OA-associated factors including matrix metalloproteinases (MMPs), inflammatory cytokines or growth factors, showed mixed results regarding the efficacy or safety of the therapeutics.[5 6] Thus, it is highly important to develop new therapeutic strategies for OA treatment, which also demands a more advanced understanding of OA.

The recent development of the CRISPR/Cas9 technology has opened an avenue to easy and efficient gene editing. In this system, Cas9 proteins and the engineered single guide RNA (sgRNA) form a complex to recognise the target DNA sequence, and introduce a double-stranded break in genomic DNA, which is hazardous and therefore subjected to DNA repair.[7 8] Two major DNA repair mechanisms can be used: error-proof homology-directed repair (HDR) and error-prone non-homologous end joining (NHEJ), of which the latter is more efficient but causes small insertions or deletions (indels) resulting in gene disruption.[9] Thus, CRISPR-mediated NHEJ can be used as a highly efficient approach to achieve permanent and complete loss-of-function of disease-causing genes.

OA involves aberrant gene upregulation in joint tissues. For instance, upregulations of nerve growth factor (NGF) and interleukin-1β (IL-1β) are found in OA, and MMP13 is a dominant collagenase expressed in OA cartilage, of which all play key roles in OA pathophysiology.[5 6 10] Therefore, ablation of these genes offers an attractive option to treat OA in a thorough and lasting manner. In this work, we explored CRISPR-mediated gene editing to treat OA, by targeting genes encoding NGF, IL-1β or MMP13 in a surgically induced OA mouse model. Our results demonstrated that targeting IL-1β or MMP13 reduced post-traumatic OA (PTOA) progression, and NGF ablation significantly palliated PTOA pain but accelerated joint damage. Importantly, combination of IL-1β- and MMP13-targeting in the setting of NGF targeting resulted in similar palliative effects on pain as NGF blockade alone, but minimised its side effects on joint structure.

MATERIALS AND METHODS

See online supplementary materials and methods.

RESULTS

NGF reduction through CRISPR/Cas9 significantly reduced OA pain but accelerated PTOA progression

We constructed multiple adeno-associated virus (AAV) vectors for each gene based on the CRISPR/Cas9 system derived from *Staphylococcus aureus*.[11] To identify effective guide sequences targeting the genes, we stably transfected mouse CD45$^-$ bone marrow stromal cells, which are mostly non-hematopoietic mesenchymal cells,[12] with the vectors, and sequenced the targeted genomic regions. Our results showed that the AAV vectors successfully generated gene-nullifying mutations (online supplementary figures 1-3). Importantly, introduction of two vectors simultaneously caused a deletion between two targeted loci, ensuring a complete loss-of-function of the gene (online supplementary figure 1 and 2). We chose AAV serotype 5 to express the CRISPR-Cas9 system in the knee joint,[13] as injection of AAV5 drove a potent, long-lasting expression of GFP in the murine joints (online supplementary figure 4). Before in vivo administration of the CRISPR-expressing AAVs, we induced PTOA in mice by partial meniscectomy. Ten days after the OA-inducing surgery, we performed intra-articular injections of two AAVs for each gene into the knee joint of the mice. Since pain is a major symptom of OA, we performed behavioural tests to determine if the pain-related behaviour was altered by CRISPR-mediated gene ablation. Our results showed that administration of AAVs targeting NGF significantly reduced pain sensitivity (figure 1A) and increased the rearing durations (online supplementary figure 5), suggesting that NGF ablation palliates OA pain and allow the mice to have more weight-bearing activities including rearing. Thus, our results confirm that NGF is a major mediator of OA pain in this animal model.

To evaluate the effects of the CRISPR therapy on joint structure, we also examined joint tissues through histology and μCT analyses. Three months after AAV injection, joint degeneration was evident in the mice receiving the control injection, as shown by AC degradation, synovial hyperplasia and subchondral sclerosis (figure 1B). In contrast to its impressive pain-palliative benefit, injection of NGF-targeting AAV did not demonstrate any positive effects in mitigating joint damage (figure 1B,C). The μCT results also revealed significant osteophyte outgrowth in the joints receiving NGF ablation, which is comparable to that in the control group (figure 1D). Six months after AAV injection, NGF ablation even had striking deleterious effects on joint structure in OA-inflicted joints, as demonstrated by more severe abrasion of AC, and marked enlargement and calcification of synovium (online supplementary figures 6 and 7). Collectively, our data showed that CRISPR-mediated NGF loss-of-function has significant pain-palliative efficacy but poses a risk of more severe cartilage degradation and ectopic bone formation.

Ablation of IL-1β or MMP13 ameliorates OA progression

We also analysed the joints receiving IL-1β-targeting or MMP13-targeting AAV by histology and μCT analyses. Our results demonstrated that CRISPR-mediated disruption of IL-1β or MMP13 significantly mitigated joint structure damage associated with PTOA progression 3 months after AAV injection (figure 1E,F), as they show significantly improved AC thickness. IL-1β antagonism also demonstrated significant efficacy in reducing synovial enlargement (figure 1E). We also looked at joint histology 6 months after AAV injection and found that ablation of MMP13 or IL-1β still had significant positive effects in attenuating pathological changes of the joint, by reducing AC destruction, decreasing synovial hyperplasia and lessening osteophyte growth (online supplementary figures 6 and 7). However, gene editing targeting MMP13 or IL-1β appeared to be not as effective as that targeting NGF in relieving OA pain as shown by the von Frey tests (figure 1G,H) and measurements of rearing (online supplementary figure 5), suggesting that MMP13 or IL-1β may not have a pivotal role as NGF in OA pain genesis and OA pain management requires additional measures than targeting joint catabolism.

CRISPR/Cas9-mediated gene editing extensively alters OA-associated signalling in multiple joint tissues

To examine the efficiency of gene ablation mediated by CRISPR/Cas9-expressing AAVs, we performed fluorescent immunohistochemistry (IHC) studies of the target proteins and found that AAV administrations successfully decreased the expression of their respective target genes, including NGF, IL-1β and MMP13 in the knee joints (figure 2A–C, online supplementary figure 8). Our quantitative reverse transcription PCR assays also demonstrated that the mRNA expression of the targeted genes was reduced in the total joint tissues (online supplementary figure 9). Notably, the ablation of the genes involves multiple tissues inside the joint, including AC, synovium, menisci and newly formed osteophytes. This suggested that intra-articular delivery of CRISPR-containing AAV led to deficiency of these OA-associated genes in the entire joint, achieving well-rounded therapeutic effects. Importantly, the data from the control group showed that the expressions of NGF, IL-1β, MMP13 and Adamts5 in the synovium and meniscus were abundant (figure 2A–C, online supplementary figure 10), suggesting that aberrant upregulation of their expressions may induce PTOA pathogenesis and progression. Specifically, expressions of IL-1β and MMP13 in joint tissue such as AC, synovium and meniscus may induce catabolic responses in AC, and NGF expression could induce neurite outgrowth in the synovium and meniscus, which fosters the formation of mechanical hypersensitivity.

As IL-1β and NGF are pivotal factors associated with OA, we asked how the therapies targeting these genes altered the downstream signalling in the joint tissues. Consistent with the change in pain-related behaviour of the mice receiving the gene editing, neurite growth in the synovium as marked by the immunostaining of β-III tubulin, a neuronal marker, was significantly decreased in the groups receiving the administration of NGF sgRNAs (figure 2D). Interestingly, NGF deletion also caused an

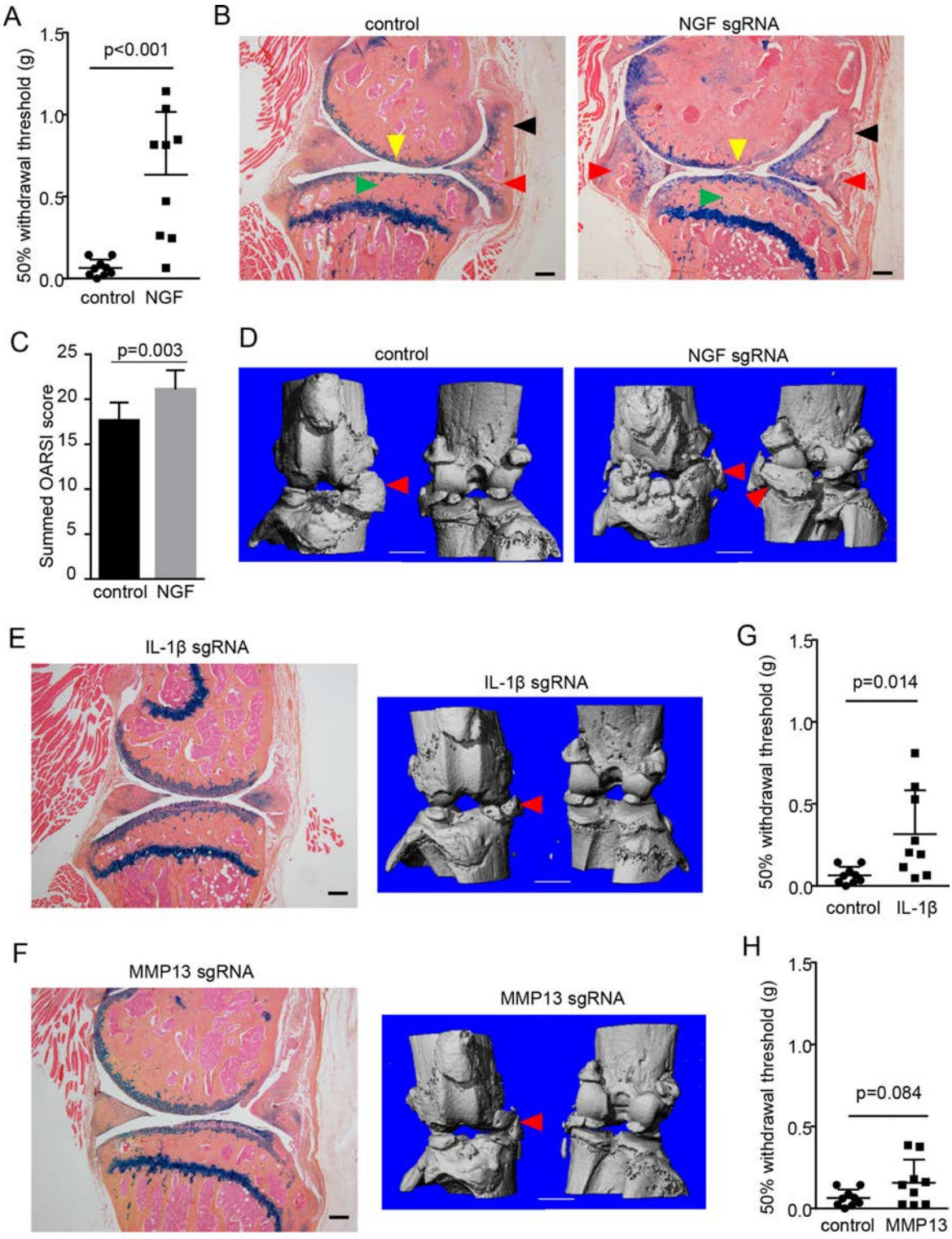

Figure 1 OA-modifying effects by CRISPR-mediated ablation of NGF, IL-1β and MMP13. (A) Results of von Frey test on the mice receiving the PMM surgery and administration of AAV that expresses control or NGF-targeting sgRNAs. n=9. Unpaired Student's t-test. (B) Representative histology images of osteoarthritic knee joints, which were collected 3 months after injections of control or NGF-targeting AAV. Yellow arrowheads, loss of AC; red arrowheads, osteochondrophytes; black arrowheads, synovial hyperplasia; green arrowheads, subchondral sclerosis. n=9. Scale bar, 200 μm. (C) OARSI scoring of knee joint AC destruction in the mice receiving the PMM surgery and control or NGF-targeting AAV. Both medial femoral condyle and medial tibial plateau were analysed on three-level sections of the joints and summed OARSI scores for the entire joint were presented. Unpaired Student's t-test. n=9. (D) Representative μCT images of osteoarthritic knee joints, which were collected 3 months after injections of control or NGF-targeting AAV. Red arrowheads, osteophytes. n=9. Scale bar, 1 mm. (E,F) Representative histological and μCT results of osteoarthritic knee joints, which were collected three months after injections of IL-1β-targeting (E) or MMP13-targeting AAV (F). (G,H) Results of von Frey tests on the mice receiving the PMM surgery and administration of AAV that expresses IL-1β- (G) or MMP13-targeting AAV (H). Unpaired Student's t-test, n=9. AAV, adeno-associated virus; AC, articular cartilage; IL-1β, interleukin-1β; MMP13, matrix metalloproteinase 13; NGF, nerve growth factor; OA, osteoarthritis; PMM, partial meniscectomy.

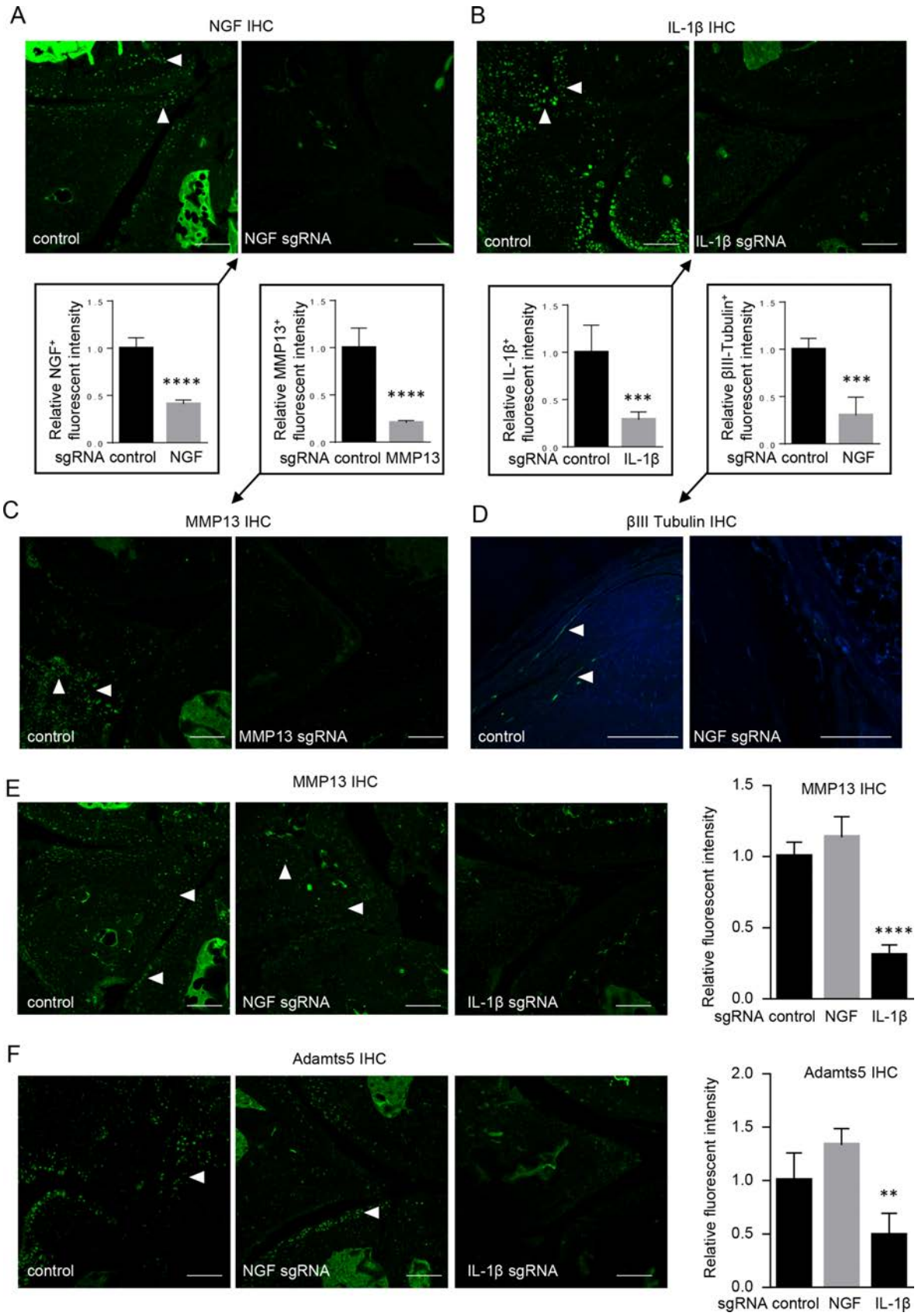

Figure 2 CRISPR-mediated gene editing attenuated OA-associated downstream signalling. (A–C) Administration of gene-ablating AAV reduced the expression of the individual targets in osteoarthritic knee joints, such as NGF (A), IL-1β (B) and MMP13 (C). (D–F) NGF-targeting AAV downregulated the expression of βIII tubulin (D), MMP13 (E) and Adamts5 (F) and IL-1β-targeting AAV reduced the expression of MMP13 (E) and Adamts5 (F) in osteoarthritic knee joints. Arrowheads, IHC-positive cells. n=5. Scale bar, 50 µm. Unpaired Student's t-test (A–D) or one-way ANOVA followed by the Tukey-Kramer test (E,F). **p<0.01, ***p<0.001, ****p<0.0001. AAV, adeno-associated virus; ANOVA, analysis of variance; IHC, immunohistochemistry; IL-1β, interleukin-1β; MMP13, matrix metalloproteinase 13; NGF, nerve growth factor; OA, osteoarthritis.

upregulation of MMP13 and Adamts5 (figure 2E,F), two major cartilage-degrading enzymes responsible for OA development as well as increased degradation products of Aggrecan (online supplementary figure 11). Thus, our results suggested that more severe cartilage destruction induced by NGF blockade is associated with dysregulation of catabolic enzymes in non-neuronal joint cells. Moreover, IL-1β deletion restrained the expressions of MMP13 and Adamts5 during PTOA progression, which pointed to the role of IL-1β as an inflammatory cytokine in promoting cartilage degradation. Together, we conclude that intra-articular gene editing targeting NGF and IL-1β act on multiple joint tissues, including AC, synovium and menisci, to affect downstream signalling pathways and to change the course of PTOA progression.

Multiplex gene editing of NGF, IL-1β and MMP13 provides enhanced benefits including both pain palliation and structural amelioration

As NGF inhibition is efficacious in OA pain mitigation but shows adverse effects on joint structure, we explored multiplex inhibitions of NGF, IL-1β and MMP13, in order to find a strategy offering the pain-palliative benefit of anti-NGF therapy without adverse joint events. IL-1β and MMP13 were chosen as the supplemental targets, because they are extracellular proteins to be more accessible by currently available biologics, and their antagonism had positive effects on joint morphology as demonstrated by our study (figure 1E,F). We performed pain-related behavioural studies, through von Frey test and measurement of rearing, and found that the multiplex therapy effectively retained pain-modifying effects of NGF blockade, as demonstrated by a significantly decreased mechanical sensitivity threshold and increased rearing durations compared with the control group (figures 1A and 3C, online supplementary figure 5). Moreover, our radiographic and histological analyses revealed that multiplex gene editing did not accelerate PTOA progression as rapidly as ablation of NGF alone did (figure 3A,B, online supplementary figure 7). Specifically, histological and μCT analyses demonstrated that AC degradation, synovial hyperplasia, osteophyte formation and subchondral sclerosis in the multiplex therapy group were significantly less than those in the control or NGF-only group (figure 3A,B, online supplementary figure 7). We also used OARSI scoring to quantify AC degradation and confirmed less destructive changes in the multiplex group (figure 3D). Thus, these results suggested that inclusion of additional targets such as IL-1β and MMP13 may offset the structural adverse effects of NGF ablation while retaining its pain-palliative benefit in the mouse model.

Next, we performed IHC studies to examine the expression levels of downstream molecules. Our data showed that both MMP13 and Adamts5 had lower expression in the multiplex group than in the control group (figure 3E–G, online supplementary figure 12). NGF ablation through CRISPR reduced the expression of NGF in the multiplex treatment group (figure 3H). Assessment of neurite growth by immunostaining β-III tubulin demonstrated that the multiplex treatment group and the NGF-only group had similar expression of β-III tubulin in the synovium, both significantly lower than in the control group (figures 2D and 3I). Together, our results suggested that the multiplex therapy attenuates the structural adverse effects of NGF blockade, possibly through decreasing inflammation and cartilage destruction, but without a compromise on pain modification offered by NGF inhibition. Further, our study proposes a strategy to treat OA that eases pain sensitisation and alleviates structural deterioration through supplementing NGF blockade with simultaneous inhibitions of catabolic and/or inflammatory factors.

DISCUSSION

In this study, we employed CRSPR/Cas9 based gene editing to treat OA, a very common, painful and debilitating disease in a surgically induced OA mouse model. Our gene editing study confirmed that NGF, IL-1β and MMP13 are promising drug targets for OA therapy, as our data demonstrated a significant improvement on joint structure or OA pain when these molecules are downregulated. Interestingly, our data of the gene editing against NGF is similar to the clinical trials that showed promising pain-palliative effects of humanised anti-NGF antibodies. However, the clinical trials exhibited an adverse structural effect presented as rapidly progressive OA, which is characterised by bone destruction,[10 14] in marked contrast to subchondral sclerosis and osteophyte outgrowth observed in our study as well as in a rat model administered with anti-NGF antibodies.[15] While the animal studies failed to reproduce the adverse events in the clinical trials of NGF blockade, our results suggest that antagonism of IL-1β and MMP13, though less impressive in OA pain palliation compared with that of NGF, could be a useful supplementation to NGF blockade, as it directly downregulates MMP13 and also reduces inflammation, thus maintaining AC and restraining the induction of catabolic factors including Adamts5 (figure 3E,G). Together, our study suggested that the symptoms of OA can be managed by a formula comprising blockade of NGF as well as inhibition of inflammation/cartilage degradation.

The target genes chosen in this study all undergo aberrant upregulation during OA pathogenesis, thus downregulation of their expressions could benefit OA treatment. Conveniently, the CRISPR/Cas9 technology provides an efficient approach to reduce gene expression by mutating genes through error-prone NHEJ. A single effective sgRNA generates small indels, which may lead to frameshift or missense mutation of the gene, or cause in-frame small mutations that may not completely silence the gene. To ensure a complete abolishment of the target genes, we introduced two sgRNAs that can produce deletions as long as hundreds of nucleotides in the targeted genes. Thus, the double sgRNA approach may avoid being obscured by incomplete gene ablations, to facilitate our evaluation of this explorative study. Notably, off-target effects could be a major concern for CRISPR/Cas9-mediated gene editing, and introduction of two sgRNAs would pose a higher risk of off-target effects. Although we did not observe apparent off-target effects in our study, it may be necessary to use only one sgRNA for clinical studies and to completely confirm that the off-target activity of the sgRNA is minimal or benign through a whole genome analysis. In addition, a proper delivery method, such as intra-articular rather than systemic administration, may greatly minimise the side-effects. Together, safe, effective sgRNAs as well as an appropriate delivery route should be vital for a successful CRISPR/Cas9-based gene editing to treat OA.

Blockade of NGF is the most promising strategy among current medications for OA pain, while it was also reported to be associated with the joint-related adverse events including bone destruction. In our study, CRISPR/Cas9-based ablation of the *Ngf* gene in the mouse joint showed enhanced ectopic bone formation. Because these joint-related adverse effects of NGF loss-of-function are in sharp contrast between humans and rodents, it is intriguing how NGF downregulation differentially induces changes in the joints of rodents and humans. An underlying mechanism could be that NGF expression in nerve is essential for the joint to retain the

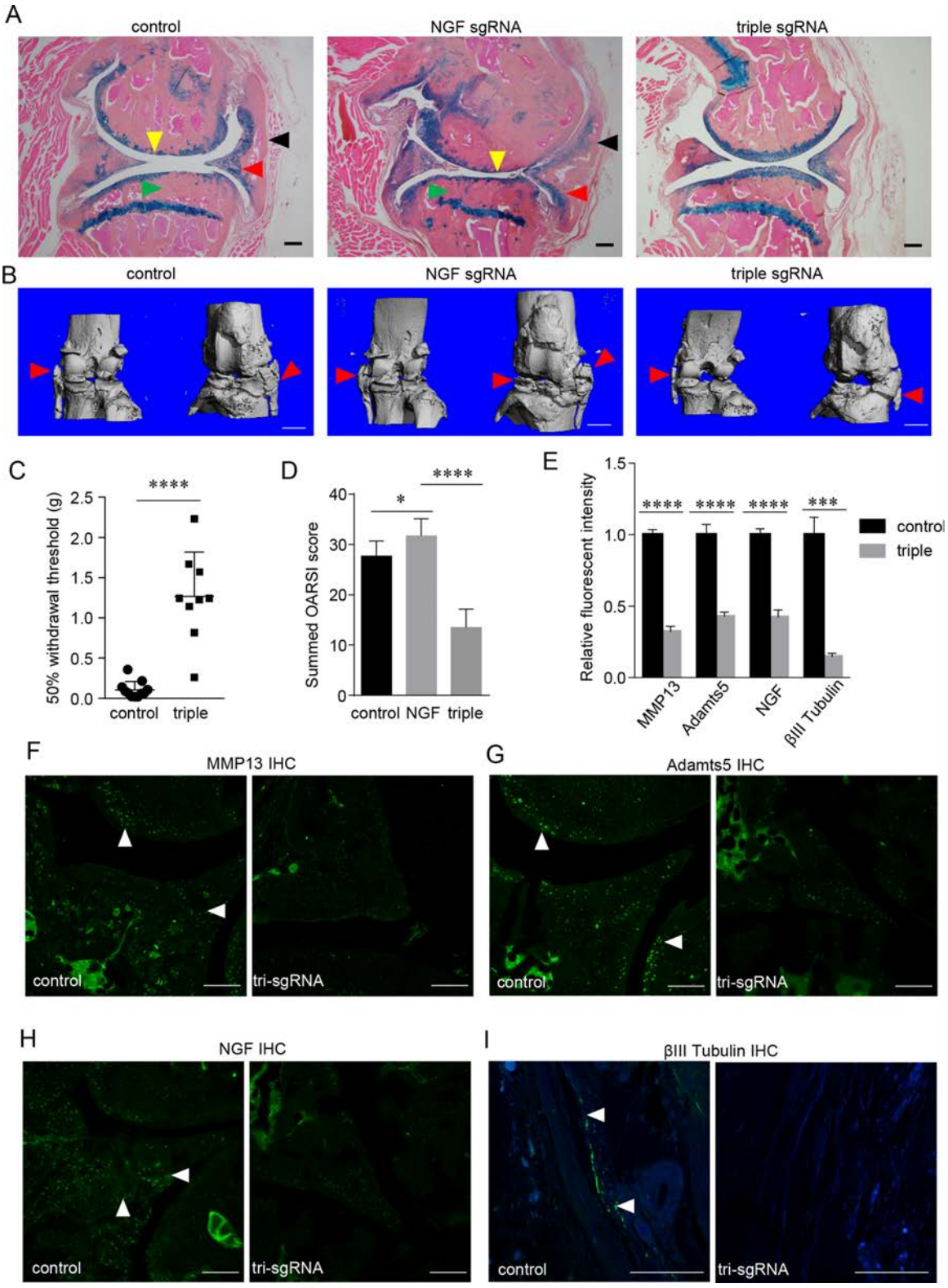

Figure 3 Concomitant loss-of-function of NGF, IL-1β and MMP13 relieves OA pain and mitigates OA progression. (A,B) Representative histology (A) and μCT (B) images of osteoarthritic knee joints, which were collected 6 months after injections of control, NGF-targeting or triple (NGF, IL-1β and MMP13)-targeting AAVs. Yellow arrowheads, loss of articular cartilage; red arrowheads, osteochondrophytes; black arrowheads, synovial hyperplasia; green arrowheads, subchondral sclerosis. n=9. Scale bar for histology, 200 μm. Scale bar for μCT, 1 mm. (C) Results of von Frey tests on the mice receiving the PMM surgery and control or triple-targeting AAV. n=9. Unpaired Student's t-test. (D) OARSI scoring of AC destruction in osteoarthritic knee joints of the mice receiving control, NGF-targeting or tri-targeting AAVs. n=9. One-way ANOVA followed by the Tukey-Kramer test. (E–I) Simultaneous deletion of NGF, IL-1β and MMP13 attenuated OA-associated matrix proteases including MMP13 (F) and Adamts5 (G) and neural genes such as NGF (H) and βIII Tubulin (I), which were quantified and summarised (E). Scale bar, 50 μm. n=5, unpaired Student's t-test. *p<0.05, ***p<0.001, ****p<0.0001. AAV, adeno-associated virus; ANOVA, analysis of variance; IL-1β, interleukin-1β; MMP13, matrix metalloproteinase 13; NGF, nerve growth factor; OA, osteoarthritis; PMM, partial meniscectomy.

ability to feel pain and other neuronal activities, which protects the joint and maintains its homeostasis. Thus, neuronal expression of NGF may have effects on non-neuronal tissues/cells through nerve activities. Nonetheless, NGF may also play important functions directly on non-neuronal joint cells, which regulate their proliferation, differentiation, survival and their anabolic/catabolic activities. A more thorough investigation for the role of NGF in the entire joint would shed new lights into development of safe and effective treatment of OA pain.

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Contributors JH designed the study and wrote the manuscript. LZ, JH and DC designed the experiments. LZ, JH, YF, JL and TY performed research. SH and GX provided expertise and feedback. JH and DC supervised the study. LZ and DC revised the manuscript.

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EPIDEMIOLOGICAL SCIENCE

Siblings of patients with rheumatoid arthritis are at increased risk of acute coronary syndrome

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ABSTRACT

Objectives To investigate a potential shared susceptibility between rheumatoid arthritis (RA) and acute coronary syndrome (ACS) by estimation of the risk of ACS among full siblings of patients with RA.

Methods By linking nation-wide Swedish registers, we identified a cohort of patients with new-onset RA 1996–2016, age- and sex-matched (5:1) general population comparator subjects, full siblings of RA and comparator subjects, and incident ACS events through 31 December 2016. We used Cox regression to estimate the HR of ACS among patients with RA and the siblings of patients with RA versus the general population, overall and stratified by RA serostatus. We explored the impact of traditional cardiovascular (CV) risk factors on the observed associations.

Results We identified 8109 patients with incident RA, and 11 562 full siblings of these. Compared with the general population, the HR of ACS in RA was 1.46 (95% CI 1.28 to 1.67) and 1.22 (95% CI 1.09 to 1.38) among their siblings. The increased risks seemed confined to seropositive RA (patients: 1.52 [1.30 to 1.79], their siblings: 1.27 [1.10 to 1.46]); no significant risk increase was observed among siblings of patients with seronegative RA (HR 1.13 [95% CI 0.92 to 1.39]). Adjustment for 19 traditional CV risk factors did not appreciably alter these associations.

Conclusion Siblings of patients with RA are at increased risk of ACS, suggesting shared susceptibility between RA and ACS, indicating the need and potential for additional cardio-preventive measures in RA (and their siblings).

Key messages

What is already known about this subject?
- Patients with rheumatoid arthritis (RA) are at increased risk of cardiovascular (CV) disease.
- Despite better inflammatory control with more efficient treatments and treatment strategies, an excess CV risk among patients with RA remains.

What does this study add?
- This study demonstrates that an excess CV risk is present also among close relatives of patients with RA, thereby suggesting shared susceptibility of RA and acute coronary syndrome (ACS).

How might this impact on clinical practice or future developments?
- Optimised RA disease control may not be enough to remove the excess CV risk in RA.
- Patients with RA (and their first-degree relatives) may benefit from additional cardio-preventive measures.

INTRODUCTION

Patients with rheumatoid arthritis (RA) are at increased risk of cardiovascular disease (CVD) including acute coronary syndrome (ACS).[1] A number of studies have demonstrated that this excess risk cannot be readily explained by traditional CV risk factors, but instead point to an association between RA disease severity and development of ACS.[2] We recently reported that despite more efficient control of inflammation in RA during recent years, and despite a general decline in ACS incidence in the general population, an excess risk for ACS among patients with RA remains.[1] These findings suggest that besides direct effects on the ACS risk exerted by the RA disease itself, there may be a shared susceptibility between RA and ACS.

If the excess risk of ACS in patients with RA was increased due to such shared susceptibility, one might expect an increased risk of ACS also in individuals without RA but with otherwise similar genetic set-up and background as the patients, such as their siblings. The objective of this study was therefore to investigate any potential shared susceptibility between RA and ACS, by estimating the risk of ACS in full siblings of individuals with versus without RA.

METHODS

Study design and setting

We performed a population-based nation-wide cohort study based on linkage of clinical and other registers with prospectively recorded information on RA, family structure and ACS. The Swedish Rheumatology Quality (SRQ) register is a clinical quality register with a current estimated coverage of around 85% of all prevalent RA followed in Swedish Rheumatology.[3] By using the unique Swedish personal identification number assigned to all permanent residents in Sweden, we linked SRQ to the following nation-wide and virtually complete registers: the Swedish Multi-generation Register (MGR), the National Patient Register (NPR), the Prescribed Drug Register (PDR), the Cause of Death Register and the Total Population Register.

The identification of our RA cohort, and subsequent register linkages, has been described in detail elsewhere.[1]

Patients with RA

In SRQ, we identified a cohort of incident RA born 1932 or later, and diagnosed with RA 1996 through 2016 within 1 year of reported RA symptom onset. RA serostatus was defined by the reporting clinician based on rheumatoid factor status.

As sensitivity analyses, and using previously devised algorithms to define incident and prevalent RA,[4] we used NPR to identify two additional RA cohorts: one with incident RA cases diagnosed between 2006 and 2016 (including also cases not followed from RA onset in SRQ), and one of all prevalent RA, irrespective of duration, with visits listing RA during 2006–2016.

Matched general population comparator subjects, and relatives of patients and comparator subjects

For each patient with RA, all full siblings born within 5 years were identified through MGR. In addition, for each patient with RA, up to five subjects from the general population, matched on age and sex, and with at least one full sibling born within 5 years, were randomly selected. All unique individuals (patients with RA, their siblings, matched general population subjects and their siblings) were required to be alive and resident in Sweden at the time of the index patient's RA diagnosis. Patients with RA and general population subjects without siblings were secluded from the study population.

Follow-up and outcome

All unique individuals were followed from the date when their index patient with RA first fulfilled the inclusion criteria in the RA cohort(s). The outcome was defined as a first-ever ACS (hospitalisation for ACS [International Classification of Diseases, Tenth Revision, ICD10: I21 or I20.0] or acute myocardial infarction listed as underlying cause of death). We censored follow-up at death, migration, first-ever RA diagnosis (for non-RA subjects) and the end of the study period (31 December 2016). All individuals with an ACS before start of follow-up were excluded.

Statistical analyses

We calculated the incidence of ACS in each cohort, and HRs of ACS comparing the RA-, sibling- and population comparator-cohorts using Cox proportional hazards model, adjusted for age, sex and calendar period of start of follow-up. All individuals with a history of ACS prior to start of follow-up were excluded from the analysis. CIs were estimated using a robust sandwich estimator to account for the correlated data structure. We contrasted the incidence of ACS among the patients with RA and their siblings to the matched general population subjects, and the incidence among RA siblings to that of the siblings of the matched general population subjects. We further estimated the risk increase in patients with RA compared with their siblings by performing within-pair analyses. We stratified by RA serostatus. As sensitivity analyses, we excluded all individuals with a history of a CV disease (CVD) (defined as ICD10: I10–I15, I20–I25, I26–I28, I30–I52, I60–I69, I70–I79 or I82) before start of follow-up, adjusted for family history of CVD before start of follow-up, and performed two subgroup analysis: one excluding all individuals with a sibling with CVD diagnosis and one of all individuals with a first-degree relative diagnosed with ischaemic heart disease (ICD10: I20–I25) at start of follow-up.

Exploratory analyses

To explore whether known CV risk factors or determinants could explain the observed increase in ACS incidence among the RA siblings, we used NPR and PDR to identify 19 medical and contextual covariates corresponding to typical general population CV risk factors or determinants such as medical histories and socioeconomic characteristics (online supplementary table 2). Since PDR was started in 2005, these analyses were restricted to individuals whose start of follow-up was 2006 or later. We first ran a Cox regression, adjusted for age, sex and calendar period, to estimate the association between each factor and incidence of ACS in the combined cohort of the general population comparators and their siblings. We then, for each of the covariates, adjusted the association between RA siblings and ACS risk for that particular risk factor. Finally, we added all covariates to the model.

By calculating the E-value, we estimated the effect size a potential unmeasured confounder would need in order to completely 'explain' the observed association between RA siblings and ACS risk.[5 6] The E-value is designed to be independent of assumptions of underlying effect sizes and prevalence of the unmeasured confounder, but assumes a symmetric effect size in the association with exposure and outcome. Therefore, we, using smoking prevalences and risk estimates for smoking and ACS from previous studies,[7 8] also estimated what the relative risk of ACS among the RA siblings would be under the extreme[9] assumption that their smoking habits were the same as their index patients' with RA, and conversely, how high the prevalence of smoking in the RA sibling cohort would have to be to fully explain the observed risk increase in that cohort. Details of these analysis are in online supplementary material 1.

Ethical permission was obtained from the Stockholm ethics review board (2015/1844-31/2).

RESULTS

For the main analysis, we identified 8109 patients with RA (66% seropositive), 11 562 of their full siblings, 38 092 general population comparator subjects and 50 793 full siblings of the latter (table 1). The proportion of RA siblings excluded due to a history of ACS (2.8%) was higher than the proportion among the siblings of the general population (2.4%), p=0.037, table 1.

During a median follow-up of 6 years, the crude incidence of ACS was 4.64 (per 1000 person-years) among RA cases, 4.74 among their siblings, 3.18 among the matched general population subjects and 3.84 among the comparator subjects' siblings. Compared with the general population, the age-, gender- and calendar-period-adjusted HR for ACS among the patients with RA was 1.46 (95% CI 1.28 to 1.67) and 1.22 (95% CI 1.10 to 1.38) among their siblings. Comparing the siblings of the patients with RA with the siblings of the general comparator subjects resulted in a similar risk estimate (HR=1.18 [95% CI 1.06 to 1.32]). The increased risk of ACS was largely confined to patients with seropositive RA (HR=1.52 [95% CI 1.30 to 1.79]) and to their siblings (HR=1.27 [95% CI 1.10 to 1.46]). The elevated risk for patients with seronegative RA was less pronounced (HR=1.34 [95% CI 1.06 to 1.70]), and nonsignificant for their siblings (HR=1.13 [95% CI 0.92 to 1.39]), figure 1. The difference in relative risks for the siblings of the seropositive and seronegative patients was, however, not statistically significant.

The within-pair analysis confirmed the increased risk of ACS in RA (HR=1.19 [95% CI 1.04 to 1.37]) but also that

Table 1 Demographics and HRs for ACS among new-onset patient with RA identified 1996–2016 in the SRQ register, their full siblings, matched general population subjects and among full siblings of the latter, and stratified by index patient with RA's serostatus. NB. The sex distribution between the patients with RA and their matched reference individuals differs compared with the siblings of the patients with RA and the siblings of the reference individuals. Therefore, the proportions and incidence of ACS are not directly comparable between these two pairs of cohorts.

	Patients with RA	Full siblings of the patients with RA	Matched reference individuals in the general population	Full siblings of matched reference individuals in the general population
Incident SRQ cohort				
N initial	8305	11 893	38 885	52 067
Excluded due to ACS event before start of study (%)	196 (2.4)	331 (2.8)	793 (2.0)	1274 (2.4)
N study	8109	11 562	38 092	50 793
Mean age (SD)	53.5 (14.3)	54.1 (14.2)	53.1 (14.3)	53.3 (14.3)
Women N (%)	5738 (70.8)	5734 (49.6)	27 035 (71)	26 760 (52.7)
Median years of follow-up	6.5 (8)	6.4 (8.2)	6.4 (8.1)	6.7 (8.2)
ACS events during study	283	410	911	1501
Incidence per 1000 person-years	4.64	4.74	3.18	3.84
HR (95% CI)*	1.46 (1.28 -1.67)	1.22 (1.09 -1.38)‡ 1.18 (1.06 -1.32)§	Ref	–
Within-pair analysis†	1.19 (1.04–1.37)	Ref	–	–
RF-positive subset				
N initial	5481	7908	25 736	34 443
Excluded due to ACS event before start of study (%)	127 (3.3)	219 (2.8)	502 (2.0)	803 (2.3)
N study	5354	7689	25 234	33 640
Mean age (SD)	53.1 (14)	53.8 (13.9)	52.8 (14)	52.9 (14)
Women N (%)	3854 (72)	3852 (50.1)	18 220 (72.2)	17 653 (52.5)
Median months of follow-up	6.9 (8.3)	6.8 (8.3)	7 (8.3)	7.2 (8.5)
ACS events during study	195	285	609	1026
Incidence per 1000 person-years	4.63	4.74	3.06	3.77
HR (95% CI)*	1.52 (1.30 -1.79)	1.27 (1.10 -1.46)	Ref	–
Within-pair analysis†	1.21 (1.02–1.44)	Ref	–	–
RF-negative subset				
N initial	2824	3985	13 149	17 624
Excluded due to ACS event before start of follow-up (%)	69 (2.4)	112 (2.8)	291 (2.2)	471 (2.7)
N study	2755	3873	12 858	17 153
Mean age (SD)	54.2 (14.9)	54.9 (14.6)	53.7 (14.9)	54 (14.8)
Women N (%)	1884 (68.4)	1882 (48.6)	8815 (68.6)	9107 (53.1)
Median months of follow-up	5.8 (7.4)	5.7 (7.6)	5.7 (7.3)	5.9 (7.6)
ACS events during study	88	125	302	475
Incidence per 1000 person-years	4.67	4.74	3.46	3.98
HR (95% CI)*	1.34 (1.06 - 1.70)	1.13 (0.92 - 1.39)	Ref	–
Within-pair analysis†	1.14 (0.89–1.46)	Ref		

*Adjusted for sex, age and calendar period of index case's diagnosis year and compared with the matched reference individuals in the general population.
†Adjusted for age, sex and birth cohort.
‡Compared to the matched reference individuals.
§Compared to the full siblings of the matched reference individuals.
ACS, acute coronary syndrome; RA, rheumatoid arthritis; RF, rheumatoid factor; SRQ, Swedish Rheumatology Quality.

this association, because of the increased risk also among RA siblings, was of lower magnitude than when patients with RA were compared with the general population. The within-pair relative risk of ACS was more evident in seropositive (HR=1.21 [95% CI 1.02 to 1.44]) rather than seronegative (HR=1.14 [95% CI 0.89 to 1.46]) RA.

Online supplementary table S1 presents the corresponding results from the sensitivity analyses. In the incident cohort, risks and HRs were similar or slightly lower than the main analysis. In the prevalent cohort, in which the mean RA duration was longer than in the incident cohorts, the HR of ACS among the patients with RA compared with their general population comparator subjects was higher (HR=1.79 [95% CI 1.67 to 1.91]), whereas the elevated HR among RA siblings (vs the siblings of the general population comparator subjects) remained significant but less pronounced (HR=1.09 [95% CI 1.02 to 1.16]); the within-pair analysis in this cohort resulted in a HR=1.63 (95% CI 1.51 to 1.77).

Removing individuals with a history of CVD before start of follow-up and adjusting for family history of CVD did not noticeably change the HRs. Subset analyses restricted to individuals without any sibling history of CVD, and without any

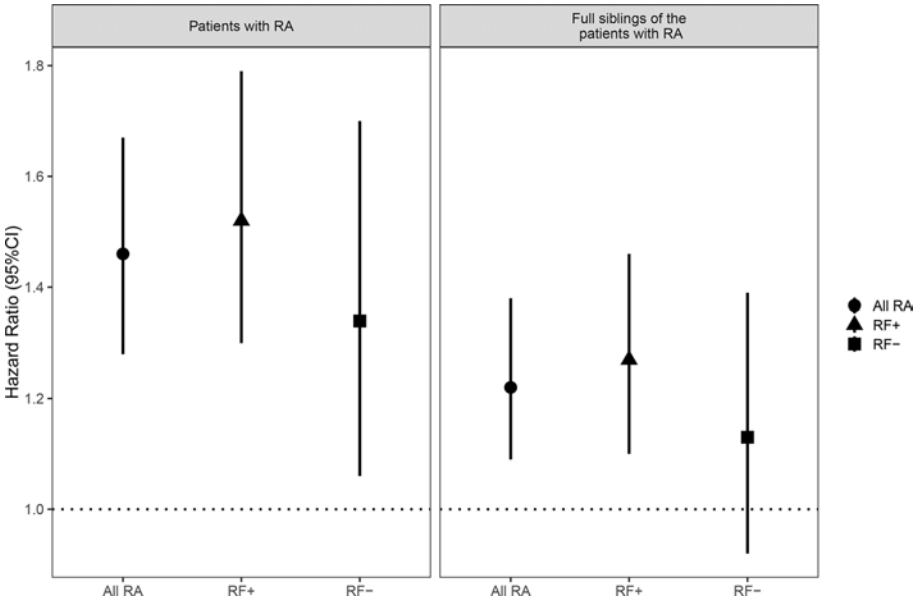

Figure 1 HRs and 95% CIs for acute coronary syndrome in Swedish patients with RA and their full siblings compared with matched reference individuals from the general population. The RA cohort was identified using the Swedish Rheumatology Quality register. HRs are presented overall and stratified by index patients with RA's serostatus.
RA, rheumatoid arthritis.

first-degree relative with IHD, respectively, the HRs decreased by less than 10% but remained significantly elevated (data not shown).

Our exploratory analyses demonstrated that 14 of the 19 CV risk factors/determinants were indeed ACS risk factors. When added to the model either one by one or all together, they did not appreciably explain the increased incidence of ACS among the RA siblings, neither overall nor when stratified by index RA serostatus (online supplementary table S2–S4).

The E-value was 1.74 with a lower bound of 1.40. In the seropositive group, the corresponding E-value was 1.86 with a lower bound of 1.43.

Finally, under the assumptions that the prevalence of ever/current smoking among the RA siblings would be the same as reported for patients with RA (67% current, 31% ever smokers, data from a Swedish study on incident RA 1996–2006[7]) rather than the general population (54% ever, 22% current smokers[7]), and that the ACS risk is increased by a factor 1.40 among past and 2.90 among current smokers,[8] we estimated that the risk for ACS in the RA sibling cohort versus the general population, in the absence of any true association between RA siblings and ACS risk, would be 1.11 (instead of 1). The same estimations indicated that for the observed overall HR of 1.22 among the RA siblings to be fully explained by an increased prevalence of smokers among the RA siblings, they would have to smoke substantially more than the patients with RA themselves.

DISCUSSION

In this study, we demonstrate that full siblings of patients with RA are at increased risk of ACS compared with the general population, and that this increase cannot readily be explained by confounding by traditional ACS risk factors or by socioeconomic factors, pointing to the existence of other shared risk factors or susceptibility between RA and ACS. Through this, we demonstrate that a substantial proportion of the increased risk of ACS in patients with RA is likely due to other factors (shared with their siblings) than the RA disease itself. Beyond our main findings, our study further demonstrates that the increased risk of ACS in patients with RA prevail also in RA diagnosed 2006 or later and followed through 2016, confirming that the level of risk increase remains elevated, is higher in patients with established versus incident RA, and largely confined to seropositive RA.

Whereas our results point to the existence of shared susceptibility, its nature (genetic or environmental) remains to be established. Initial reports suggested an association between the CIITA gene, RA and myocardial infarction.[10] Subsequent reports have, however, failed to replicate this association.[11–15] One report has also linked the shared epitope alleles to myocardial infarction risk.[16] Our results suggest that adjustment for medical or contextual CV risk factors did not remove the observed increase in sibling risk. Smoking is a risk factor for both RA and ACS, and to some extent familial.[17] In studies of twin-pairs discordant for RA, the twin affected with RA is more likely to be a smoker than its co-twin.[9] By contrast, our exploratory analyses indicated that to fully explain the increased ACS risk in RA siblings, we would need to make extreme assumptions about smoking among the siblings such that they were more often smokers than their index-patient with RA, an assumption that is neither plausible nor supported by previous literature.[9] Thus, and since smoking is a risk factor for many of the ACS risk factors adjusted for in our exploratory analyses (online supplementary table S4), we conclude that unmeasured confounding from smoking may to some part, but is unlikely to fully, explain the observed risk increase among the RA siblings.

Our study has a few limitations. Despite large numbers of patients with RA we still had limited precision and thus cannot formally exclude that the adjustments for our 19 risk factors actually did remove some of the association under study. Some of the ACS risk factors (hypertension, chronic obstructive pulmonary disease, mild renal insufficiency) used in this analysis are treated in primary and not specialist care and thus not covered by NPR. For this reason, we used information on dispensed drugs (from PDR, with full coverage) as an additional source to define these risk factors. Another limitation is the lack of individual-level smoking data.

We are not aware of any previous large-scale studies of ACS risks among close relatives of patients with RA. Indeed, the main strength of our study is the population-based, nationwide setting, the use of registers with high coverage of patients with RA, their first-degree relatives and the occurrence of ACS all of which could be determined independently of each other rather than, say, by self-report. For the same reasons, we believe the results from our study may be generalisable to similar subjects also outside of Sweden.

In conclusion, from an etiological point of view, our findings indicate a shared susceptibility to RA and ACS that is not readily explained by traditional CV risk factors but point to a need to further explore the nature of this association, be it genetic or environmental. From a clinical point of view, our findings serve as a reminder that reducing or removing RA-specific inflammation may in itself not be sufficient to remove the entire excess risk of ACS in RA. Instead, additional cardio-preventive measures, such as optimisation of traditional CV risk factors, may be (particularly) important in these patients and among their siblings.

Contributors HW had full access to all of the data used for the analysis in this study and takes full responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: JA, HW and TF. Acquisition of data: JA, TF and HW. Statistical analysis: HW, TF and JA. Analysis and interpretation of data: all authors. Drafting of manuscript: HW and JA. Critical revision of manuscript and final approval given: all authors. Obtained funding: JA. Study supervision: JA.

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Competing interests JA has or has had research agreements with Abbvie, Astra-Zeneca, BMS, Eli Lilly, MSD, Pfizer, Roche, Samsung Bioepis, and UCB, mainly in the context of safety monitoring of biologics via ARTIS/Swedish Biologics Register. Karolinska Institutet has received remuneration for JA participating in advisory boards arranged by Pfizer and Lilly.

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EPIDEMIOLOGICAL SCIENCE

Common mental disorders within chronic inflammatory disorders: a primary care database prospective investigation

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ABSTRACT

Objective There is inconsistent evidence about the association between inflammatory disorders and depression and anxiety onset in a primary care context. The study aimed to evaluate the risk of depression and anxiety within multisystem and organ-specific inflammatory disorders.

Methods This is a prospective cohort study with primary care patients from the UK Clinical Practice Research Datalink diagnosed with an inflammatory disorder between 1 January 2001 and 31 December 2016. These patients were matched on age, gender, practice and index date with patients without an inflammatory disorder. The study exposures were seven chronic inflammatory disorders. Clinical diagnosis of depression and anxiety represented the outcome measures of interest.

Results Among 538 707 participants, the incidence of depression ranged from 14 per 1000 person-years (severe psoriasis) to 9 per 1000 person-years (systemic vasculitis), substantively higher compared with their comparison group (5–7 per 1000 person-years). HRs of multiple depression and anxiety events were 16% higher within inflammatory disorders (HR, 1.16, 95% CI 1.12 to 1.21, p<0.001) compared with the matched comparison group. The incidence of depression and anxiety was strongly associated with the age at inflammatory disorder onset. The overall HR estimate for depression was 1.90 (95% CI 1.66 to 2.17, p<0.001) within early-onset disorder (<40 years of age) and 0.93 (95% CI 0.90 to 1.09, p=0.80) within late-onset disorder (≥60 years of age).

Conclusions Primary care patients with inflammatory disorders have elevated rates of depression and anxiety incidence, particularly those patients with early-onset inflammatory disorders. This finding may reflect the impact of the underlying disease on patients' quality of life, although the precise mechanisms require further investigation.

Key messages

What is already known about this subject?

- Several cross-sectional studies suggested but did not establish a contributory role of inflammation in the initiation of depression and anxiety within patients diagnosed with chronic inflammatory disorders.

What does this study add?

- In a prospective cohort study with 538 707 patients from primary care, a significant increment in the onset of new depression and anxiety events was documented within organ-specific and multisystemic inflammatory disorders.
- The incidence of depression or anxiety varied with the age at inflammatory disorder onset.

How might this impact on clinical practice or future developments?

- The elevated risk of depression and anxiety means clinicians should be vigilant for early symptoms of depression or anxiety in this highly at-risk group of patients.
- The risk was greater among patients with younger age at inflammatory disorder onset, supporting tailored preventative approaches early in the course of a chronic disorder.
- The study, however, does not demonstrate a causal relationship between inflammation and depression and anxiety.

INTRODUCTION

A growing body of evidence indicated that low-grade inflammation may play an influential role in the onset of depression and anxiety.[1] Past research has linked upregulated proinflammatory cytokines and increased levels of acute-phase reactants with changes in neurotransmitter and neuroendocrine functioning related to psychiatric disorders.[2 3] This evidence supports a link between depression and anxiety and inflammatory disorders (eg, rheumatoid arthritis [RA], psoriasis, ankylosing spondylitis [AS]), and cross-sectional studies are in line with this suggestion.[4–7] Evidence from prospective studies exploring the role of inflammatory disorders in depression and anxiety onset was, however, inconsistent.[8 9] Little is known about the incidence of depression or anxiety across clinically diverse inflammatory disorders. Differences in genetic influences and treatment choices across inflammatory disorders may lead to variation in depression or anxiety onset.[10 11] The genetic association with human leucocyte antigen alleles, for instance, was stronger within AS compared with

RA.[10] Quantifying the extent to which the link between inflammatory disorders and depression varies by individual disorders may suggest mechanisms underlying specific relationships and ultimately facilitate targeted preventative approaches. There is substantive variation in the age of onset across individual inflammatory disorders that may also lead to differential association with depression or anxiety,[12 13] in turn more prevalent in early adult years. The incidence of depression or anxiety, thus, may be lower across disorders with late age at onset (eg, RA) than those with early age at onset (eg, Crohn's disease [CD]). The detection of disparities in mental health burden could guide treatment choice and effective tailoring of healthcare resources. The aim of the present study was to implement a prospective cohort study within a large primary care database to test the hypothesis that the incidence of depression or anxiety varied across specific inflammatory disorders. It was also hypothesised that depression or anxiety risk was greatest within people with an early age at disorder onset.

METHODS

Data

A prospective, matched cohort study design was implemented in the Clinical Practice Research Datalink (CPRD), one of the world's largest electronic medical records databases. CPRD collects routine primary care data on over 14 million patients (≈6.7 million active) from around 675 practices throughout the UK National Health Service (NHS). All patients in the NHS are registered with a general practice that provides all their primary care and coordinates secondary and community care. Important diagnostic and therapy information from referrals to secondary or community care services is captured by primary care records. Patients included in the CPRD are broadly representative of the UK's wider population in terms of age, gender and ethnicity.[14] The validity and accuracy of CPRD diagnostic and prescription data have been demonstrated across a wide range of disorders, including cancer,[15] stroke,[16] chronic obstructive pulmonary disease (COPD),[17] depression and anxiety,[18] RA,[19] inflammatory bowel disorders,[20] and autoimmune disorders.[21 22]

Study population

A cohort of primary care patients aged >18 years with a first-ever diagnosis of a chronic inflammatory disorder (psoriasis, CD and ulcerative colitis [UC], RA, systemic lupus erythematosus [SLE], AS, and systemic vasculitis [SV]) recorded between 1 January 2001 and 30 September 2016 who were free from depression or anxiety disorders at the time of inflammatory disorder diagnosis were sampled from the CPRD. The date of diagnosis was defined as the index date. The index date for patients transferring into the practice was their practice registration date, and the practice up-to-standard date was used if a practice joined the database during the recruitment period. The end of recruitment was the earliest of 30 September 2016 or the date of death or the date transferred out of the practice. Patients below the age of 18 at the time of diagnosis were excluded from the study sample because the presentation and course of inflammatory disorders might be different in younger people.[23]

All diagnoses were derived from the medical codes recorded by family physicians in patients' electronic health records. These patients were matched (a 1:2 ratio of inflammatory-exposed to two matched non-exposed) on age (year of birth), gender, practice and index date with a group of patients without a chronic inflammatory disorder selected for this study during the recruitment period. Matched controls were assigned the index date of the inflammatory disorder diagnosis of the matched case. Similar to the inflammatory patients, matched controls with a diagnosis of depression or anxiety before the assigned index date were excluded from the analyses. Patients with psoriasis are commonly classified into severe if they were prescribed a systemic therapy (ie, methotrexate, azathioprine, ciclosporin, hydroxyurea) or phototherapy (psoralen and ultraviolet A) during the study period, or into mild psoriasis if no such treatment was recorded.[24 25] This classification has been validated with similar databases[26–28] and has also been used in this study. Data were extracted from the CPRD in September 2017.

Outcome

The study primary outcome measures were a new Read medical code for a diagnosis of depression or anxiety used as binary variables (yes/no).[29] The date of the first outcome code following an inflammatory disorder diagnosis was referred to as the outcome index date. Depression was broadly defined to include single episode of depression, recurrent depression events and bipolar depressive events to allow for the possibility that chronic inflammation is implicated across the wider spectrum of the depressive disorder. In keeping with other studies,[30] anxiety was broadly defined to include generalised anxiety disorders, phobias, panic attacks and panic disorders.

Covariates

Factors known to be associated with chronic inflammation and depression or anxiety were adjusted for in the analyses. These covariates included age (continuous), gender (male vs female), body mass index (<18.5, 18.5–25, >25 to <30, 30 to <35, and ≥35 kg/m^2), index of multiple deprivation (quintiles), blood pressure (BP) (<120 mm Hg, normal; 120–139 mm Hg, borderline; ≥140 mm Hg, hypertension), smoking (ex or current vs never), drinking (ex or current vs never), physical comorbidities (yes/no) (ie, cancer, diabetes, stroke, coronary heart disease, dementia, epilepsy, COPD, liver disorders, kidney disorders, insomnia), and stressful life events (eg, stress at home or at work), together with prescription of statins, antihypertensive, antidiabetic and hypnotics. Previous studies[31 32] linked corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) with increased risk of depression and were therefore also included as covariates. For each potential confounder, the value closer to the index date and within the 5-year period prior to a chronic inflammation diagnosis was included. For instance, if a patient had two BP measures within 5 years prior to the baseline (eg, at 4 years and at 2 years prior to baseline), the value closer to the study baseline (eg, at 2 years) was included in the analyses. Expanding the baseline period to available data was found to enhance covariate sensitivity by capturing data that would otherwise be missed.[33]

Statistical analysis

The analyses were conducted in a time-to-event framework. Failure was classed as a new diagnosis of depression or anxiety. Participants contributed person-time to the analysis from the study start date (the later of the start of the participant's record in CPRD or the diagnosis date for a chronic inflammatory condition). Follow-up ended at the earliest of the study outcome date, the end of a participant's registration, the last date of CPRD data collection or the date of death. All participants had at least 12 months of follow-up recorded and had at least one medical event recorded from the study start date to the study end date.

A Cox proportional hazards model for clustered data based on the matched pairs was implemented with the use

of a multiple-failure events to allow for the possibility that each patient may experience more than one outcome event.[34] This approach permits analysis of data for each of multiple outcomes in a single model, allowing the most efficient use of each patient's data and reducing problems of multiple testing.[24] The multiple-failure model avoids the need to censor records at earlier outcome events or to test hypotheses separately for each outcome. Robust variance estimator was used to adjust for the dependency introduced by the matching variables. This approach is considered[35] superior to matched stratification as it allows for unbiased estimation of HRs. Because confounding by matching variables cannot be excluded,[36] the estimation models adjusted for matching variables (age, gender, practice, index year) and all study covariates listed above. A similar estimation was performed to estimate whether depression or anxiety onset varied with the age (<40, 40–49, 50–59 and 60 or over) at inflammatory disorder diagnosis. Additional analyses estimated the specific associations between each inflammatory condition with depression and anxiety in separate Cox regression models with robust estimate variance. The analyses used the Efron method to handle tied events. Forest plots were used to present measures of association for age subgroups and individual inflammatory disorder. A random-effects meta-analysis was implemented to evaluate heterogeneity by chronic inflammatory disorder and overall.[37] The proportionality assumption was tested and confirmed using Schoenfeld residuals. As this was an exploratory study, no adjustment for multiple comparisons was made, and therefore marginally significant results may be type I errors. Several sensitivity analyses were conducted. First, alternative follow-up times were used by starting the follow-up immediately after the inflammatory disorder diagnosis. Second, depression and anxiety were redefined to include both a clinical diagnosis code plus a relevant prescription (ie, antidepressant or anxiolytic drugs, respectively). Third, stratification by matched pairs was implemented to account for the matching. Fourth, to test the robustness of psoriasis findings, data on systemic therapy were used to classify patients with RA and SV (the only sufficiently powered disorders) into mild (no systemic therapy) and severe (systemic therapy). The effect of competing risk on mortality was also assessed. Multiple imputation by chained equation was used to handle missing data. The analyses were implemented using Stata V.15.

RESULTS

The analyses included 180 163 patients with chronic inflammatory disorders (see table 1) who were individually matched for age, gender, practice and index date with 358 544 control patients without a diagnosis of chronic inflammation. The median duration of follow-up was around 4 years for patients and controls. While clinical diagnosis and therapy data were comprehensive, among lifestyle factors missing information ranged from around 6% for smoking to 22% for alcohol status. Selective baseline characteristics of study participants are described in table 1 (see Table S1 in the online supplementary (online supplementary file 1)material for full data description).

Figure 1 shows that across all inflammatory disorders, the incidence of both depression or anxiety was greater within cases compared with the matched controls. The highest incidence rate was observed within severe psoriasis (14 per 1000 person-years), followed by those diagnosed with CD and AS (12 per 1000 person-years). Similar trends emerged with regard to the incidence of anxiety (see online supplementary figure S1)).

Table 2 presents the results of the analyses by study outcomes indicating increased hazard rates of depression and anxiety across all chronic inflammatory disorders. The strongest association was observed for severe psoriasis, being associated with a 71% increased rate of new depression onset (HR 1.71, 95% CI

Table 1 Participants' characteristics at baseline assessment*

	RA	Psoriasis		CD	UC	SLE	Vasculitis	AS	Total	
		Mild	Severe						Exposed	Unexposed
n	37 399	84 184	6528	10 453	23 291	3604	14 177	10 363	180 163	358 544
Follow-up†	4 (2–8)	5 (2–8)	6 (3–9)	4 (2–8)	4 (2–8)	4 (2–8)	4 (2–7)	5 (2–9)	4 (2–8)	4 (2–7)
Age, M (SD)	60 (16)	49 (18)	49 (16)	46 (18)	54 (19)	51 (16)	65 (17)	51 (17)	53 (18)	53 (18)
Female	24 929 (67)	43 757 (52)	3474 (53)	5678 (54)	12 664 (54)	2936 (81)	8974 (63)	6301 (61)	108 713 (57)	216 287 (57)
Cancer	3164 (8)	4319 (5)	301 (5)	525 (5)	1969 (8)	261 (7)	1673 (12)	609 (6)	12 821 (7)	24 050 (6)
CKD	4875 (13)	5936 (7)	529 (8)	895 (9)	2836 (12)	436 (12)	2574 (18)	927 (9)	19 008 (10)	28 645 (8)
Diabetes	4224 (11)	6349 (8)	564 (9)	694 (7)	2297 (10)	259 (7)	1934 (12)	751 (7)	17 072 (9)	26 822 (7)
CHD	3985 (11)	5415 (6)	403 (6)	688 (7)	2345 (10)	242 (7)	2082 (15)	753 (7)	15 913 (8)	25 606 (7)
COPD	2131 (6)	2532 (3)	200 (3)	348 (3)	1110 (5)	105 (3)	935 (7)	269 (3)	7630 (4)	10 428 (3)
Stress	8006 (21)	14 598 (17)	1239 (19)	1880 (18)	4505 (19)	832 (23)	2917 (21)	2244 (22)	36 221(19)	57 083 (15)
Hypertension	14 177 (39)	25 138 (33)	1993 (33)	2402 (25)	6945 (31)	1063 (30)	6472 (47)	3071 (31)	61 261 (34)	112 226 (34)
Obesity	9710 (29)	18 926 (27)	1837 (34)	1792 (20)	4339 (21)	714 (23)	3284 (26)	2122 (23)	42 724 (26)	69 536 (23)
Smoking	27 738 (76)	55 278 (69)	4364 (70)	7236 (82)	17 927 (80)	2419 (69)	11 124 (81)	7365 (74)	133 451 (73)	262 379 (76)
Alcohol	8060 (24)	12 930 (19)	1103 (20)	1826 (21)	4195 (22)	827 (26)	3254 (26)	1783 (20)	33 924 (21)	62 263 (21)
AHT	19 205 (51)	28 043 (33)	2422 (37)	3337 (32)	10 013 (43)	1553 (43)	8520 (60)	4004 (39)	77 097 (41)	128 197 (34)
Statins	9953 (27)	13 820 (16)	1161 (18)	1493 (14)	5220 (22)	621 (17)	4585 (32)	1749 (17)	38 602 (20)	64 919 (17)
Hypnotics	5985 (16)	9492 (11)	934 (14)	1366 (13)	3361 (14)	559 (16)	2407 (17)	1592 (15)	25 696 (14)	37 263 (10)
NSAIDs	31 894 (85)	46 984 (56)	4477 (69)	6089 (58)	14 115 (61)	2513 (70)	10 080 (71)	8571 (83)	142 723 (66)	181 991 (48)
Steroids	16 467 (44)	14 390 (17)	1893 (29)	3499 (33)	7022 (30)	1287 (36)	8444 (60)	2490 (24)	55 492 (29)	54 104 (14)

Figures are numbers and percentages unless otherwise specified.
*For ease of presentation some of the covariates data are not presented here.
†Figures represent median and IQR.
AHT, antihypertensive therapy; AS, ankylosing spondylitis; CD, Crohn's disease; CHD, coronary heart disease; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; NSAIDs, non-steroidal anti-inflammatory drugs; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; UC, ulcerative colitis.

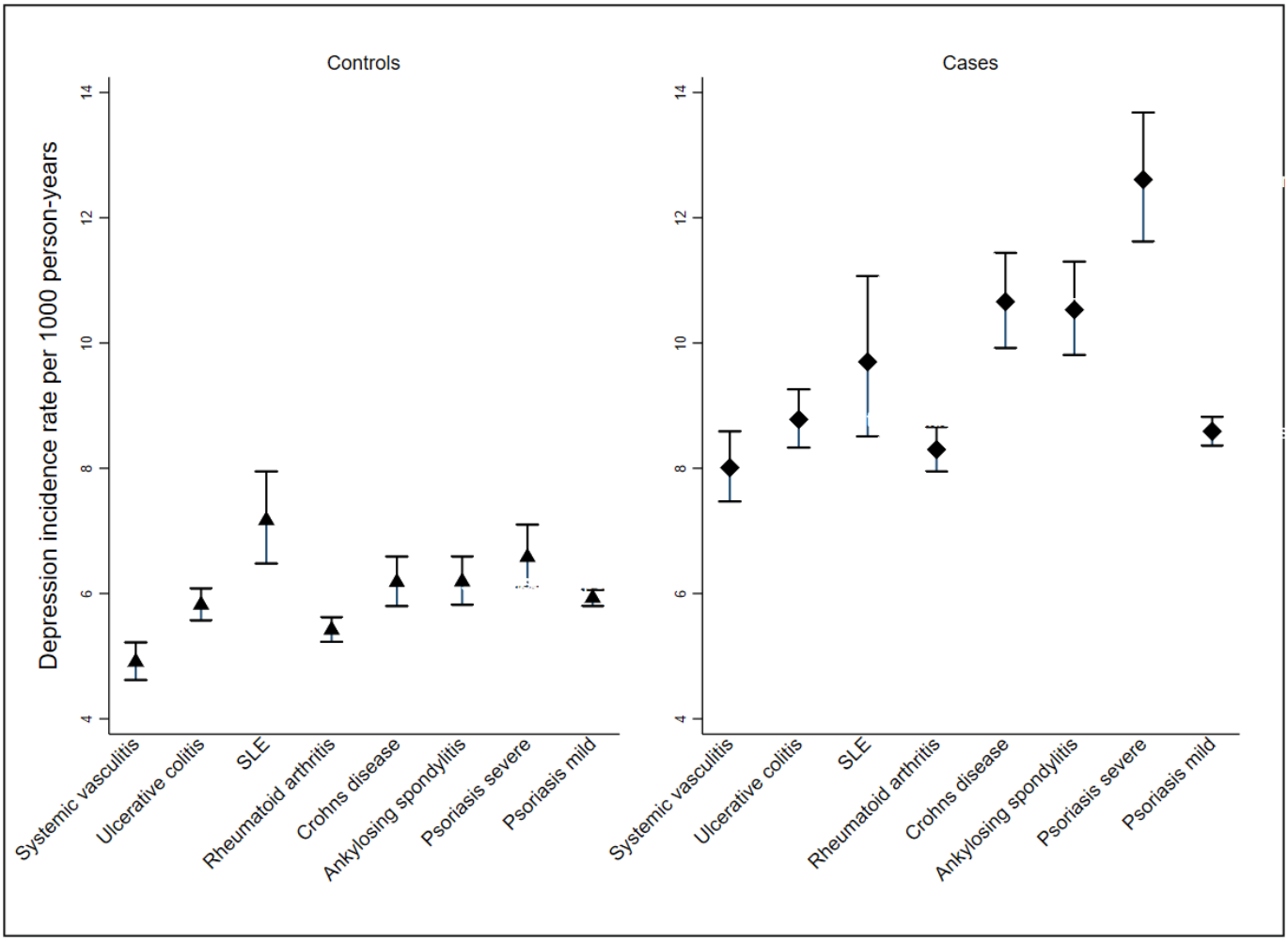

Figure 1 Incidence of depression by condition for participants with chronic inflammatory disorders and matched controls. SLE, systemic lupus erythematosus.

1.52 to 1.93, p<0.0001) compared with the matched comparisons. Regarding new anxiety onset, patients diagnosed with AS presented with the largest hazard rates (HR 1.36, 95% CI 1.23 to 1.51, p<0.0001) compared with their matched comparison group. Age-related analyses revealed higher depression and anxiety incidence among persons with early inflammatory disorder onset. Kaplan-Meier survival curves are presented in online supplementary figure S2.

Figure 2 displays the results from the multiple outcome models, with patients being allowed to experience either depression or anxiety in a random order. Compared with the matched group, patients with an inflammatory disorder experienced a 16% (HR 1.16, 95% CI 1.12 to 1.21, p<0.001) increased risk of depression or anxiety events. Patients diagnosed with CD presented with the highest HR (1.23, 95% CI 1.13 to 1.33, p<0.001), while those with mild psoriasis with the lowest HR (1.08, 95% CI 1.03 to 1.13, p<0.001). Age-based analyses (figure 3) indicated that the pooled hazard rate for multiple depression or anxiety incidence was 1.71 (95% CI 1.59 to 1.84, p<0.001) among patients with early inflammatory disorder onset (<40 years of age), which declined to 0.93 (95% CI 0.85 to 1.01, p=0.080) among those with late disorder onset (≥60 years) (see online supplementary figure S3 for findings among the 40–49 and 50–59 years age groups).

Sensitivity analyses

Sensitivity analyses using a more stringent criteria for depression and anxiety definition (eg, clinical diagnosis plus corresponding drug prescriptions) resulted in modestly higher estimates, validating the robustness of the main findings (online supplementary Figure S4 and Table S1). Analyses stratified by matched pairs endorsed the estimates and associations of the study findings. Systemic therapy-based sensitivity analyses indicated that both severe RA (HR 1.43, 95% CI 1.28 to 1.59, p<0.001) and SV (HR 1.65, 95% CI 1.20 to 2.25, p<0.001) presented greater risk of depression incidence relative to mild RA (HR 1.36, 95% CI 1.25 to 1.49, p<0.01) or mild SV (HR 1.42, 95% CI 1.27 to 1.60, p<0.001).

DISCUSSION

The main aim of the present study was to provide a comprehensive understanding about the burden of common mental disorders across specific inflammatory disorders within a primary care context. Within a large prospective design, several clinically diverse inflammatory disorders presented with a consistently elevated risk of depression and anxiety incidence. In particular, a 16% overall increased risk of multiple depression and anxiety events emerged across seven specific chronic inflammatory disorders (RA, psoriasis, CD, UC, SLE, SV and AS). Associations were observed between incident depression with each specific

Table 2 Adjusted HR (95% CI) for depression and anxiety incidence among persons with inflammatory disorders diagnosis compared with the matched comparison group

	Overall sample HR (95% CI)	Age at diagnosis, HR (95% CI)			
		<40	40–49	50–59	≥60
Depression incidence					
Psoriasis mild	1.30 (1.26 to 1.35)	1.59 (1.48 to 1.71)	1.32 (1.20 to 1.45)	1.01 (0.92 to 1.12)	0.88 (0.81 to 0.95)
Psoriasis severe	1.71 (1.52 to 1.93)	2.00 (1.61 to 2.48)	1.77 (1.39 to 2.24)	1.21 (0.94 to 1.57)	0.87 (0.66 to 1.13)
Rheumatoid arthritis	1.38 (1.29 to 1.47)	2.40 (2.07 to 2.79)	1.93 (1.68 to 2.22)	1.40 (1.23 to 1.59)	1.06 (0.96 to 1.17)
Systemic lupus erythematosus	1.28 (1.06 to 1.56)	1.27 (0.91 to 1.78)	1.53 (1.09 to 2.14)	1.02 (0.68 to 1.54)	0.91 (0.65 to 1.28)
Ankylosing spondylitis	1.44 (1.30 to 1.60)	1.93 (1.59 to 2.33)	1.62 (1.30 to 2.01)	1.30 (1.02 to 1.65)	1.07 (0.87 to 1.30)
Systemic vasculitis	1.46 (1.31 to 1.62)	2.52 (1.98 to 3.20)	2.37 (1.83 to 3.09)	1.73 (1.37 to 2.20)	1.23 (1.07 to 1.42)
Ulcerative colitis	1.39 (1.29 to 1.49)	1.81 (1.60 to 2.05)	1.31 (1.09 to 1.56)	1.44 (1.22 to 1.70)	0.96 (0.84 to 1.09)
Crohn's disease	1.47 (1.32 to 1.63)	1.84 (1.55 to 2.19)	1.59 (1.26 to 2.00)	1.28 (0.99 to 1.65)	0.92 (0.73 to 1.14)
Anxiety incidence					
Psoriasis mild	1.28 (1.24 to 1.33)	1.51 (1.40 to 1.63)	1.08 (0.97 to 1.21)	1.03 (0.93 to 1.15)	0.85 (0.78 to 0.93)
Psoriasis severe	1.33 (1.17 to 1.50)	1.40 (1.10 to 1.80)	1.31 (0.98 to 1.75)	1.04 (0.77 to 1.41)	0.84 (0.63 to 1.14)
Rheumatoid arthritis	1.10 (1.03 to 1.18)	1.51 (1.26 to 1.81)	1.20 (1.01 to 1.43)	0.93 (0.79 to 1.09)	0.80 (0.71 to 0.90)
Systemic lupus erythematosus	1.28 (1.06 to 1.55)	1.25 (0.78 to 1.83)	1.61 (1.07 to 2.42)	1.03 (0.63 to 1.67)	0.78 (0.49 to 1.22)
Ankylosing spondylitis	1.36 (1.23 to 1.51)	1.54 (1.25 to 1.90)	1.33 (1.05 to 1.70)	1.32 (1.03 to 1.70)	1.08 (0.88 to 1.32)
Systemic vasculitis	1.19 (1.07 to 1.32)	1.52 (1.15 to 2.02)	1.45 (1.04 to 2.02)	1.24 (0.94 to 1.64)	1.01 (0.86 to 1.18)
Ulcerative colitis	1.34 (1.24 to 1.44)	1.57 (1.35 to 1.83)	1.21 (0.991.48)	0.94 (0.76 to 1.17)	0.86 (0.74 to 1.00)
Crohn's disease	1.35 (1.21 to 1.50)	1.24 (1.02 to 1.49)	0.97 (0.73 to 1.28)	1.03 (0.76 to 1.39)	0.78 (0.61 to 1.00)

Adjusted for age, gender, deprivation, blood pressure, body mass index, smoking, alcohol, coronary heart disease, stroke, cancer, diabetes, dementia, epilepsy, chronic kidney disease, liver disease, chronic obstructive pulmonary disease, sleep disorders, antihypertensive therapy, statins, hypnotics, corticosteroids, non-steroidal anti-inflammatory drugs and antidiabetics.

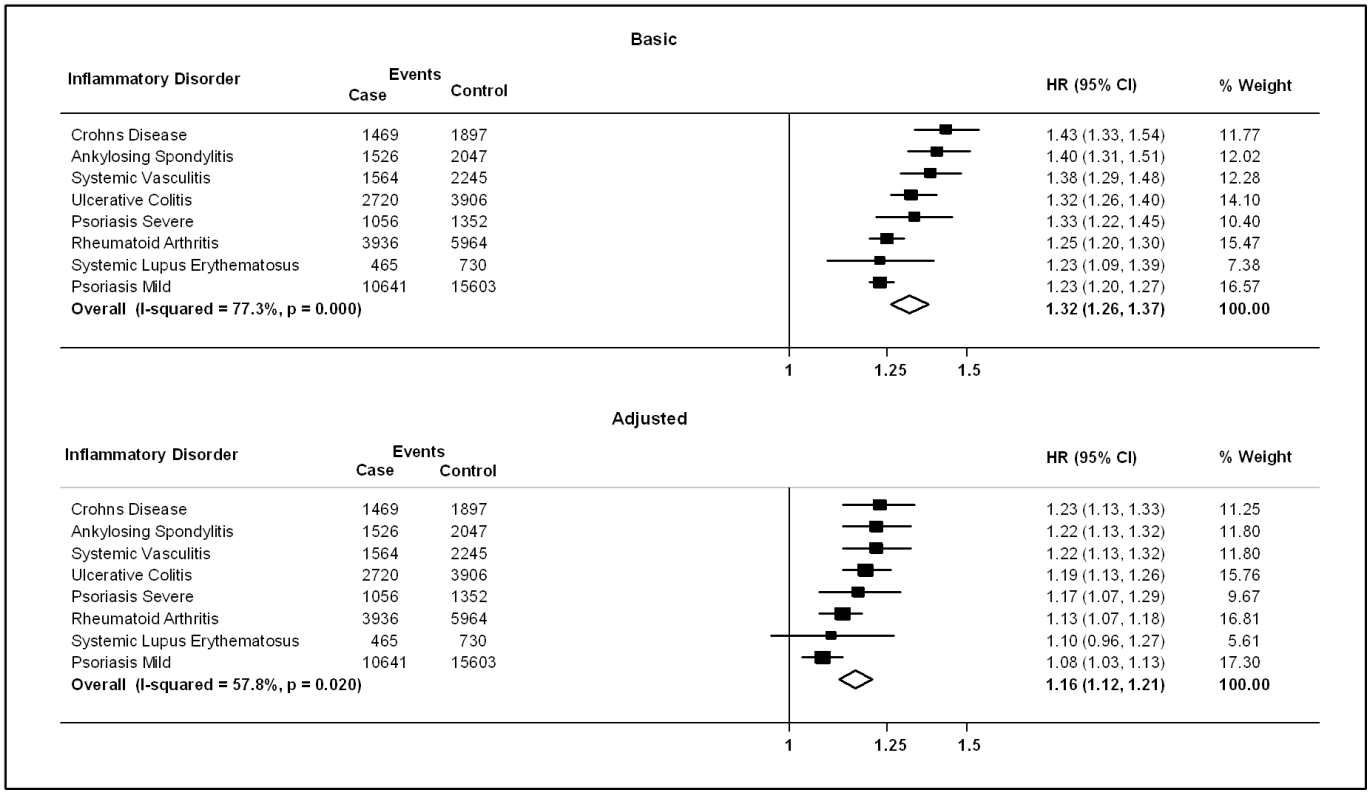

Figure 2 Forest plot displaying random-effects meta-analysis of the influence of specific inflammatory disorders on the incidence of multiple depression and anxiety outcomes. Basic, adjusted for age and gender. Adjusted, adjusted for age, gender, deprivation, blood pressure, body mass index, smoking, alcohol, coronary heart disease, stroke, diabetes, cancer, dementia, epilepsy, chronic kidney disease, liver disease, chronic obstructive pulmonary disease, sleep disorders, antihypertensive therapy, statins, hypnotics, corticosteroids, non-steroidal anti-inflammatory drugs and antidiabetics. HR, hazard rate.

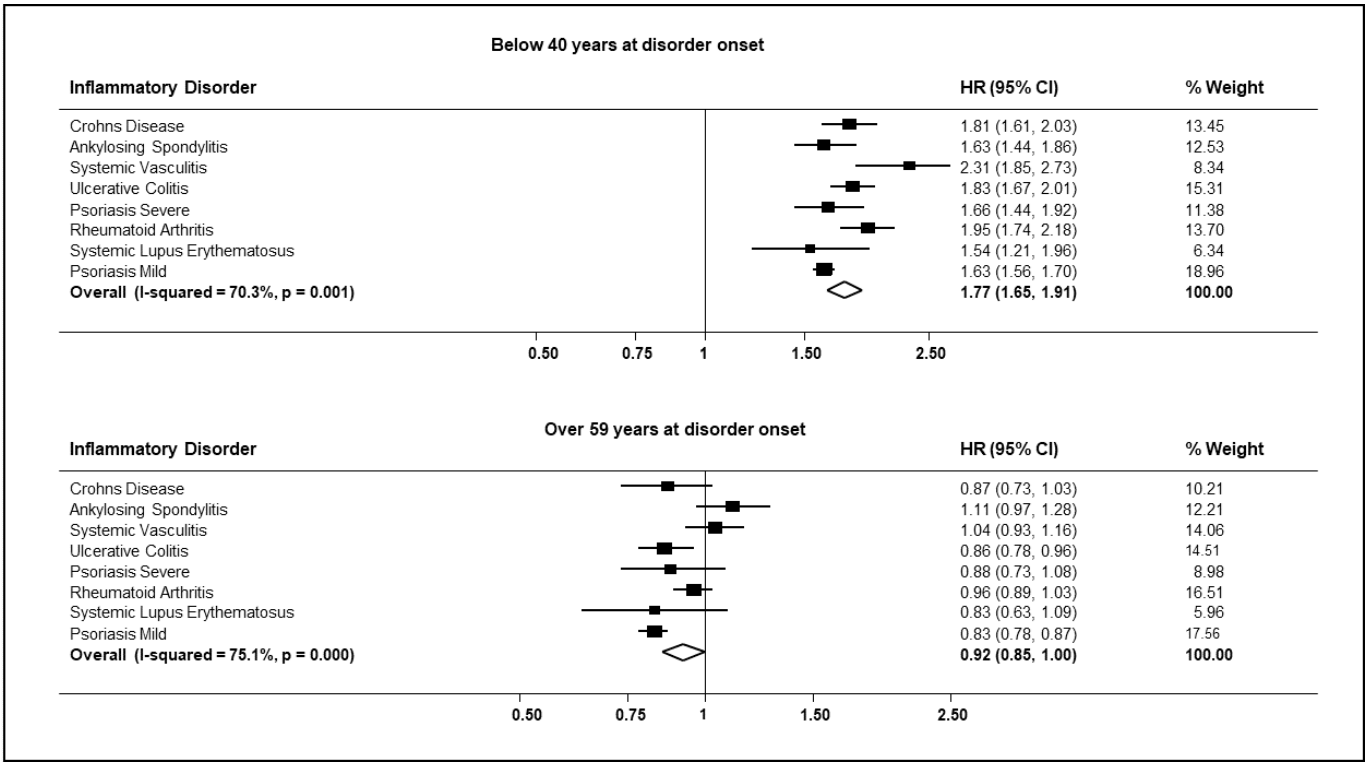

Figure 3 Forest plot displaying random-effects meta-analysis of the influence of age at inflammatory disorders onset on the incidence of multiple depression and anxiety. Adjusted for age, gender, deprivation, blood pressure, body mass index, smoking, alcohol, coronary heart disease, stroke, diabetes, cancer, dementia, epilepsy, chronic kidney disease, liver disease, chronic obstructive pulmonary disease, sleep disorders, antihypertensive therapy, statins, hypnotics, corticosteroids, non-steroidal anti-inflammatory drugs and antidiabetics. HR, hazard rate.

inflammatory disorder, although the effect size was of lower magnitude than suggested by findings based on secondary care-based populations.[4] The reason for this discrepancy may be that a smaller proportion of patients with inflammatory disorders, those with most severe or active disease, are seen in secondary care.[38] In our study, the incidence of depression and anxiety was higher for patients with severe psoriasis relative to those with mild psoriasis. This suggestion was substantiated in sensitivity analyses among RA and SV disorders.

The pooled incidence of depression and anxiety was considerably increased (71% increment) among primary care patients with early-onset inflammatory disorder (<40 years of age) and less so (−7%) among those with late disorder onset (≥60 years of age). Early-onset inflammatory disorders are associated with more widespread inflammation, increased frequency of active disease, and more aggressive disease manifestation and treatment compared with late-onset disorder.[39] Whether the increased incidence of depression or anxiety within early disorder onset was caused by increased disease activity or delay in disorder diagnosis and treatment (or their combined effect) needs further exploration.

All seven chronic disorders analysed in this study are connected by common underlying inflammatory mechanism, and the consistently elevated rates of depression and anxiety incidence across them might support a potential role of inflammation in the pathogenesis of these disorders, although this suggestion was not directly tested in this study. The main alternative hypothesis that cannot be excluded from this study design is that depression and anxiety may represent emotional responses to the experience of living with a distressing and often debilitating inflammatory disorder. The psychosocial and physical effects of the inflammatory disorder might therefore contribute to the onset of depressive and anxiety symptoms. For example, increased depression and anxiety incidence among primary care patients with early disorder onset, as found in this study, may reflect these patients presenting with more extensive and severe manifestations of the inflammatory disorder.[40] The elevated rates of depression events among patients with severe psoriasis relative to those with mild psoriasis seem to be in line with a disease response hypothesis. Pain, disfigurement, loneliness and stigma associated with severe inflammatory disease indicators (eg, eruptive psoriasis, multiple nail lesions), for example, could worsen patients' sleep quality and prevent them from full social participation, leading to the onset of depressive symptoms.[41]

The results of the present study raise important questions about the assessment and management of common mental health disorders among younger patients diagnosed with specific inflammatory disorders. Irrespective of whether psychological problems are the consequence of the emotional reaction to disease and disability or of a common inflammatory aetiology, there seems a clear association between inflammatory disorders and depression or anxiety, especially for younger early-onset patients. Routine assessments of patients' mental health could lead to improved prevention and treatment outcomes. If further research supports the common inflammatory aetiology hypothesis, then clinical intervention might target the inflammatory response itself. Renewed interest in the potential effectiveness of immunomodulatory therapies (eg, new biologics, methotrexate) for the prevention of treatment-resistant depression may indicate one way forward.

Previous prospective studies explored the association between depression and anxiety and specific inflammatory disorders.[9 42] Marrie *et al*,[9 43] for example, documented somewhat higher incidence rates of depression and anxiety among patients with RA

and inflammatory bowel disorders (IBD). Marie *et al*'s[9 43] studies did not adjust for differences in chronic illnesses (eg, cardiovascular disease, diabetes, chronic kidney disease) at baseline, did not account for matching in their analyses, used a different case definition (eg, exclusion of cases within a 5-year period from index date) and relied on a more local population. These variations may account for the observed differences in effect size between ours and Marrie *et al*'s[9 43] findings. Meesters *et al*[44] also documented higher incidence rates of depression events among patients with AS from primary care compared with our findings, possibly due to previous study failure to adjust for other covariates apart from age and gender. An earlier study found no increased risk of depression among patients diagnosed with CD or UC[42]: this may reflect the previous study's lack of a comparison group or shorter follow-up (<5 years). Recent studies[6 27] indicated greater incidence of depression among patients diagnosed with severe psoriasis relative to those with mild psoriasis, as observed in this study. The decline in depression incidence with age at disorder onset is in line with an earlier systematic review among patients with RA,[4] and extends previous findings to anxiety.

Strengths and limitations

The present study has several strengths, including nationally representative primary care population, prospective study design, and clinically valid diagnoses of inflammatory disorders, depression and anxiety. The inclusion of primary care patients with systemic and organ-specific inflammatory disorders ensures the generalisability of the study findings to real-world clinical practice. While our data possibly contain all diagnoses issued within primary care, it may be less complete with regard to diagnoses made in secondary or community care.[45] Nine out of ten adults with mental health disorders are supported in primary care in the UK, implying that only a small number of cases are not captured by the CPRD. The use of antidepressant and anxiolytic therapies as sensitivity analyses may have also mitigated against diagnostic bias, given that drug prescribing is often considered a reliable marker for case identification.[46] Clinicians may be more alert (or ask different questions) to depressive or anxiety symptoms among patients with inflammatory disorders due to increased contact with the healthcare system, and thus more likely to identify relevant cases. The mean number of primary care consultations, however, was similar between inflammatory patients and matched controls (data not shown). The precise timings of the onset of exposure or outcome measures cannot be determined precisely in observational data, precluding robust causal inferences. To mitigate against this concern, the analyses excluded outcome measures that occurred during the first 12 months following an inflammatory disorder diagnosis. Our large study sample comprised a heterogeneous group of patients with distinctive underlying disease severity and symptomatology, potentially masking subgroups of patients that could present with clinically significant mental disorders. This suggestion is supported by our finding with regard to severe psoriasis and age at inflammatory disorder diagnosis. A method of analysis that did not allow for matching might give slightly wider CIs and larger p values than a matched analysis.[47] Sensitivity analyses adjusting for matching validated the study main findings. We cannot exclude the possibility that the comparison group included patients diagnosed with other less common inflammatory disorders (eg, bullous skin diseases, Sjogren syndrome). This concern may have attenuated the true risk of depression or anxiety within chronic inflammatory disorders. The study's primary aim was to model initial inflammatory disorder status (eg, psoriasis, RA, SLE) and therapy (eg, NSAIDs, corticosteroids), along with patients' sociodemographic and clinical data, to patients' overall risk for future depression or anxiety onset. The analyses, however, did not model potential postdiagnosis mediators and moderators for depression or anxiety onset, including temporary changes in underlying disease severity, treatment choices and inflammatory responses. These are clinically relevant questions that deserve detailed investigation with future prospective studies. The study only differentiated between mild and severe psoriasis. The smaller sample of patients within the rest of inflammatory disorders precluded a similar classification. This concern also applied to patients with psoriatic arthritis that were classified as psoriasis. Given that the definition of severe of psoriasis was based on disease-modifying antirheumatic drug exposure, however, it is possible that patients with psoriatic arthritis were included in the severe psoriasis subgroup. Sensitivity analyses within RA and SV disorders endorsed psoriasis severity results increasing confidence in the robustness of the study findings. Future studies with larger IBD, SLE and AS samples are also required to confirm the link between inflammatory disorder severity and study outcomes. Missing data on lifestyle covariates can compromise the results of statistical analysis, but use of multiple imputation and appropriate sensitivity analyses should have mitigated some of this risk. A larger proportion of women were diagnosed with AS in this study, which is contrary to other studies showing higher AS rates among men.[48] The study findings about the incidence of depression or anxiety may, thus, not be generalisable to the wider AS population. This concern was likely caused by the matching of patients and controls on gender, leading to intentional non-representativeness. In analytical studies where the aim is to explore the exposure–outcome association (as in this study), however, population representativeness is not considered necessary or desirable.[49] Richiardi *et al*,[50] for instance, suggested that non-representativeness increases the power to assess main effects and effect modification, and that valid statistical inferences can be made when adjusting for confounders. Primary care patients diagnosed with an inflammatory disorder were at greater risk of new depression and anxiety onset compared with matched patients without an inflammatory disorder, a risk that was particularly elevated among patients with early onset of chronic inflammatory disorder. These findings may reflect either a response to the physical effects of living with a chronic inflammatory disorder, or a role of inflammation in the genesis of depression and anxiety. The latter hypothesis deserves further attention as it may offer the opportunity for new therapeutic approaches to anxiety and depression, but first the question of whether depression is a consequence of inflammation or is a reaction to experiencing a chronic illness deserves further exploration. Studies evaluating modifiable mediators for depression and anxiety incidence across specific inflammatory disorders are also warranted.

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Contributors AD, FM, AB, KD, CP, DA, RS, SH and MH were responsible for study design. AD was responsible for data acquisition. AD analysed the data and wrote the manuscript. All authors critically revised the manuscript and approved the final version.

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Competing interests None declared.

Patient consent for publication Not required.

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EPIDEMIOLOGICAL SCIENCE

Comorbidity and long-term outcome in patients with congenital heart block and their siblings exposed to Ro/SSA autoantibodies in utero

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ABSTRACT

Objective Congenital heart block (CHB) may develop in fetuses of Ro/SSA autoantibody-positive women. Given the rarity of CHB, information on comorbidity and complications later in life is difficult to systematically collect for large groups of patients. We therefore used nation-wide healthcare registers to investigate comorbidity and outcomes in patients with CHB and their siblings.

Methods Data from patients with CHB (n= 119) and their siblings (n= 128), all born to anti-Ro/SSA-positive mothers, and from matched healthy controls (n= 1,190) and their siblings (n= 1,071), were retrieved from the Swedish National Patient Register. Analyses were performed by Cox proportional hazard modelling.

Results Individuals with CHB had a significantly increased risk of cardiovascular comorbidity, with cardiomyopathy and/or heart failure observed in 20 (16.8%) patients versus 3 (0.3%) controls, yielding a HR of 70.0 (95% CI 20.8 to 235.4), and with a HR for cerebral infarction of 39.9 (95% CI 4.5 to 357.3). Patients with CHB also had a higher risk of infections. Pacemaker treatment was associated with a decreased risk of cerebral infarction but increased risks of cardiomyopathy/heart failure and infection. The risk of systemic connective tissue disorder was also increased in patients with CHB (HR 11.8, 95% CI 4.0 to 11.8), and both patients with CHB and their siblings had an increased risk to develop any of 15 common autoimmune conditions (HR 5.7, 95% CI 2.83 to 11.69 and 3.6, 95% CI 1.7 to 8.0, respectively).

Conclusions The data indicate an increased risk of several cardiovascular, infectious and autoimmune diseases in patients with CHB, with the latter risk shared by their siblings.

Key messages

What is already known about this subject?

► No population-based health register studies have been performed to investigate comorbidity and long-term outcome in congenital heart block (CHB).

What does this study add?

► Patients with CHB have a significantly increased risk of cardiovascular disease, including heart failure, cardiomyopathy and cerebral infarction.
► Autoimmune diseases are significantly more frequent in individuals with CHB, as well as in siblings of patients with CHB.

How might this impact on clinical practice or future development?

► Our data support a close follow-up of cardiac function in patients with CHB, and that autoimmune conditions should be considered in both patients and their siblings.

INTRODUCTION

Complete congenital heart block (CHB) without associated cardiac malformation is a rare condition, affecting 1 in 23,000 births in the general population.[1] The association between CHB and maternal anti-Ro/SSA and anti-La/SSB autoantibodies is well established,[2–4] with CHB occurring in 1%–2% of anti-Ro/SSA-exposed fetuses in several studies,[4–6] although lesser figures have been reported in studies confined to women with systemic lupus erythematosus (SLE).[7] Women carrying the autoantibodies are often diagnosed with SLE or Sjögren's syndrome (SS) but can also be asymptomatic.[8–10] During pregnancy, the autoantibodies are transported across the placenta and may induce neonatal lupus, including a complete third-degree atrioventricular (AV) block.[11–13] The majority of children with CHB require a pacemaker at an early age to improve cardiac function.[14] However, pacemaker treatment may potentially carry negative effects, and right ventricular pacing has been suggested to associate with subsequent development of dilated cardiomyopathy.[15–17]

Given the rarity of CHB, information on comorbidity and complications later in life is difficult to systematically collect for large groups of patients, and the literature on long-term follow-up and comorbidity in children with CHB is limited. We and others have previously observed that patients with CHB are growth-restricted during the first years of life,[18 19] and that there is an increased prevalence of impaired neurodevelopment[19] and neuro-psychiatric abnormalities[20] in this group of individuals. Recent studies on CHB, including several case reports[21–26] and a questionnaire-based study,[27] have also indicated that CHB might be a risk factor for the development of rheumatic and

Table 1 Characteristics of investigated cohorts

	Patients with CHB	General population comparators	Siblings of patients with CHB	Siblings of general population comparators
n (females/males)	119 (63/56)	1190 (630/560)	128*	1,071*
Age at inclusion† mean (SD), years	7.5 (10.7)	7.4 (10.6)	11.7 (14.1)	10.0 (12.8)
Follow-up time mean (SD), years	17.1 (8.0)	16.9 (8.2)	18.4 (7.3)	18.2 (8.0)
Complete atrioventricular block‡, n	119	0	0	0

*Information on sex not available.
†Inclusion refers to first year of data included in the current study.
‡Pacemaker treatment is described in online online supplementary table 4.
CHB, congenital heart block.

autoimmune disease. Whether there may also be a risk for the siblings without CHB is not clear,[27] although familial aggregation of autoimmune diagnoses[28 29] suggests that also the siblings could have an increased risk to develop autoimmune disease.

To systematically assess morbidity and long-term outcome in individuals with or without CHB born to anti-Ro/SSA autoantibody-positive mothers, we established a cohort of CHB individuals and their siblings based on 115 families in which anti-Ro/SSA antibody-positive mothers had given birth to at least one child with CHB. Ten control families were identified for each index family using Swedish population registers, and healthcare data were subsequently obtained through national healthcare registers. The incidence of International Classification of Diseases 10th revision (ICD10) diagnoses in exposure and matched control groups was then analysed to assess the risk of comorbidity and future disease development in children with CHB and their siblings.

MATERIALS AND METHODS

Study population and data sources used to detect outcomes during follow-up

Individuals in the present study were previously identified through a population-based strategy and included in a cohort of Swedish patients with CHB.[30] From this cohort, all individuals with CHB (n=119) and their siblings without CHB (n=128) born to anti-Ro/SSA antibody-positive mothers (n=115) between year 1948 and 2010 were included for analysis (table 1). Siblings were defined as individuals sharing both parents. For each patient with CHB, 10 controls (n=1,190) from the general population matched for sex, year and month of birth, as well as region of birth were randomly selected from the Swedish Total Population Register at Statistics Sweden (www.scb.se). Siblings (n=1,071) of the controls were identified through the Swedish national multi-generation register at Statistics Sweden, and served as controls to the siblings of the patients with CHB. Diagnoses received by patients with CHB, siblings and controls during the period of observation were obtained from the Swedish National Patient Register (inpatient care 1987–2010 and non-primary outpatient care 2001–2010; www.socialstyrelsen.se). For these periods, the register is nationwide, with a coverage of 99% for hospitalisations and 80% for outpatient care. The latter lower coverage is mainly due to lower reporting rates from private care.[31] All diagnoses are coded according to the ICD, and the data analysed in the current study are based on the 9th and 10th ICD editions (http://www.who.int/classifications/icd/en/). Diagnoses based on the 9th ICD edition were converted to their corresponding ICD-10 versions, thus enabling an aggregate analysis of comorbidity throughout the observational period. The cohorts were followed from birth or 1st of January 1987 (whichever came last) until death or 31st December 2010, whichever came first.

The study was approved by the Regional Ethics Committee in Stockholm, and written informed consent was obtained from all participating individuals from the CHB families or their parents if <18 years old.

Statistical analysis

Statistical analysis was performed using STATA MP V. 13.0 (StataCorp LP, College Station, TX, USA). Statistical significance was defined by an alpha level of 0.05. Q-values were calculated to account for false discovery rates, only observations with q<0.2 are reported.

Cox regression was used to estimate HRs of disease during follow-up time. Significance parameters were defined when the sum of events in the exposure and control groups was ≥5 in CHB and ≥10 in siblings and their respective controls. In comparisons between unmatched samples, such as siblings of individuals with CHB versus siblings of controls, HRs adjusted for differences in age. HRs above 100 or below 0.01 are reported as >100 and<0.01, respectively. If no event was present in the exposure or control group, no CI is reported. A Nelson-Aalen estimator was used to calculate cumulative hazard rates of disease.

RESULTS

Demographics of the investigated cohort

In the present study, we included all patients with CHB (n=119) and their siblings without CHB (n=128, referred to hereafter as 'CHB siblings') enrolled in a population-based CHB cohort and born to Ro/SSA antibody-positive mothers,[30] and assigned them to 1,190 and 1,071 controls, respectively (table 1). Data were extracted from the Swedish National Patient Register for all individuals. The mean age at inclusion in the study was 7.5 years (median 0, range 0–38.6 years) for individuals with CHB, and 11.7 years (median 3.3, range 0–45.7 years) for CHB siblings (table 1, online supplementary table 1). The mean follow-up time was 17.1±8.0 years in the CHB group and 18.4±7.3 years in the CHB sibling group, with a total exposure of 2036 and 2352 patient-years for patients with CHB and their siblings, respectively, and 20 078 and 19 534 comparator-years for the respective control groups.

Comorbidity and long-term outcome in CHB in relation to organ system and aetiology

To investigate comorbidity and long-term health status in individuals affected by CHB, we first analysed the occurrence of diagnoses included in the ICD blocks of chapters I–XIV in CHB individuals and matched controls (table 2). A higher proportion

Table 2 ICD blocks associated with significant HRs for patients with CHB

ICD chapter*	ICD block	HR (95% CI)	Q-value	Incidence rate per 1,000 person-years (95% CI)		No. events(%)	
				Patients with CHB†	Controls‡	Patients with CHB†	Controls‡
I	Other bacterial diseases (A30–A49)	7.6 (3.2 to 18.1)	<0.01	4.6 (2.4 to 8.9)	0.6 (0.3–1.1)	9 (7.6%)	12 (1.0%)
I	Other infectious diseases (B99–B99)	9.9 (2.5 to 39.7)	0.05	2.0 (0.7 to 5.3)	0.2 (0.1–0.5)	4 (3.4%)	4 (0.3%)
III	Certain disorders involving the immune mechanism (D80–D89)	14.8 (2.5 to 88.4)	0.11	1.5 (0.5 to 4.6)	0.1 (0.0–0.4)	3 (2.5%)	2 (0.2%)
IV	Metabolic disorders (E70–E90)	4 (1.6 to 10.3)	0.13	3.0 (1.4 to 6.7)	0.8 (0.5–1.2)	6 (5.0%)	15 (1.3%)
V	Disorders of psychological development (F80–F89)	5.0 (1.7 to 14.6)	0.11	2.5 (1.0 to 6.0)	0.5 (0.3–0.9)	5 (4.2%)	10 (0.8%)
IX	Other forms of heart disease (I30–I52)	>100 (191.4 to 690.2)	<0.01	203.5 (169.6 to 244.1)	0.7 (0.5–1.2)	116 (97.5%)	15 (1.3%)
IX	Cerebrovascular diseases (I60–I69)	10.1 (2.5 to 40.2)	0.05	2.0 (0.7 to 5.3)	0.2 (0.1–0.5)	4 (3.4%)	4 (0.3%)
IX	Diseases of veins, lymphatic vessels and lymph nodes, not elsewhere classified (I80–I89)	3.9 (1.7 to 8.8)	0.05	4.1 (2.0 to 8.1)	1.1 (0.7–1.6)	8 (6.7%)	21 (1.8%)
X	Acute upper respiratory infections (J00–J06)	2.2 (1.4 to 3.3)	0.02	15.1 (10.3 to 22.0)	6.8 (5.7–8.1)	27 (22.7%)	129 (10.8%)
X	Influenza and pneumonia (J09–J18)	4.3 (2.2 to 8.2)	<0.01	6.7 (3.9 to 11.6)	1.6 (1.1–2.2)	13 (10.9%)	31 (2.6%)
X	Other acute lower respiratory infections (J20–J22)	3.8 (1.8 to 8.2)	0.04	4.6 (2.4 to 8.8)	1.2 (0.8–1.8)	9 (7.6%)	24 (2.0%)
XI	Other diseases of the digestive system (K90–K93)	4.4 (1.7 to 11.4)	0.10	3.1 (1.4 to 6.8)	0.7 (0.4–1.2)	6 (5.0%)	14 (1.2%)
XII	Infections of the skin and subcutaneous tissue (L00–L08)	5.7 (3.0 to 11.0)	<0.01	7.1 (4.2 to 12.0)	1.3 (0.8–1.9)	14 (11.8%)	25 (2.1%)
XII	Other disorders of the skin and subcutaneous tissue (L80–L99)	3.5 (1.6 to 7.9)	0.09	4.0 (2.0 to 8.0)	1.2 (0.8–1.7)	8 (6.7%)	23 (1.9%)
XIII	Systemic connective tissue disorders (M30–M36)	11.8 (4.0 to 35.1)	<0.01	3.5 (1.7 to 7.4)	0.3 (0.1–0.7)	7 (5.9%)	6 (0.5%)

*I Certain infectious and parasitic diseases, III Diseases of the blood and blood-forming organs and certain disorders involving the immune mechanism, IV Endocrine, nutritional and metabolic diseases, IV Endocrine, nutritional and metabolic diseases, V Mental and behavioral disorders, IX Diseases of the circulatory system, X Diseases of the respiratory system, XI Diseases of the digestive system, XII Diseases of the skin and subcutaneous tissue, XIII Diseases of the musculoskeletal system and connective tissue, XIII Diseases of the musculoskeletal system and connective tissue.
†Patients with CHB, n=119.
‡General population comparators, n=1,190.
CHB, congenital heart block; ICD, International Classification of Diseases.

of patients with CHB than controls received diagnoses within the circulatory system ICD chapter during the observation period. This chapter indeed includes the diagnosis of AV block within the ICD block 'Other forms of heart disease' (I30-I52), and although this diagnosis was present in all CHB individuals as part of the cohort inclusion criteria, it was also re-assigned to 97% of CHB individuals during the observation period. The AV-block diagnosis is therefore retained in the tables and accounts, at least in part, for a HR of >100 for the ICD block 'Other forms of heart disease'. Nevertheless, HRs>1 were also observed for two other ICD blocks within the circulatory system ICD chapter, and these blocks were related to disorders not manifested in the heart itself but in the vascular tissue, with HRs ranging from 3.9 to 10.1 (table 2). We further found that patients with CHB had an elevated risk of systemic connective tissue disorders compared with controls (HR 11.8, 95% CI 4.0 to 35.1). In addition, individuals with CHB appeared at higher risk of infectious diseases, including general illnesses and infections of the respiratory system or the skin. Finally, we observed that patients with CHB were at higher risk of developing psychological disorders, metabolic disorders and diseases of the digestive system.

To assess the risk of these diagnoses in the CHB siblings, we next compared their occurrence between CHB siblings and siblings of the controls (table 3). No significant hazard ratios were observed for any of these ICD blocks. We however observed that the incidence of systemic connective tissue disorders was considerably higher among CHB siblings compared with controls (3.1% vs 0.4%, n=4 in each group, HR 12.7). Similarly, the number of events of 'Other forms of heart disease' (I30-I52) observed in CHB siblings (3.9%, n=5) was higher than in the control group (1.0%, n=11) (HR 3.0, 95% CI 1.0 to 8.9, corresponding to a p value of 0.067).

Defining comorbidity and long-term outcome at the three-character ICD code level

To more precisely define the observed comorbidities, we next assessed risk at the level of the discrete three-character ICD codes included in the blocks for which patients with CHB had presented significant hazard ratios. We found that patients with CHB had an increased risk of disease across multiple cardiovascular diagnoses (table 4). Overall, 20 (16.8%) patients with CHB were diagnosed with cardiomyopathy and/or heart failure during the observation period (HR 70.0, 95% CI 20.8 to 235.4). More specifically, there were 14 events of cardiomyopathy in the CHB group compared with none in the control group (HR >100), and 10 events of heart failure among patients with CHB versus three among controls (HR 34.4, 95% CI 9.5 to 125.2). In addition, a diagnosis of 'other arrhythmias' (I49) was present in 55 (46.2%) patients with CHB versus 5 (0.4%) controls (HR >100, 95% CI 66.0 to 415.0). This included sick sinus syndrome (SSS, I49.5) present in 5 (4.2%) patients with CHB and 1 (0.1%) control, while the majority of cases (50 CHB individuals and four controls) were classified as 'other specified cardiac arrhythmias'

Table 3 HR and incidence of disease for siblings of patients with CHB in ICD blocks associated with significant HRs for patients with CHB

ICD Chapter*	ICD Block	HR (95% CI)	Incidence rate per 1,000 person-years (95% CI)		No. events(%)	
			Siblings†	Controls‡	Siblings†	Controls‡
I	Other bacterial diseases (A30-A49)	1.2 (0.3–5.4)	0.9 (0.2 to 3.4)	0.8 (0.5–1.3)	2 (1.6)	15 (1.4)
I	Other infectious diseases (B99-B99)	<0.01 (N/A§)	0	0.1 (0.0–0.4)	0 (0)	1 (0.1)
III	Certain disorders involving the immune mechanism (D80-D89)	<0.01 (N/A§)	0	0.2 (0.0–0.5)	0 (0)	3 (0.3)
IV	Metabolic disorders (E70-E90)	1.5 (0.4–5.3)	1.3 (0.4 to 4.0)	0.9 (0.6–1.5)	3 (2.3)	18 (1.7)
V	Disorders of psychological development (F80-F89)	1.3 (0.3–5.7)	0.9 (0.2 to 3.4)	0.8 (0.5–1.3)	2 (1.6)	16 (1.5)
IX	Other forms of heart disease (I30-I52)	3.0 (1.0–8.9)	2.2 (0.9 to 5.2)	0.6 (0.3–1.0)	5 (3.9)	11 (1)
IX	Cerebrovascular diseases (I60-I69)	1.4 (N/A¶)	0.4 (0.1 to 3.0)	0.3 (0.1–0.6)	1 (0.8)	5 (0.5)
IX	Diseases of veins, lymphatic vessels and lymph nodes, not elsewhere classified (I80-I89)	0.5 (0.1–2.0)	0.9 (0.2 to 3.4)	1.6 (1.1–2.3)	2 (1.6)	31 (2.9)
X	Acute upper respiratory infections (J00-J06)	0.7 (0.4–1.3)	5.3 (3.0 to 9.3)	7.7 (6.5–9.1)	12 (9.4)	141 (13.2)
X	Influenza and pneumonia (J09-J18)	1.1 (0.4–3.2)	1.7 (0.6 to 4.6)	1.4 (0.9–2.0)	4 (3.1)	27 (2.5)
X	Other acute lower respiratory infections (J20-J22)	0.6 (0.1–2.8)	0.9 (0.2 to 3.4)	1.2 (0.8–1.8)	2 (1.6)	24 (2.2)
XI	Other diseases of the digestive system (K90-K93)	1.3 (0.3–5.1)	1.3 (0.4 to 4.0)	0.9 (0.5–1.4)	3 (2.3)	17 (1.6%)
XII	Infections of the skin and subcutaneous tissue (L00-L08)	1.2 (0.3–3.9)	1.3 (0.4 to 4.0)	1.3 (0.9–1.9)	3 (2.3%)	25 (2.3)
XII	Other disorders of the skin and subcutaneous tissue (L80-L99)	1.9 (0.7–5.2)	2.1 (0.9 to 5.2)	1.2 (0.8–1.8)	5 (3.9)	24 (2.2)
XIII	Systemic connective tissue disorders (M30-M36)	12.7 (N/A¶)	1.7 (0.6 to 4.6)	0.2 (0.1–0.5)	4 (3.1)	4 (0.4)

*I Certain infectious and parasitic diseases, III Diseases of the blood and blood-forming organs and certain disorders involving the immune mechanism, IV Endocrine, nutritional and metabolic diseases, IV Endocrine, nutritional and metabolic diseases, V Mental and behavioral disorders, IX Diseases of the circulatory system, X Diseases of the respiratory system, XI Diseases of the digestive system, XII Diseases of the skin and subcutaneous tissue, XIII Diseases of the musculoskeletal system and connective tissue, XIII Diseases of the musculoskeletal system and connective tissue.
†Siblings of patients with CHB, n=128.
‡Siblings of general population comparators, n=1,071.
§Confidence intervals not enclosed when no events were observed in either cases or controls.
¶In analyses with unmatched samples, confidence intervals were not calculated when the total number of events did not equal or exceed 10.

(I49.8), and could not be further defined. Nine (7.6%) patients with CHB developed atrial fibrillation and flutter (HR 46.7, 95% CI 10.1 to 216.1), of which five either had a previous or subsequent diagnosis of heart failure or cardiomyopathy.

Notably, 4 (3.4%) patients with CHB experienced a cerebral infarction, compared with one individual (0.08%) in the control group (HR 39.9, 95% CI 4.5 to 357.3) (table 4). Two of the patients had previously been diagnosed with cardiomyopathy, and one individual had records of pacemaker treatment before the cerebral infarction. No records of previous or subsequent atrial fibrillation or flutter were found in patients with CHB with cerebral infarction.

We further observed that patients with CHB had a significantly increased risk of the ICD diagnosis code 'other systemic involvement of connective tissue' (M35) (HR 7.5, 95% CI 1.7 to 33.4), with 3 (2.5%) patients with CHB versus 3 (0.3%) control individuals diagnosed with such conditions. Other individual 3-character ICD codes within the 'systemic connective tissue disorder' block did not fulfil criteria for analysis due to few events, or the estimated HR was not significant.

The analysis at the three-character ICD code level also confirmed the increased risk of multiple infectious diseases in patients with CHB previously noted at the ICD block level, including bacterial infections, sepsis, tonsillitis, upper respiratory tract infections, bronchitis and pneumonia, as well as infections of the skin and subcutaneous tissue (table 4). The proportion of infections occurring before the age of 1 year did not significantly differ between patients with CHB and controls (p=0.41, online supplementary table 2. Skin involvement was also apparent from the observation of an increased risk of atrophic skin disorders (L90). Of the disorders of psychological development, pervasive developmental disordered was more common in patients with CHB than controls. Notably, the siblings of patients with CHB did not present significant hazard ratios for any of the discrete diagnoses cited above (online supplementary table 3).

Effects of pacemaker implantation on cardiovascular morbidity and infections

Considering that pacemaker implants have been shown to associate with various morbidities,[17 32–36] we assessed the effect of pacemaker treatment on cardiovascular morbidity and infections in individuals with CHB, performing Cox proportional hazard modelling with pacemaker surgery as time-varying covariate (figure 1, online supplementary table 4). n=107 (90%) individuals with CHB had records indicating pacing treatment during the observational period. A protective effect of pacemaker treatment was observed regarding the risk of developing cerebral infarction (HR 0.1; 95% CI 0.01 to 0.8) and other cardiac arrhythmias (HR 0.4; 95% CI 0.1 to 0.9). Pacemaker treatment was however associated with an increased risk of developing cardiomyopathy and/or heart failure after pacemaker implantation (HR 3.8, 95% CI 1.1 to 12.6). In addition, pacemaker implants were associated with an increased risk of infections (A00-B99 and L00-08) (HR 5.5; 95% CI, 2.7 to 11.3) (figure 1).

Cumulative risk of autoimmune disease

We observed a significantly increased risk of several conditions at the three character ICD code level related to autoimmunity

Table 4 Three-character ICD codes within blocks in table 2 associated with significant hazard ratios for patients with CHB

ICD block	Three-character ICD code	HR (95% CI)	Q-value	Incidence rate per 1,000 person-years (95% CI)		No events n (%)	
				Patients with CHB*	Controls†	Patients with CHB*	Controls†
Other bacterial diseases	Other sepsis (A41)	>100 (N/A‡)	<0.01	2.5 (1.0 to 6.0)	0	5 (4.2)	0 (0)
	Bacterial infection of unspecified site (A49)	14.9 (2.5–89.1)	0.05	1.5 (0.5 to 4.6)	0.1 (0.0–0.4)	3 (2.5)	2 (0.2)
Disorders of psycho-logical development	Pervasive developmental disorders (F84)	7.4 (1.7–33.2)	0.11	1.5 (0.5 to 4.6)	0.2 (0.1–0.5)	3 (2.5)	4 (0.3)
Other forms of heart disease	Cardiomyopathy (I42)	>100 (N/A‡)	<0.01	7.1 (4.2 to 12.0)	0	14 (11.8)	0 (0)
	Atrioventricular and left bundle-branch block (I44)	>100 (N/A‡)	<0.01	195.6 (162.7 to 235.2)	0	113 (95)	0 (0)
	Other conduction disorders (I45)	>100 (N/A‡)	<0.01	8.8 (5.4 to 14.3)	0	16 (13.4)	0 (0)
	Paroxysmal tachycardia (I47)	51.0 (6.0–436.4)	<0.01	2.5 (1.1 to 6.1)	0.0 (0.0–0.4)	5 (4.2)	1 (0.1)
	Atrial fibrillation and flutter (I48)	46.7 (10.1–216.1)	<0.01	4.6 (2.4 to 8.8)	0.1 (0.0–0.4)	9 (7.6)	2 (0.2)
	Other cardiac arrhythmias (I49)	>100 (66.0–415.0)	<0.01	52.4 (40.2 to 68.3)	0.2 (0.1–0.6)	55 (46.2)	5 (0.4)
	Heart failure (I50)	34.4 (9.5–125.2)	<0.01	5.1 (2.7 to 9.4)	0.1 (0.0–0.5)	10 (8.4)	3 (0.3)
Cerebrovascular diseases	Cerebral infarction (I63)	39.9 (4.5–357.3)	0.02	2.0 (0.7 to 5.3)	0.0 (0.0–0.4)	4 (3.4)	1 (0.1)
Acute upper respiratory infections	Acute tonsillitis (J03)	3.7 (1.8–7.7)	<0.01	5.1 (2.8 to 9.5)	1.4 (0.9–2.0)	10 (8.4)	27 (2.3)
Acute	Acute upper respiratory infections of multiple and unspecified sites (J06)	2.3 (1.3–4.0)	0.04	8.5 (5.2 to 13.9)	3.6 (2.9–4.6)	16 (13.4)	71 (6)
Influenza and pneumonia	Bacterial pneumonia, not elsewhere classified (J15)	3.6 (1.3–9.9)	0.18	2.5 (1.0 to 6.0)	0.7 (0.4–1.2)	5 (4.2)	14 (1.2)
pneumonia	Pneumonia, organism unspecified (J18)	6.4 (2.5–16.5)	<0.01	3.5 (1.7 to 7.4)	0.5 (0.3–1.0)	7 (5.9)	11 (0.9)
Other acute lower respiratory infections	Acute bronchitis (J20)	9.2 (3.7–22.6)	<0.01	4.6 (2.4 to 8.8)	0.5 (0.3–0.9)	9 (7.6)	10 (0.8)
Infections of the skin and subcutaneous tissue	Other local infections of skin and subcutaneous tissue (L08)	20.1 (6.1–66.9)	<0.01	4.0 (2.0 to 8.0)	0.2 (0.1–0.5)	8 (6.7)	4 (0.3)
Other disorders of the skin and subcutaneous tissue	Atrophic disorders of skin (L90)	8.0 (2.2–29.9)	0.03	2.0 (0.7s to 5.3)	0.2 (0.1–0.6)	4 (3.4)	5 (0.4)
Systemic connective tissue disorders	Other systemic involvement of connective tissue (M35)	7.5 (1.7–33.4)	0.11	1.5 (0.5 to 4.6)	0.2 (0.1–0.5)	3 (2.5)	4 (0.3)

*Patients with CHB, n=119.
†General population comparators, n=1,190.
‡Confidence intervals not enclosed when no events were observed in either cases or controls.

and a trend for increased risks of others in patients with CHB (tables 2 and 4, and data not shown). To assess the aggregated risk of autoimmune disease, we created a composite outcome variable of common autoimmune diagnoses (thyroid disease, multiple sclerosis, psoriasis, arthritis and systemic rheumatic disease). The ICD codes included in the measure are specified in the legend of figure 2. This variable was then analysed using a Nelson-Aalen estimator to investigate the age-wise accumulation of autoimmune disease, and hazard ratios were defined by Cox proportional regression. Both patients with CHB and their siblings presented a significantly higher frequency of autoimmune diseases as defined by the composite variable than their respective controls, with hazard ratios for autoimmune disease of 5.7 for patients with CHB ($p<0.01$), and 3.6 ($p<0.01$) for their siblings (figure 2, online supplementary table 5). Changing the composite outcome variable to only include the systemic

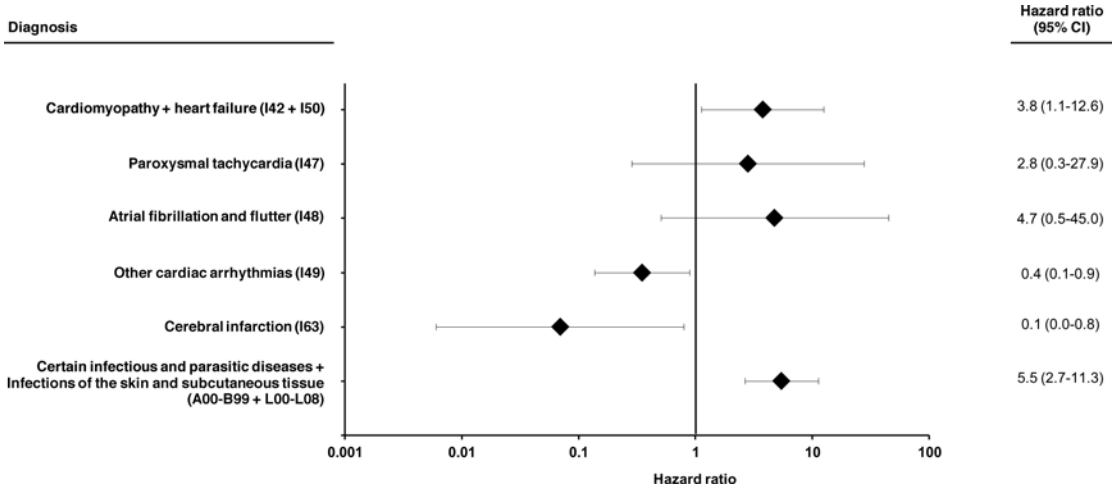

Figure 1 Influence of pacemaker treatment on the risk of comorbidities. Hazard ratios and 95% CI for indicated diagnoses with pacemaker surgery as time-varying covariate.

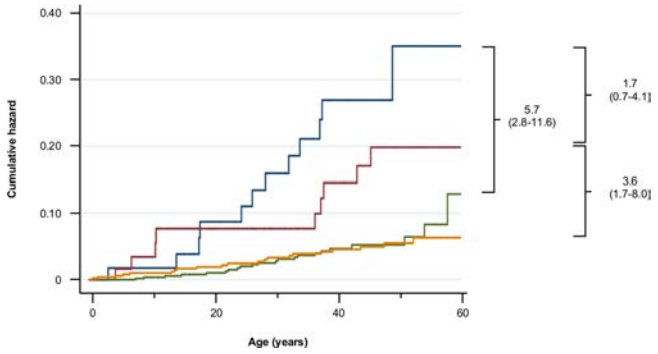

Figure 2 Risk of developing autoimmune disease for patients with CHB and their siblings. Cumulative hazard rates of autoimmune disease as assessed by a composite score and plotted for patients with cHb (blue line) and controls (green line), as well as CHB siblings (red line) and controls' siblings (yellow line). the autoimmune disease composite score was defined as any of: thyroid diagnoses (E03, E04, E06), multiple sclerosis (G35), psoriasis (L40), systemic rheumatic diseases (M32, L93, M33, M34, M35.0), and arthritis diagnoses (M02, M03, M05, M08). Brackets indicate inter-group comparisons using Cox regression with corresponding hazard ratio (95% CI).

rheumatic diseases, or the thyroid diseases, or the arthritic diseases, all demonstrated significantly increased hazard ratios for patients with CHB, while only the thyroid disease composite variable remained significant for siblings of patients with CHB (online supplementary table 6). During the follow-up time, 13 (11%) of patients with CHB developed an autoimmune disease, compared with 24 (2%) of the controls. Six (5%) siblings of patients with CHB versus 33 (3%) of sibling controls received a diagnosis of autoimmune disease. Although the proportion of CHB siblings developing an autoimmune disease was smaller than that seen in the group of CHB individuals, the difference was not statistically significant.

DISCUSSION

Information on comorbidity and complications later in life for individuals affected by CHB is scarce due to the rareness of the condition. We therefore conducted a study based on data available in nationwide Swedish healthcare registers to systematically survey long-term outcome in patients with CHB that had been identified in a population-based manner.[30] In our cohort, patients with CHB displayed an increased risk of cardiovascular comorbidities. Specifically, we observed an increased risk of cardiomyopathy and/or heart failure in CHB individuals, confirming findings from previous studies.[14 17 35–37] Although we also found an association between pacemaker implantation and an increased risk of developing cardiomyopathy/heart failure, as previously observed,[36] it should be noted that our study does not allow investigation of a possible causal relationship, and that it has also been reported that the vast majority of patients with CHB already have an impaired heart function before receiving pacemaker treatment.[37] We also observed that patients with CHB had elevated risks of atrial fibrillation and flutter, as well as of SSS. Importantly, the increased risk of cardiovascular morbidity was not just related to conditions affecting the heart itself, but also included an increased risk of other cardiovascular diseases, with patients with CHB displaying a substantially increased risk to develop cerebral infarction. Our analyses revealed that pacing therapy may provide a degree of protection, with three of the four individuals who experienced cerebral infarction not having received pacemaker treatment. It is also worth noting that siblings of patients with CHB had a higher incidence of heart disease than their controls. Our data indicate conduction disorders and tachyarrhythmias, but a statistical relationship could not be established due to the low number of events in this limited cohort. It is important to keep in mind that the higher frequency of minor cardiac abnormalities may also relate to reporting bias, as siblings of patient with CHB may more often be subject to investigation of cardiac function than the general population.

Our study further demonstrates that patients with CHB are more likely of being diagnosed with infections. Several reasons may underlie this increased risk. One explanation, although perhaps less probable, is that individuals with CHB represent a group with an inherently increased susceptibility to infectious disease. The occurrence of infections secondary to pacemaker implantation surgery is well known,[33 38] thus constituting a likely explanation, and our analysis of complications secondary to pacemaker implantation indeed confirmed this association. Moreover, the increased prevalence of prematurity reported in individuals with CHB,[1 39] which itself is associated with an increased risk of infections,[40] may also explain some of the risk. Although we were not able to account for the impact of preterm births, the age-wise distribution of infections did not suggest a significant role of prematurity. In addition, it may be assumed that patients with an increased healthcare exposure and more frequent healthcare visits, such as patients with CHB, are more likely to receive diagnoses for infections compared with the general population.

Our findings also implicate that patients with CHB have an increased frequency of psychological developmental disorders, consistent with previous studies reporting an increased prevalence of neuro-psychiatric dysfunction in this group.[19 20] Moreover, we also observed an increased incidence of diagnoses related to metabolic disorders and diseases of the digestive system. However, reporting bias may influence the estimated risk of morbidity in patients with CHB, and false discoveries are likely more probable in disorders with few observed cases such as the above.

The patients with CHB included in this study were all born to anti-Ro/SSA antibody-positive mothers, the majority of whom had a diagnosis of autoimmune disease either at the time of pregnancy or later in life.[10 30 41–43] In line with previous reports of familial aggregation of autoimmune diseases,[28 29 44] we observed that both patients with CHB and their siblings had an increased risk of developing systemic connective tissue disorders and/or autoimmune diseases. Although the frequency of autoimmune diseases in patients with CHB was not statistically different from that observed in their siblings, the proportion of patients with CHB developing autoimmune disease was nevertheless greater, with the cumulative incidence about 2-fold higher among patients with CHB than their siblings. A relatively low recurrence rate of CHB despite the persisting presence of maternal anti-Ro/SSA antibodies[45–47] indicates that fetal genetic susceptibility may modulate pathogenetic mechanisms and influence CHB development.[48–50] Similarly, genetic differences could underlie a higher frequency of autoimmune disease in patients with CHB compared with their siblings, as well as the overall increased risk for these two groups in relation to their general population comparators.

Limitations of this study include potential errors related to the registers used with regard to validity and exhaustiveness. This constraint is however somewhat mitigated by the setup, as the information collected for both exposure and control groups is subject to the same limitations. Another drawback is the fact that we only included CHB individuals alive at the time of entry into

the cohort, as its original establishment was designed to include biological sample collection, leading to a survival bias. Further, as discussed above, we were not able to assess or control for the impact of prematurity, which may contribute to the risk of some morbidities.[18 51] Finally, despite the national coverage and long-term follow-up at a mean of almost two decades, the overall limited number of events makes statistical estimates less stable. We nonetheless think that our dataset, allowing long-term follow-up of a relatively large number of patients born with CHB, is unique, and carries substantial value in understanding the health challenges for this group of patients.

In all, our findings suggest a non-negligible burden of comorbidity for patients with CHB, which is most apparent within cardiovascular, infectious and chronic inflammatory or autoimmune disorders, with the latter risk also shared by their siblings.

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Contributors JM, SS and MWH conceived the study, with support from MF and AE for the study design. HE, GB, SES recruited patients and recorded clinical data. ASA and MWH extracted register data. JM analyzed the data with input from HE, GB, SES and MWH. JM wrote the first version of the manuscript with input from HE, AA, GB, SES and MWH, and all authors participated in the editing until its final version.

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Incidence of inflammatory polyarthritis in polymyalgia rheumatica: a population-based cohort study

The relationship between polymyalgia rheumatica (PMR) and inflammatory polyarthritis (IP) remains a source of debate in rheumatology: although both conditions have been classified separately as distinct entities, they share many clinical features.[1–4] It remains unclear whether synovitis in IP is part of a spectrum of PMR, or if the symptoms of PMR are early manifestations of a distinct diagnosis of IP. Alternatively, the arthritis that develops in PMR might represent a phenotypic transformation in susceptible individuals.

We examined the risk of IP following the diagnosis of PMR in the data from the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk study, a prospective population-based cohort.[5] Incident cases of PMR were identified retrospectively among 24 068 volunteers enrolled after 2002 by (1) free-text questionnaire responses at baseline, 18 months, and at 3, 10 and 13 years; (2) linkage to hospital electronic discharge summaries containing International Classification of Diseases codes; and (3) linkage to keyword searches (*polymyalgia or rheumatica*) of outpatient clinic letters. To be identified as PMR, participants were required to have received at least two prescriptions for oral glucocorticoids for PMR within 6 months following the index date of diagnosis. Our approach to classifying cases of PMR follows the methodology validated in the Clinical Practice Research Datalink.[6] Cases were excluded from analysis if the diagnosis in the case record was refuted or changed within the first 6 months to an alternative diagnosis other than IP. Case assignment was carried out independently by two rheumatologists (MY, RAW). Anti-citrullinated protein antibody (ACPA) testing (Axis-Shield CCP2 antigen-plate DIASTAT kit (Axis-Shield, Dundee, UK), where >5 IU/mL was considered positive) was performed at inclusion in the EPIC-Norfolk study, supplemented by case record review. The rate of development and predictors of onset IP were examined using competing risks Cox regression analysis.

We identified 322 incident cases of PMR (median age at diagnosis: 75.3 years, minimum 51.5 years, maximum 93.8 years; median erythrocyte sedimentation rate (ESR) at diagnosis: 54 mm/hour; 73.2% female). In 1855 person-years of follow-up, 32 participants (63% female) were diagnosed with IP. The cumulative incidence of IP at 6 months and at 1, 2, 5 and 10 years was 2.2% (95% CI 1.0 to 4.3), 3.5% (95% CI 1.9 to 6.0), 6.5% (95% CI 4.1 to 9.6), 8.4% (95% CI 5.6 to 11.9) and 12.9% (95% CI 8.8 to 17.9), respectively, taking into account censoring for losses to follow-up and the competing risk of death. Clinical features at PMR onset associated with subsequent IP included the presence of any clinically apparent small joint synovitis, younger age and positive ACPA serology (table 1). There was a trend for greater risk for IP in men compared with women in the first 5 years following a diagnosis of PMR, 13.1% (6.9 to 21.2) vs 6.6% (3.8 to 10.5), but the difference did not reach statistical significance. A sensitivity analysis in which cases were confined to those that fulfilled current classification criteria for PMR and rheumatoid arthritis shows similar associations but with a stronger association with synovitis (table 1).

The findings are consistent with the emergence of IP as a distinct diagnosis in patients initially diagnosed with PMR. The heightened risk of IP following a diagnosis of PMR, which is greatest in the first 2 years but extends for up to a decade, indicates a need for long-term clinical vigilance. While ACPA is a predictor of subsequent IP emergence, the majority who developed IP in this cohort were ACPA-negative at the time of IP diagnosis (81%), suggesting that autoantibody tests might have limitations for identifying those at risk. Classification criteria should not be used for diagnosis and our data support this assertion. Ultimately, clinicians must remain vigilant for diagnostic transformation when managing patients with PMR.

Table 1 Predictors of IP/RA using Cox modelling

	All cases of PMR (n=322) PMR changing to IP (n=32)		Cases of PMR meeting EULAR/ACR criteria (n=292) PMR changing to RA (n=12)	
Clinical feature	**Sub-HR and 95% CI**	**P values**	**Sub-HR and 95% CI**	**P values**
Age at time of PMR diagnosis >75.9 years	0.33 (0.15 to 0.74)	0.007	0.49 (0.15 to 1.62)	0.244
Male sex	1.72 (0.84 to 3.53)	0.139	1.47 (0.45 to 4.84)	0.524
Ever smoked*	1.77 (0.84 to 3.72)	0.131	1.72 (0.50 to 5.93)	0.393
RhF-positive†	3.46 (0.89 to 13.51)	0.074	NA‡	
ACPA-positive*†	3.14 (1.16 to 8.54)	0.025	NA‡	
Wrist synovitis at time of PMR diagnosis*	3.41 (0.54 to 21.50)	0.192	8.55 (1.05 to 69.74)	0.045
Small joint synovitis at time of PMR diagnosis*	3.11 (1.08 to 8.93)	0.035	6.17 (1.34 to 28.43)	0.019

*Adjusted estimates accounting for age and sex.
†ACPA and RhF were measured at inclusion in the EPIC-Norfolk study.
‡As the absence of rheumatoid factor and/or anti-CCP antibodies forms part of the EULAR/ACR criteria for PMR and their presence is included in the EULAR/ACR criteria for RA, these have not been included in the model.
ACPA, anti-citrullinated protein antibody; ACR, American College of Rheumatology; EULAR, European League Against Rheumatism; IP, inflammatory polyarthritis; NA, not applicable; PMR, polymyalgia rheumatica; RA, rheumatoid arthritis; RhF, rheumatoid factor.

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Competing interests None declared.

Patient consent Not required.

Ethics approval The study complies with the Declaration of Helsinki. The Norwich District Health Authority Ethics Committee approved the study and all participants gave written informed consent.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement EPIC-Norfolk custodians and collaborators are committed to open and collaborative analysis. Requests for data sharing are reviewed by the committee and forms can be found at http://www.srl.cam.ac.uk/epic/contact/index.shtml.

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Serum IgG N-glycans act as novel serum biomarkers of ankylosing spondylitis

Ankylosing spondylitis (AS) is a chronic inflammatory disease with poorly defined aetiologies and no curative treatments. The average delay in the diagnosis of AS is 6–8 years.[1] Human leukocyte antigen B27 (HLA-B27) is a key laboratory marker for AS presenting in at least 90% of patients with AS.[2] However, 63%–90% of patients with reactive arthritis[3] and 19.2% of patients with psoriatic arthritis (PsA)[4] are also positive for HLA-B27, indicating low specificity of HLA-B27. The risk of development of AS in an HLA-B27-positive individual is only 2%–10%,[5] which suggests the limited value of HLA-B27 in supporting an AS diagnosis. Moreover, reported serum biomarkers for AS have generally exhibited low sensitivity or specificity[6] (<60%). Novel serum biomarkers with high prediction capacity remain needed.

The changed IgG glycosylation in autoimmune and inflammatory conditions, as well as the broad roles for specific IgG glycoforms in maintaining immune homeostasis, have been well documented.[7 8] However, specific glycan biomarkers on IgG for AS have not been fully identified. In our previous study, a specialised microfluidic titanium dioxide-porous graphitised carbon chip was developed; this approach enabled the quantification of low-abundance and trace acidic glycans that are often biologically important species. In glycomic analyses of serum IgG in patients with rheumatoid arthritis (RA), two sulfated N-glycans were identified as promising biomarkers for seronegative RA.[9] In the current study, we used this glycomic approach to analyse serum IgG in patients with AS and identified potential N-glycan biomarkers of AS for the first time.

Eighty patients who exhibited definite AS that fulfilled the modified New York criteria (1984) from three hospitals in China and 80 age-matched and gender-matched healthy volunteers were enrolled in this study. The determined levels of individual N-glycans[9] were used as variations for the classification. In total 160 samples were divided into a training set (n=56) and a validation set (n=104) (online supplementary table 1).

By using the feature selection methods in WEKA,[9] 11 neutral and 6 acidic N-glycans were selected as potential biomarkers for the classification of AS (online supplementary table 2). Two of the 17 biomarkers, 5_5_1_0 and 6_5_0_3-a (figure 1A,D), demonstrated relatively high prediction capacity for AS, with area under the curve (AUC), sensitivity and specificity greater than 70% for both the training and validation sets (figure 1B,E). Of note, significantly higher AUCs (0.823 and 0.911), sensitivities (75% and 86.5%) and specificities (82.1% and 80.8%) in training and validation sets, respectively, were observed for a combination of these two N-glycan biomarkers (online supplementary table 2). Univariate analysis showed significant differences in the levels of these two markers between the control and AS groups (figure 1C,F), while no significant alterations were observed in patients with PsA (online supplementary figure 1, online supplementary tables 3 and 4). Moreover, we noted a correlation between the levels of glycan 5_5_1_0 and erythrocyte sedimentation rate (ESR) ($|r|=0.42$, $p=0.0001$), and observed more significant reduction of this glycan in the subgroup with elevated ESR (online supplementary figure 2). No such correlation was observed for glycan 6_5_0_3-a ($|r|=0.11$, $p=0.3328$). Influence from impurity (IgA and IgM) was proved to be slight (<5%; online supplementary table 5).

In conclusion, we identified N-glycan-based biomarkers for patients with AS for the first time. Two N-glycans which are overwhelmingly from IgG exhibited relatively high sensitivity and specificity for the classification of AS. Given the crucial roles of N-glycans of IgG for immune homeostasis and inflammation, the identified biomarkers could serve as additional measures of disease phenotype, predict patients' responsiveness to treatment and provide new insight into the pathogenesis for AS. We anticipate that large-scale studies on the roles of N-glycans in AS could be profoundly conducted further.

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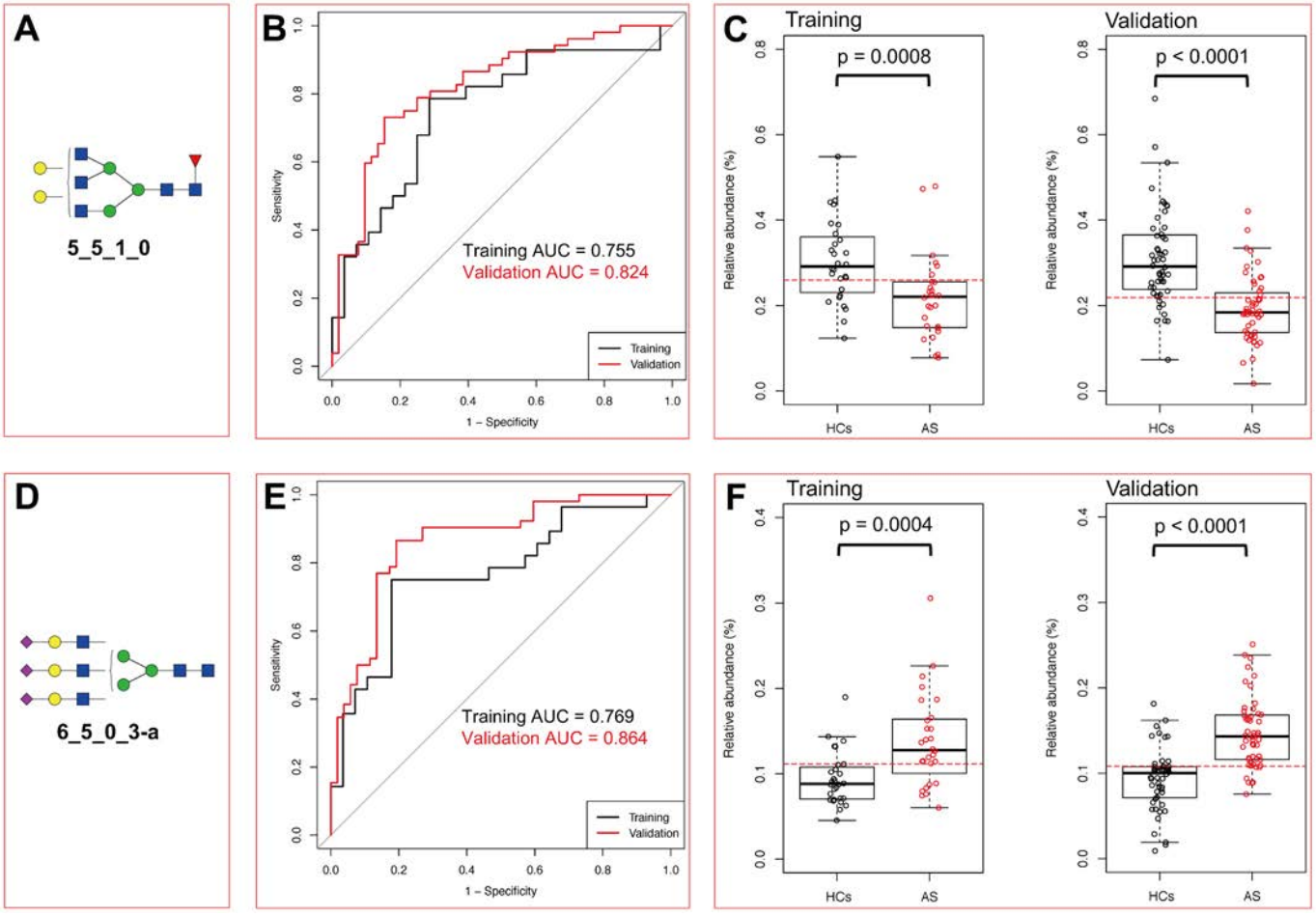

Figure 1 Performance and relative abundances of the two potential N-glycan biomarkers for ankylosing spondylitis (AS) in the training set (AS, n=28; healthy controls (HCs), n=28) and validation set (AS, n=52; HCs, n=52). A and D show the symbols depicting N-glycan biomarkers identified in the current study. B and E show the receiver operating characteristic curves of biomarkers for the classification of AS and HCs. C and F show the boxplots for the levels of the biomarkers in AS and HCs. The red dotted lines in the figures represent the cut-off values determined based on the maximum values generated using the formula, sensitivity+specificity – 1, in our analyses. A and D were drawn using GlycoWorkbench V.2.1 stable (build: 157) (developed by Alessio Ceroni, KAI Maass, and David Damerell, European carbohydrates database, Europe), and B, C, E and F were drawn using RStudio V.1.0.153 (RStudio, Boston, USA). AUC, area under the curve.

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Normal vaginal microbiome in women with primary Sjögren's syndrome-associated vaginal dryness

Dryness of epithelial surfaces is characteristic for patients with primary Sjögren's syndrome (pSS). Vaginal dryness is frequently reported by pSS-women and is associated with sexual dysfunction.[1 2] Recently, we showed that dysbiosis of the oral microbiome is largely similar between oral dryness patients with and without pSS when compared with healthy controls.[3 4] The objective of our current study was to assess whether the vaginal microbiome of women with pSS-associated vaginal dryness differs from controls.

In a case-control design, we compared the vaginal microbiome of 10 premenopausal pSS-women with that of 10 age-matched premenopausal women without pSS, who underwent general anaesthesia for a laparoscopic procedure. Exclusion criteria were genital inflammatory or infectious comorbidity, endometriosis and use of disease modifying antirheumatic drugs, corticosteroids, vaginal oestrogens or an intrauterine contraceptive device. All patients with pSS fulfilled the 2016 ACR/EULAR classification criteria. All participants completed a questionnaire on vaginal symptoms. Patient-reported vaginal dryness was scored using a Numeric Rating Scale (NRS, range 0–10). Vaginal health was assessed with the Vaginal Health Index (VHI).[5] From all participants, a cervicovaginal lavage (CVL) and endocervical swab (ES) were collected. DNA from all samples was isolated. The V3-V4 region of the bacterial 16S rRNA gene was amplified. Paired-end sequencing was performed on an Illumina MiSeq platform. For details, see online supplementary methods.

After inclusion, one patient with pSS was diagnosed with Chlamydia in the ES and two control women with endometriosis at laparoscopy. These women were excluded, resulting in nine pSS-women and eight controls for further analyses (table 1).

As expected, scores for vaginal dryness, dyspareunia and use of lubricants were higher in pSS-women.[2] Furthermore, pSS-women scored significantly lower on the total VHI-score.[5] Vaginal pH-values were normal in patients with pSS. Microbiota composition of CVL and ES samples were highly similar within individuals, with 95% being explained by individuality (*adonis*, $p<0.001$; figures 1A). Disease (pSS vs control) did not affect overall vaginal microbiota composition in both CVL and ES samples (*adonis*, $p>0.05$; figure 1B). Despite the small sample size, we were able to identify in both groups (pSS and controls), four of the five vaginal community state types (CSTs) previously described (figure 1C–E).[6] Distribution of CSTs and distribution of the three most prevalent genera (ie, *Lactobacillus*, *Gardnerella* and *Streptococcus*) showed similar patterns in pSS-women and controls (figure 1F,G). Also, the mean relative abundance of these three genera did not differ between pSS-women and controls ($p>0.05$). Patient-reported vaginal dryness severity (NRS-score) did not correlate with the relative abundance of the three most prevalent genera (Spearman, $p>0.05$). The small number of patients with pSS did not allow us to analyse associations between vaginal microbiota and disease activity.

Our results indicate that the vaginal microbiome in pSS-women with vaginal dryness is similar to that of controls, which contrasts the observed difference in vaginal microbiota composition between postmenopausal women with and without vaginal dryness.[7] The different outcomes may be explained by different underlying causes of vaginal dryness (ie, pSS in premenopausal vs loss of oestrogen in postmenopausal women).[7] Under the influence of oestrogen, glycogen is deposited in the epithelium of the vagina.[8] Lactobacilli use the breakdown products of glycogen to produce lactic acid, which contributes to the low vaginal pH and thereby inhibits the growth of other bacteria.[8]

Table 1 Study population characteristics

Characteristic	pSS N=9	Control N=8	P values*
Age, mean (SD)	38 (9)	40 (4)	0.6
anti-SSA antibody positive, n (%)	7 (78)	na	
anti-SSB antibody positive, n (%)	6 (67)	na	
Disease duration in years, mean (SD)	8 (7)	NA	
Smoking, n (%)	3 (33)	4 (50)	0.8
Pack years, mean (SD)	0.7 (2)	0.7 (1)	0.4
Numeric Rating Scale on dryness (0–10)			
Eyes, mean (SD)	7 (1)	2 (2)	**0.001**
Mouth, mean (SD)	7 (1)	1 (2)	**<0.001**
Vagina, mean (SD)	6 (2)	1 (2)	**0.002**
Use of lubricants, n (%)	5 (56)	0 (0)	**0.05**
Dyspareunia, n (%)	9 (100)	2 (25)	**0.01**
Vaginal Health Index† total score, mean (SD)	19 (3)	23 (2)	**0.02**
pH posterior fornix, mean (SD)	4.6 (0.7)	4.7 (0.5)	0.6
Current medication			
Oral contraceptives, n (%)	6 (67)	3 (38)	0.5
Current NSAIDs, n (%)	2 (22)	0 (0)	0.5
ESSDAI—total, mean (SD)	6 (3)	NA	
ESSPRI—dryness, mean (SD)	6 (1)	NA	
ESSPRI—fatigue, mean (SD)	6 (3)	NA	
ESSPRI—pain, mean (SD)	3 (3)	NA	
ESSPRI—total, mean (SD)	5 (2)	NA	
Reason for laparoscopic procedure in controls			
BRCA1 or BRCA2 mutation, n	NA	6	
Refertilisation, n	NA	2	
Mucous cyst of the adnex, n	NA	1	

Bold values indicate a p value of 0.5 or lower.
*Vaginal Health Index (VHI) scoring system: see online supplementary figure s1.
†χ^2 test and Wilcoxon rank sum test were used for categorical and numerical data, respectively.
ESSPRI, EULAR Sjögren's syndrome patient-reported index; NA, not applicable; na, not assessed; NSAIDs, non-steroidal anti-inflammatory drugs; pSS, primary Sjögren's syndrome; SSA, Sjögren's syndrome antigen A; SSB, Sjögren's syndrome antigen B; SSDAI, EULAR Sjögren's syndrome disease activity index.

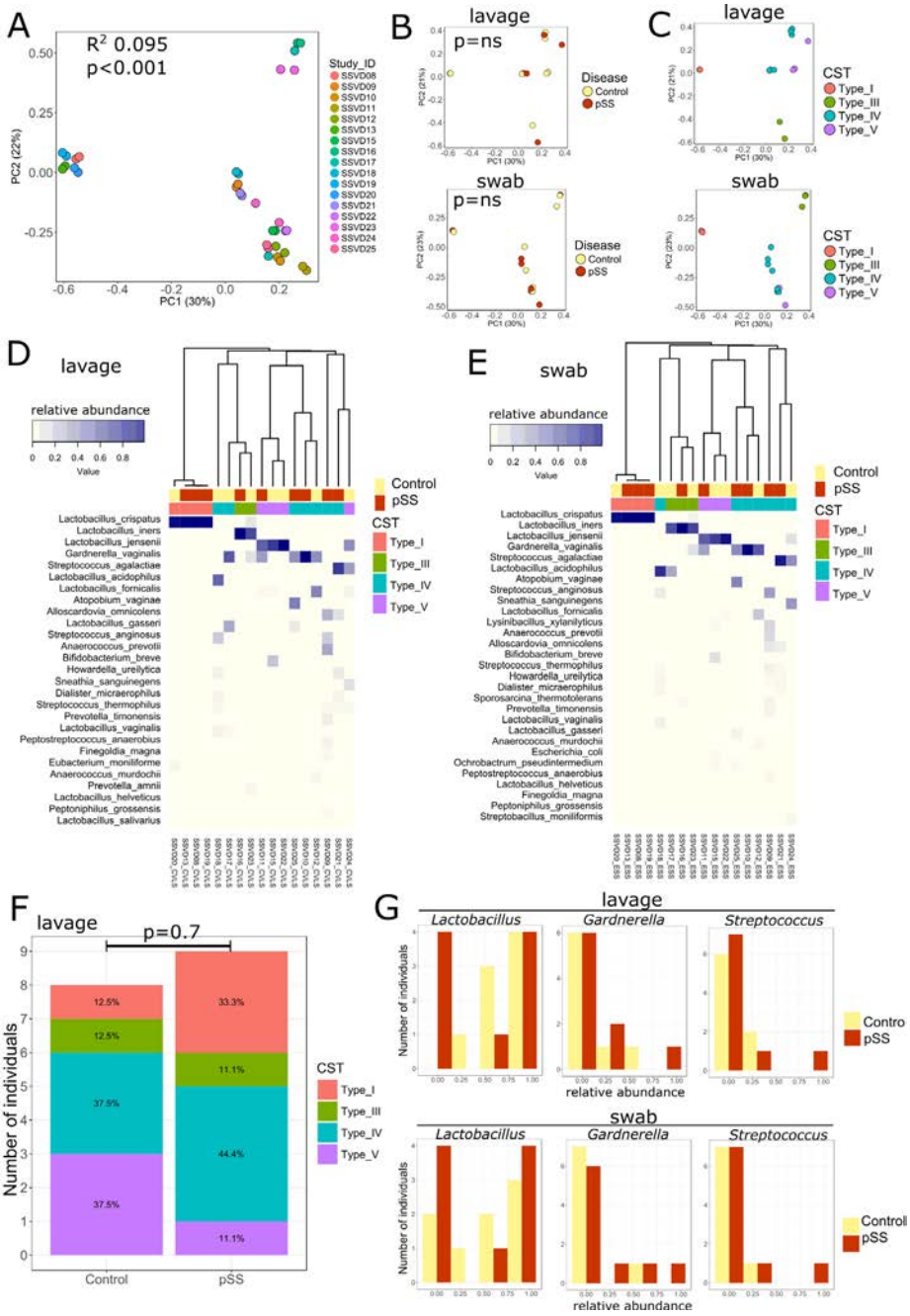

Figure 1 Vaginal microbiota composition in premenopausal pSS-women with and controls. (A) Principal coordinate analysis of CVL and ES samples shows high similarity within individuals (overlapping dots are separated slightly for enhanced clarity, see online supplementary figure S2 for original image). clustering of pSS-women or control women is observed based on vaginal microbiota composition in CVL (lavage) or ES (swab) samples. (C) CVL and ES samples show evident clustering based on the four CSTs. (D and E) CST-I, dominated by *Lactobacillus crispatus*, CST-III, dominated by *Lactobacillus iners*, CST-IV, a heterogeneous non-lactobacilli dominated type and CST-V, which is dominated by *Lactobacillus jensenii* were identified using Bray-Curtis distance clustering, based on the relative abundance of bacterial species with a relative abundance >0.1%. (F) Distribution of CSTs did not differ between pSS-women and controls (Fisher's exact test). (G) Histograms of the three most abundant genera show similar patterns in pSS-women and controls. CST, community state type; CVL, cervicovaginal lavage; ES, endocervical swab; pSS, primary Sjögren's syndrome.

Apparently, the unique vaginal microbiome—dominated by acid producing lactobacilli—is less dependent on dryness than the oral microbiome. Oral dryness is associated with higher *Lactobacillus* relative abundance, which contributes to oral diseases (ie, dental caries and Candida infection). In the vagina, lactobacilli represent a healthy microbiome and are essential for the low vaginal pH.[8] Our study suggests that pSS-associated vaginal dryness in premenopausal women does not negatively influence homeostasis of the vaginal ecosystem.

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Contributors TAvdM designed the study, analysed all data (clinical and microbiome) and wrote the manuscript. JFvN designed the study, recruited the patients, did the study logistics and reviewed the manuscript. HJMH performed the 16S rRNA sequencing and reviewed the manuscript. SCL performed the DNA isolation and reviewed the manuscript. KvdT and MJEM performed the vaginal health scoring, collected the samples and reviewed the manuscript. MJEM, FGMK, AV and HB helped in the design of the study, interpretation of data and reviewed the manuscript.

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Neutrophils are not consistently activated by antineutrophil cytoplasmic antibodies in vitro

Antineutrophil cytoplasmic antibody (ANCA) vasculitis is characterised by autoantibodies against myeloperoxidase (MPO) and proteinase 3 (PR3). The evidence that ANCA are pathogenic comes from in vitro studies in which IgG from patients with anti-MPO or anti-PR3 antibodies activate neutrophils to undergo respiratory burst and degranulation. Furthermore, murine monoclonal antibodies against human MPO and PR3 and a chimeric humanised anti-PR3 monoclonal antibody activate neutrophils. The paradigm of neutrophil activation by ANCA has therefore become established.[1] Further support for the pathogenicity of ANCA comes from in vivo studies in which injection of anti-MPO antibodies causes focal necrotising crescentic glomerulonephritis in mice.[2]

We assessed the effect of purified ANCA on the activation of TNFα primed neutrophils using 10 control IgGs, 11 MPO-ANCA and 9 PR3-ANCA using two different assays of the neutrophil respiratory burst (full methods are in a online supplementary file 1). We found no significant difference in two separate neutrophil donors (figure 1A-C). We also used assays for four markers of neutrophil degranulation and found no differences in two neutrophil donors (figure 1D-G). The results are not due to inactivity of the purified ANCA IgG preparations. Aliquots of the same ANCA and control IgG batches were used in a recent publication where we demonstrated clear effects of these ANCA IgG preparations on monocytes, in experiments performed with during the same period of time.[3]

Our data challenge the established paradigm of neutrophil activation by ANCA. It is not clear why our results differ from others, but note that most previous publications have included small numbers which might lead to chance effects and selection bias. The ability of ANCA to activate neutrophils may be affected by affinity. We did not measure affinity or explore this possibility. We reviewed the literature to find publications in which six or more MPO-ANCA or PR3-ANCA IgG samples were compared with a similar number of control IgG samples and found only two. Franssen *et al* compared IgG purified from 17 PR3-ANCA positive patients, 14 MPO-ANCA positive patients and 16 controls. The patients were consecutive, eliminating selection bias.[4] These authors found no significant effect of MPO-ANCA IgG on neutrophil respiratory burst using the DHR 123 and ferricytochrome C assays, and no effect on degranulation as measured by glucuronidase and lactoferrin release. There was an effect for PR3-ANCA which, although statistically significant, was small in magnitude. In all cases, the level of activation was much less than with N-formylmethionine-leucyl-phenylalanine. Harper *et al* compared 23 MPO-ANCAs, 15 PR3 ANCAs and 8 control IgGs using ferricytochrome C, calcium flux and MPO release assays.[5] Both MPO-ANCA and PR3-ANCA caused significant activation compared with control IgG. However, in contrast to the study by Franssen *et al*, MPO-ANCA had a greater effect.

A recent report consistent with our data suggests that ANCA IgG does not activate neutrophils in vitro.[6] Kraaij *et al* showed that serum from patients with ANCA vasculitis induced neutrophil extracellular traps (NET) formation, but this was unaffected by IgG depletion. In addition, purified IgG was unable to induce NET formation. This suggested that factors in the serum of patients with vasculitis, other than IgG, could activate neutrophils. This raises the possibility that the purity of IgG preparations could have influenced results in previous studies. We emphasise that our data do not exclude a role for neutrophils in the pathogenesis of ANCA vasculitis. ANCA may have direct or indirect effects on neutrophils in vivo that are not evident using in vitro assays of activation. We also acknowledge that there are many previous publications suggesting that ANCA do activate neutrophils in vitro and encourage other investigators to re-examine this question.

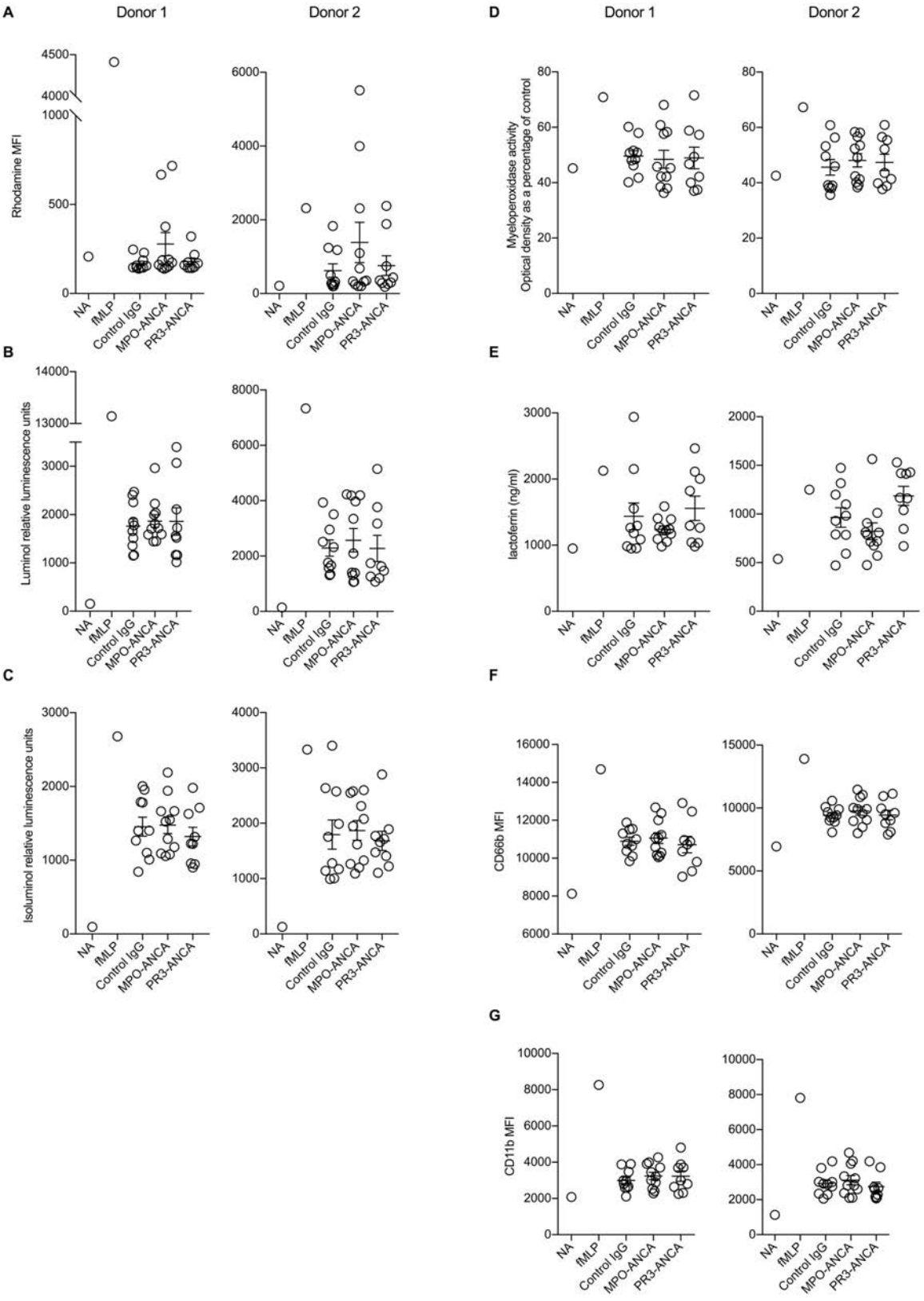

Figure 1 ANCA does not stimulate the neutrophil respiratory burst or degranulation in vitro. Ten control IgG, 11 MPO-ANCA and 9 PR3-ANCA were tested, with experiments performed in two neutrophil donors. The respiratory burst was assessed with (A) a dihydrorhodamine 123 assay of hydrogen peroxide generation, (B–C) luminol and isoluminol-based assays of total and extracellular superoxide generation. Degranulation products measured were (D) soluble MPO (azurophilic granules), (E) soluble lactoferrin (specific granules), (F) cell surface CD66b (specific granules) and (G) cell surface CD11b (secretory, gelatinase and specific granules). In (B–C), data shown are the peak response. For fMLP, this occurred at approximately 2 min, whereas the peak response to IgG was at approximately 30 min. There were no significant differences between the groups for any of the assays. ANCA, antineutrophil cytoplasmic antibody; fMLP, N-formylmethionine-leucyl-phenylalanine; NA, not activated.

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Role of linoleic acid in autoimmune disorders: a Mendelian randomisation study

Autoimmune diseases are a major challenge for global health. From an evolutionary biology perspective, reproduction, particularly androgens, trades off against immune activity.[1] Correspondingly, trials suggest androgens improve rheumatoid arthritis (RA).[2 3] As such, dietary factors promoting reproduction might prevent or treat autoimmune disorders. Linoleic acid (LA) is a major n−6 polyunsaturated fatty acid (PUFA) in widely used polyunsaturated vegetable oils, such as sunflower, corn, soybean and cottonseed oil. In animal experiments, LA stimulates the synthesis of testosterone.[4] Observationally, endogenous LA is inversely associated with RA[5] and systemic lupus erythematosus (SLE),[6] but these findings have not been confirmed in randomised controlled trials. Comparing autoimmune disorders according to naturally occurring LA-related genetic variants, that is, Mendelian randomisation (MR), provides a means of obtaining unconfounded estimates of causal effects.

We obtained strong, independent genetic predictors of LA using (1) the three most significant uncorrelated single-nucleotide polymorphisms (SNPs) and (2) seven uncorrelated SNPs ($r^2<0.01$) in genes (*FADS1*, *FADS2* and *NTAN1*) relevant to PUFA metabolism from a genome-wide association study (GWAS) in 8631 adults of European ancestry, mean age 60 years, 55% women.[7] We applied these genetic predictors of LA to the largest publically available European ancestry consortium GWAS and the UK Biobank GWAS of RA and SLE. For RA, we used the Rheumatoid Arthritis Consortium (14 361 cases, 43 923 controls).[8] RA diagnosis was on the 1987 criteria of the American College of Rheumatology (~90%) or a clinical evaluation by a professional board-certified rheumatologist. For SLE, we used the ImmunoBase Consortium (7219 cases, 15 991 controls)[9] mainly based on the Health and Retirement Study aged 50+ years, ~41% men.[10] Genetic associations with RA (n=4412) and SLE (n=342) were also meta-analysed with summary data from the UK Biobank in white British (n=408 961), adjusted for age, sex and four principal components, which used Saige to control for unbalanced case–control ratios and sample relatedness.[11]

We combined SNP-specific estimates using inverse variance weighting, and as sensitivity analysis used a weighted median, MR Egger and MR PRESSO,[12] which are more robust to pleiotropy. We used multivariable MR to handle known pleiotropic associations of rs526126 (*FADS2*) with serum docosapentaenoic acid, an n−3 PUFA.

Genetically instrumented LA was inversely associated with RA (OR 0.97, 95% CI 0.95 to 0.98) and SLE (OR 0.95, 95% CI 0.92 to 0.99) (table 1). The associations were generally robust to sensitivity analysis and different outcome GWAS although MR Egger had wider CIs which sometimes included the null value (table 1). MR Egger did not indicate of directional pleiotropy.

Consistent with implications of evolutionary biology theory, our novel study suggests LA protects against RA and SLE. Applying MR to large publicly available GWAS enables examination of the independent role of LA cost-efficiently, overcoming

Table 1 Mendelian randomisation estimates of associations of genetically predicted linoleic acid with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) using different analysis methods and data sources

		Using 3 SNPs with top significance						Sensitivity analysis using a different SNP selection*			
Outcome	Data source	Method	OR	95% CI	P values	Heterogeneity p†	MR-Egger intercept p‡	Method	OR	95% CI	P values
RA	Rheumatoid Arthritis Consortium	IVW	**0.96**	**0.94 to 0.98**	**<0.001**	0.44		Multivariable MR	**0.97**	**0.96 to 0.99**	**<0.001**
		WM	**0.96**	**0.94 to 0.98**	**<0.001**			WM	**0.96**	**0.94 to 0.98**	**<0.001**
		MR Egger	0.97	0.94 to 1.004	0.09		0.40	MR PRESSO	**0.97**	**0.94 to 0.99**	**0.01**
	Meta-analysis with UK Biobank	IVW	**0.97**	**0.95 to 0.98**	**<0.001**	0.24		Multivariable MR	**0.98**	**0.97 to 0.997**	**0.02**
		WM	**0.97**	**0.95 to 0.98**	**<0.001**			WM	**0.97**	**0.96 to 0.99**	**0.004**
		MR Egger	0.97	0.93 to 1.01	0.16		0.88	MR PRESSO	0.98	0.95 to 1.003	0.08
SLE	ImmunoBase Consortium	IVW	**0.95**	**0.91 to 0.99**	**0.02**	0.84		Multivariable MR	**0.96**	**0.93 to 0.99**	**0.01**
		WM	**0.95**	**0.91 to 0.99**	**0.02**			WM	**0.95**	**0.92 to 0.99**	**0.01**
		MR Egger	0.95	0.89 to 1.01	0.12		0.97	MR PRESSO	**0.96**	**0.93 to 0.995**	**0.03**
	Meta-analysis with UK Biobank	IVW	**0.95**	**0.92 to 0.99**	**0.01**	0.91		Multivariable MR	**0.96**	**0.93 to 0.99**	**0.01**
		WM	**0.95**	**0.92 to 0.99**	**0.02**			WM	**0.96**	**0.93 to 0.99**	**0.01**
		MR Egger	0.96	0.90 to 1.01	0.14		0.92	MR PRESSO	**0.97**	**0.93 to 0.999**	**0.03**

The associations with p value <0.05 were labelled with bold.
*Seven SNPs on functionally relevant genes in genome-wide association study of linoleic acid were used.
†According to the heterogeneity test, IVW with fixed-effects model was used.
‡The intercept can be interpreted as an estimate of the average pleiotropic effect across the genetic variants where a corresponding p value of <0.05 indicates the presence of directional pleiotropy across the genetic variants included in the analyses.
IVW, inverse variance weighting; SNP, single-nucleotide polymorphism; WM, weighted median.

the challenge of separating the role of n−6 PUFA from other interacting nutrients in observational studies.

MR studies are more suitable for testing for causation than indicating the exact size of causal effects. However, relatively small effects may still be an important determinant of population health, particularly for LA, the major dietary PUFA in most commonly used vegetable oils.

From a public health perspective, our findings suggest that dietary intake of LA, such as from vegetable oils, might reduce the risk of autoimmune disorders, with relevance to primary prevention of autoimmune disorders. Clarifying the role of LA and its underlying pathways would be worthwhile, with relevance to dietary recommendations, and identifying effective new interventions for autoimmune disorders.

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Swimming against the stream: the fishbowl discussion method as an interactive tool for medical conferences: experiences from the 11th European Lupus Meeting

Based on both historical development and functional advantages, such as in emergencies, hierarchical aspects dominate all levels of medicine. Doubtlessly, the high degree of respect for experts and their opinions may impede exchange between different levels of hierarchy. At medical conferences, discussions usually take place between experts, while patients, young doctors and students only listen and rarely actively participate.

For the 11th European Lupus Meeting in Düsseldorf in March 2018, we have tried the fishbowl method to increase participation at all levels. Fishbowl is an interactive and dynamic technique with a group of discussants sitting in an inner circle that contains an additional empty chair and surrounded by the audience in an outer circle.[1] The empty chair can be occupied by any member of the outer circle at any time to join the discussion immediately. After the additional discussant of the auditorium has made his/her statement, he or she will leave the chair again to create an opportunity for another member of the audience (figure 1).

Imagine the circle (the fishbowl) as a protected space in focus, with the audience outside the 'bowl' observing the group. What happens if any of the 'fish' out in the ocean can, at any time, join the discussion by swimming into the bowl?

Ten fishbowl discussion groups were formed in advance of the European Lupus Meeting 2018, each of which was assigned a topic. The groups consisted of a moderator (a systemic lupus erythematosus (SLE) expert), a patient with SLE, a fellow in training and two more international SLE experts. The 'fishbowl'-round discussing SLE clinical trial design, for instance, comprised an experienced Lupus Europe patient representative, a well-prepared rheumatology fellow, a rheumatologist with significant experience in SLE clinical trials and an industry-based trial expert. Each discussion lasted 1 hour and was protocolled on flip charts by another fellow and one more SLE expert who finally summarised the session together with the moderator.

The method was evaluated in an online survey distributed via email to all attendees after the conference. We assessed opinions regarding the effectiveness of the method and feelings as active participants. Of 733 conference attendees, a total of 169 persons completed the survey. Forty-seven had participated as members of the inner circle (8 moderators, 14 experts, 5 patients, 20 fellows) and 122 as members of the audience (among these experts, trainees and patients). Only 15.5% had heard about the method before and even fewer (6.6%) had previously participated in a fishbowl round (see online supplementary table S1).

Of the 122 respondents reporting the audience's point of view, 39 members had participated actively, of which close to half felt comfortable (28.7%) or even very comfortable (16.6%) with their role on the empty chair. Of all participants, 78.5% would recommend the method for future conferences. In the evaluation of the method, the majority agreed or strongly agreed on fishbowl discussions being effective (73.5%), more diverse than other methods (72.7%) and efficient to include otherwise hesitant individuals to participate (64.0%) (figure 2). Opinions did not differ between those in the inner circle and those in the audience. The major point of criticism was the suboptimal acoustics, whereas the fishbowl method itself was not substantially criticised.

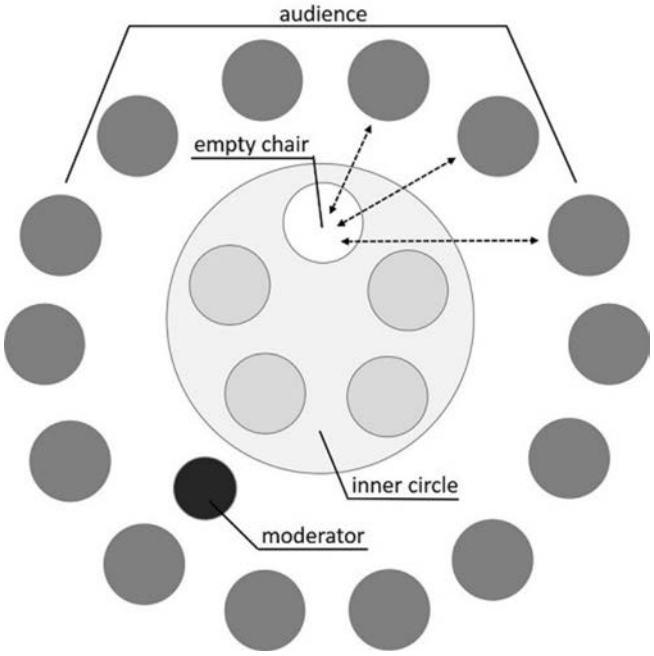

Figure 1 The fishbowl method. A group of discussants are located in the inner circle containing an additional empty chair. The auditorium is sitting in an outer circle surrounding the discussants. The empty chair can be occupied at any time by a member of the audience and will be abandoned again after his/her statement to provide space for the ensuing member of the audience.

Overall, the fishbowl discussions were excellently received and positively evaluated. In contrast to conventional discussion rounds, that is, grand debates, fishbowl discussions stimulate active participation of 'non-experts' and inclusion of other opinions. The cost of time-consuming preparation pays off with a diverse and highly effective method for scientific exchange. Other novel methods like 'world café' or 'open space' aim at a high level of interaction as well but focus rather on small groups than on large ones. In the end, the choice of discussion methods depends on the approach and the goal of the discussion. It turns out that in fishbowl also the otherwise hesitant participants easily move 'into the bowl' and take an active part in the discussion—swimming with the current, against it or even swirling.

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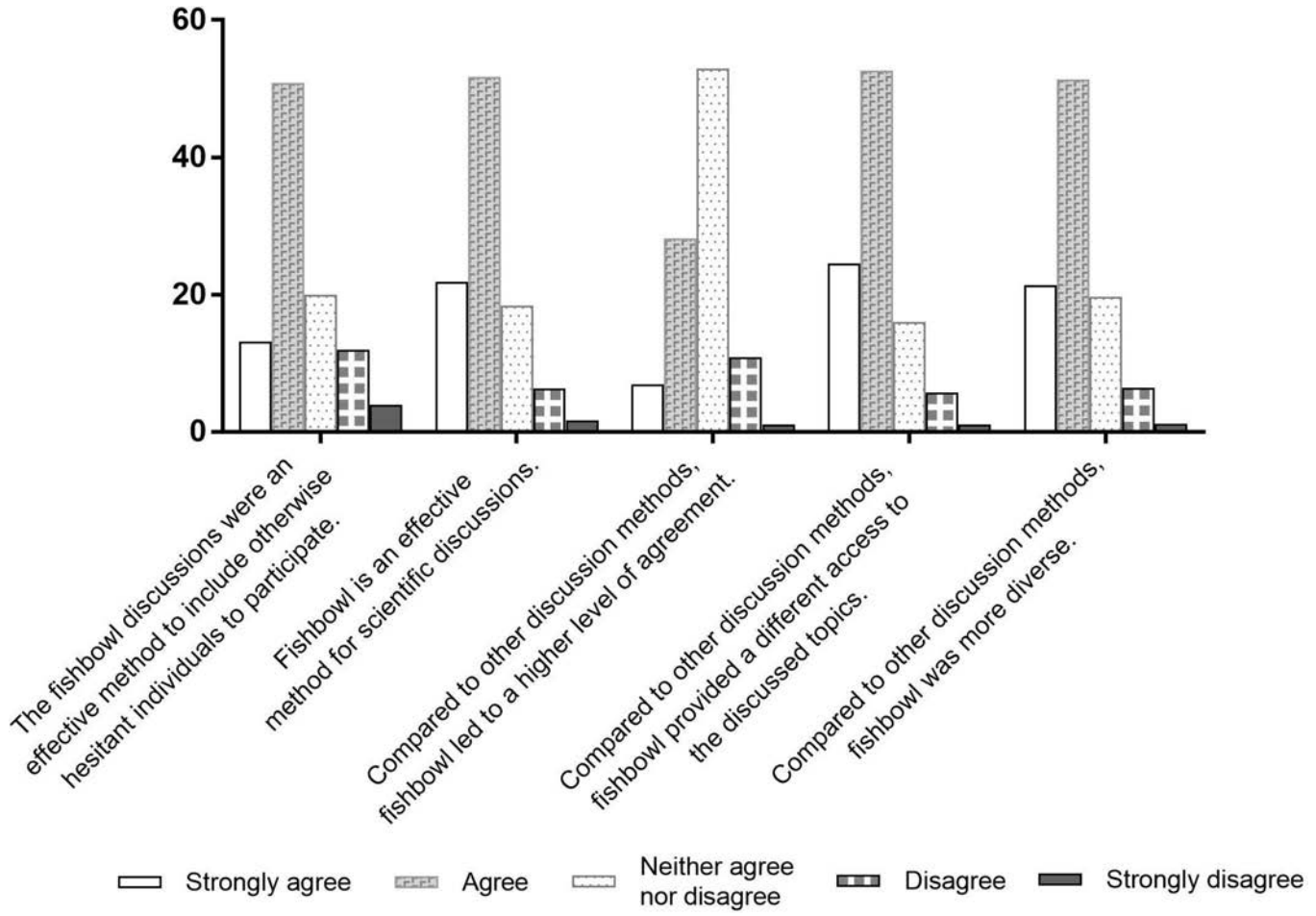

Figure 2 Levels of agreement of all participants (%).

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Lack of standardisation of ANA and implications for drug development and precision medicine

The recent article by Pisetsky *et al*[1], showing data derived from a comparison between different antinuclear antibody (ANA) assays in a cohort of patients with established systemic lupus erythematosus (SLE), addresses several important and current aspects of ANA detection. In addition, the study touches on clinical trials for ANA-associated rheumatic diseases (AARDs) and raises several relevant points that will be discussed in this letter.

WHAT IS AN ANA?

One of the fundamental questions around ANA testing is: "What is an ANA and what are diagnostic tests actually measuring?". In other words, there is no clear definition of what is and what should be included in ANA testing.[2] For example, technically antibodies to cytoplasmic antigens do not belong to ANA, but can help in the diagnosis of certain AARDs and are in some countries reported as ANAs. This is important in the context of SLE as about 15%–30% of patients with SLE have anti-ribosomal antibodies that typically present with a cytoplasmic staining pattern. However, the cytoplasmic pattern need to be clearly defined as patients with other autoimmune diseases might also present with a cytoplasmic pattern (eg, myositis or autoimmune liver disease), although with different staining pattern.[3]

METHODS FOR ANA DETECTION AND SENSITIVITY AND SPECIFICITY OF ANA TESTING

Although the ANA indirect immunofluorescence (IIF) test has been recommended as the method of choice,[4] the method is not without limitations.[5] In addition, novel solid phase assays (SPAs) have significantly improved[6] and are increasingly being used as the screening test of choice in high-throughput laboratories for the detection of ANA.[5] However, it remains a matter of debate whether or not SPA such as the multiplex used in the present study should be regarded as an ANA screen or more as a screening assay for AARD (with a clear distinction in the name). The performance of IIF versus SPA also depends on the autoimmune disease under consideration in the clinic. In two recent studies on a large population of patients, it was demonstrated that IIF on HEp-2 cells performs better for systemic sclerosis, but SPAs are superior for Sjogren's syndrome (SjS) and inflammatory myositis[7 8] Lastly, Bossuyt and Fieuws[9] showed that adding a SPA to the IIF HEp-2 testing algorithm increased the diagnostic utility for SLE, SjS (all samples on both assays) and SSc (all samples by IIF and positives by SPA). Since IIF on HEp-2 cells lacks sensitivity for several clinically relevant autoantibodies including but not limited to SS-A/Ro60, Ro52/TRIM21, ribosomal P and Jo-1[2] and SPAs contain a limited number of antigens, it is not surprising that the agreement is limited. Furthermore, between 10% and 20% of apparently healthy individuals have been reported to be ANA IIF positive with an established association with antibodies to DFS70,[2 5] as well as other unknown targets. It is not unlikely that this 10%–20% of 'false positive' rate also occurs in patients with SLE, but will be considered true positive as it fits well to the disease state. However, those patients might have a different clinical phenotype and also require different clinical care. One potential strategy is to define ANA positivity for clinical trial enrolment (and drug prescription) by a positive result in different methods (eg, SPA and IIF).

STANDARDISATION EFFORTS

Despite efforts and advances in the field of ANA test standardisation (mostly driven by the International Consensus on ANA Pattern[3]), ANA testing in clinical practice remains challenging. One major area of discussion is the screening dilution used for ANA by IIF, which is directly linked to the sensitivity of the assay. Recently, the new SLE criteria were published recommending a screening dilution of 1:80 versus the 1:40 used in the present study.[10] Using 1:80, potentially even more samples might have been negative in the study by Pisetsky *et al*.[1] Although the serum dilution is relevant, other factors such as the conjugate strength and specificity, stringency of washing steps as well as the microscope light sources and optics play an important role in the variability. The sensitivity of slides from different manufacturers differ not only in terms of the overall sensitivity butalso in regards to the analytical sensitivity of the individual ANA fine-specificities.[11] These differences are attributed to the way the cells are grown, immobilised and fixed on the slides. Ideally, monospecific patient samples and/or human monoclonal antibodies should be used in titration studies to fully assess the analytical sensitivity by fine-specificity for all manufacturers. Preliminary data are available for anti-Rib-P antibodies, which show significant inter-manufacturer sensitivity variations.[11] Besides the slides and other reagents, a very important aspect for the detection of ANA is the subjectivity in interpretation.[5] Although the study by Pisetsky *et al* used trained technicians in a single laboratory, interobserver variability was not clearly addressed, a very important factor as reported in a recent study.[12] Consequently, automated interpretation systems (available from several manufacturers) are highly recommended to reduce variability and subjectivity, which is of particular importance in a global clinical trial setting. This also facilitates unbiased image acquisition and documentation of results, which is also important for clinical trials.

REPORTED DISCORDANCE OF METHODS

The number of patients included in this study[1] was small (n=103), and no confidence intervals (CIs) for the frequency of negative results were provided, which makes it difficult to fully assess the level of disagreement of the individual assays. In more detail, ANA negativity in the 103 patients with SLE ranged widely from 4.9% to 22.3%, but was also accompanied by large 95% CIs (IIF Kit 1=22.3% (14.9%–31.1%), IIF Kit 2=9.7% (4.2%–15.8%), IIF Kit 3 4.9% (0.8%–9.2%), ELISA 11.7% (5.7%–18.3%), Multiplex 13.6% (7.3%–20.7%)). Therefore, taking into account CIs, only the difference between IIF Kits 1 and 3 showed significance. However, the key message that there is variation among ANA assays is not altered as other studies have shown similar results.[11]

LUPUS AS A HETEROGENEOUS GROUP OF SYMPTOMS

It is widely appreciated that SLE can manifest in various forms and that autoantibody profiles can subdivide patients into more homogeneous groups.[13–15] Also, it is possible that stratification of patients powered by machine learning techniques will lead to a novel, molecular-based nomenclature of disease that will likely improve patient outcome.[16] For clinical trials in patients with AARD, ANA, even if clearly defined, is unlikely to provide the full insight into meaningful disease subsets of patients who respond to a particular treatment. Along those lines, autoantibodies might not provide a robust reflection of pathogenic pathways where other biomarkers such as cytokines, inflammatory

proteins or complement components can provide further insights into potential treatment strategies.[15]

COMPANION OR COMPLEMENTARY DIAGNOSTICS

As pointed out by Pisetsky *et al*, biomarkers have significant potential to help stratify patients with SLE into more meaningful subsets and are often referred to theranostic biomarkers. From a practical and regulatory perspective, it is important to clearly differentiate between companion and complementary diagnostics. Companion diagnostics have to be included into early clinical trials and will eventually become a prerequisite for the associated drug and are therefore listed in the drug label. Since the test has to be part of the regulatory submission, only the tests that have been included might be used. By contrast, complementary diagnostics can be established after the commercialisation of a drug and 'only' rely on the in vitro diagnostic regulation.

CONCLUDING REMARKS

Taken together, the report by Pisetsky *et al* touches important aspects in the context of ANA testing and the high visibility of this article will hopefully trigger new initiatives for better understanding of the variability of ANA tests and the consequences. Ideally, those initiates should include rheumatologists, autoimmunologists, standardisation organisations as well as experts from diagnostic and pharmaceutical companies. Such an approach could lead to more precise and commutable testing, improved clinical trials, reduced healthcare expenditures and ultimately to better patient care and outcome.

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Response to: 'Lack of standardization of ANA and implications for drug development and precision medicine' by Mahler

We appreciate Dr Mahler's[1] comments on our paper, 'Assay variation in the detection of antinuclear antibodies in the sera of patients with established SLE',[2] by Pisetsky and colleagues, and the thoughtful discussion on the technical issues that affect the testing for antinuclear antibodies (ANA) by immunofluorescence assays with HEp-2 cells (IFA). We agree that our sample size was relatively small and that we did not present confidence limits on the frequency of positive responses. Rather than attempting to revalidate the assays, we designed our study to correspond to the 'real world' situation that might occur in a clinical trial or clinical practice. In the real world, whatever the purpose of ANA testing, it is likely that an IFA will be performed by a single reader on a single occasion using only one kit; our study highlights the kit issue and the variable results obtained when the same sample is assayed with multiple kits. While our study involved only one reader, reader variability is well recognised and has provided the impetus to develop less operator-dependent tests including ELISAs, multiplex bead-based assays and computer-based imaging.

As our paper and Dr Mahler's discussion indicate, testing depends on context. Indeed, there are important differences in the use of the IFA to screen for an ANA-associated rheumatic disease in the clinic, on one hand, or to subset patients with systemic lupus erythematosus (SLE) in a clinical trial, on the other. Assay variability can be problematic in both settings; we believe that assay reliability is especially relevant in the treatment setting for SLE, whether to determine trial eligibility or prescription of a medication approved for 'active autoantibody positive' disease. Dr Mahler is right to point out the differences between a companion diagnostic and complementary diagnostic. As more clinical trials for new agents for SLE incorporate the testing for ANA (and anti-DNA) to assess eligibility and to inform labelling, this difference is critical.

In view of the importance of serology in establishing eligibility of patients for clinical trials as well as product labelling, we believe that regulatory agencies need to recognise the important issues with assay variability with current ANA tests and to develop guidance on the best approach to use serology in the development of new therapies for SLE.

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'A20 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly recognised NF-kB-mediated autoinflammatory disease'

We have read with interest the article by Aeschlimann[1] and colleagues about clinical phenotypes and disease course of 16 American patients with A20 haploinsufficiency (HA20).

We would like to share our experience of a French family of three new related patients with juvenile onset Behçet's disease associated with HA20 (figure 1).

P1, a 48-year-old woman, was the first patient to be diagnosed with HA20 in July 2017 in our unit. She carries the heterozygous loss of function c.[994G>T] p.Glu332* truncating mutation, a mutation never described in the ovarian tumour domain of *TNFAIP3*. Since she was 6 years old, she had recurrent episodes of fever associated with bipolar ulcers, abdominal pain, hips and knees arthralgia, back pain, dry cough and asthenia. She was diagnosed with Behçet's disease in 2004 after a severe episode of abdominal pain with sigmoidal ulcers on colonoscopy. She also developed several anal fissures, two knee monarthritis, two lower limbs thrombophlebitis and one bilateral episcleritis. Laboratory tests showed polyclonal hypergammaglobulinaemia, elevated C reactive protein during episodes of fever and positive anti-neutrophil cytoplasmic antibodies (ANCA) without specificity.

She was initially treated with colchicine, partially and temporarily efficient. Non steroidal anti-inflammatories (NSAI) and corticosteroids were inefficient. On August 2017, we introduced a biotherapy, anti-interleukine 1 (anakinra 100 mg/day subcutaneously), which was very efficient in the first 2 weeks of treatment but was complicated by a pneumonia and lost efficacy afterwards. On October 2017, after a pneumococcal unconjugated vaccine dose, she developed high fever and a severe inflammatory swelling of the arm at site of injection that lasted 3 weeks (figure 2). She is currently receiving anti-TNFα (etanercept 25 mg/week) in association with colchicine, with moderate efficacy.

Concomitantly, her two children, P2 and P3, were diagnosed carriers of the same mutation in our unit in 2017.

P2, her 22-year-old son, had a history of recurrent fever, abdominal pain with diarrhoea and vomiting since he was 6 years old. He also developed oral recurrent ulcers and then

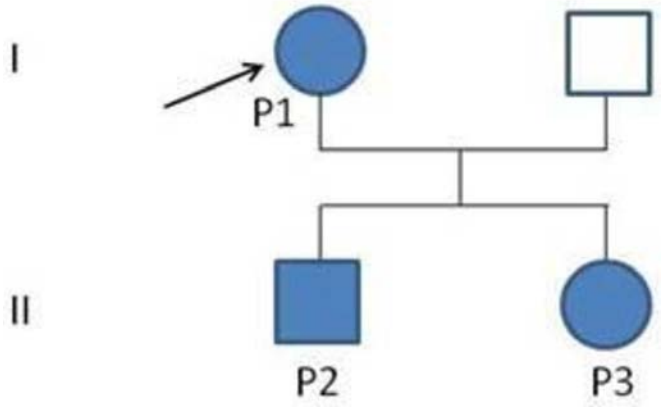

Figure 1 Pedigree of a French family diagnosed with HA20. The arrow indicates the proband. Filled in blue symbols indicate subjects carrying a p.Glu332* mutation in *TNFAIP3*. Men are indicated by squares, and women are indicated by circles.

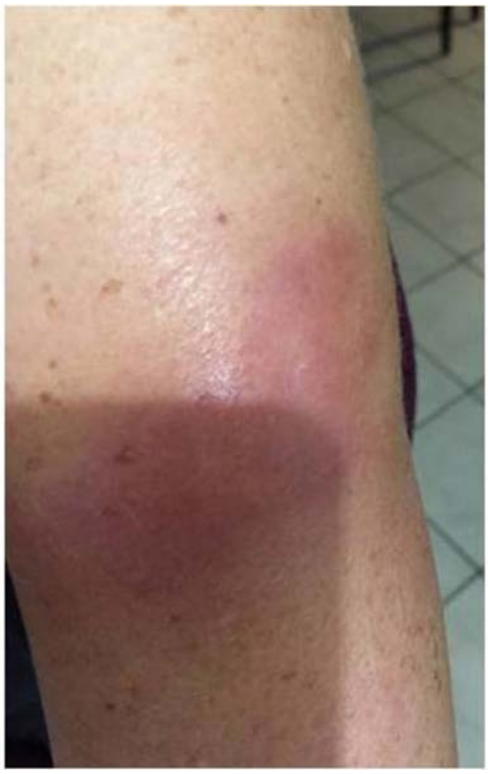

Figure 2 Photograph from the severe inflammatory swelling of the arm that occurred at site of pneumococcal unconjugated vaccine injection in patient P1.

a Hashimoto's thyroiditis and a vitiligo. He is currently treated with colchicine, with a good efficacy.

P3, her 15-year-old daughter, was diagnosed with Behçet's disease when she was 6 months old on bipolar ulcers associated with digestive disorders (abdominal pain, diarrhoea, vomiting and rectal bleeding). She also had recurrent fever, knees and hands arthralgia, several arthritis, pseudofolliculitis, urticaria and recurrent pharyngitis. She is currently treated with colchicine and mesalazine, with a good efficacy.

The family illustrates the common clinical features of this Behçet-like genetic autosomal dominant disorder recently described in association with *TNFAIP3* mutation, that is, recurrent oral and genital ulcers, digestive disorders, arthralgia/arthritis and recurrent fever starting in early childhood. Autoimmune disorders can coexist as in patient P2. Evolution of the disease is inconstant and unpredictable. Response to colchicine is inconstant and pharmacological control of inflammatory disorders can be tricky, as in patient P1.

Patients presenting with Behçet-like disease starting in early childhood, especially if there is a family history of similar symptoms, should be screened for *TNFAIP3* mutation, as clinical course and response to treatment in this genetic disorder differ from common Behçet's disease.

Florian Berteau,[1] Bénédicte Rouvière,[1] Alice Nau,[1] Rozenn Le Berre,[1,2] Guillaume Sarrabay,[3,4] Isabelle Touitou,[3,4] Claire de Moreuil[1,5]

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REFERENCE

1 Aeschlimann FA, Batu ED, Canna SW, *et al*. A20 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly recognised NF-kB-mediated autoinflammatory disease. *Ann Rheum Dis* 2018;77:728–35.

Response to: 'A20 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly recognised NF-kB-mediated autoinflammatory disease' by Aeschlimann *et al*

We thank Dr Berteau *et al*[1] for sharing their experience of a family diagnosed with HA20 based on a Behçet-like disease phenotype and an autosomal-dominant inheritance pattern.

The presentation and disease course described in the mother and her two children support the findings described in our cohort[2] and in a recent large cohort of Japanese patients with HA20.[3] An interesting observation presented by Dr Berteau *et al* is the development of high fever and a severe local inflammatory swelling following immunisation with an unconjugated anti-pneumococcal vaccine in the mother. While we did not observe such reactions in our cohort, they have also been reported in several Japanese patients with HA20[3] and in other autoinflammatory diseases such as the cryopyrin-associated periodic fever syndrome.

This letter reinforces our observations and adds a new mutation c.[994G>T] p.Glu332* to the list of already known heterozygous *TNFAIP3* mutations. In addition, it again highlights the need for clinicians to suspect HA20 in patients with Behçet-like disease phenotypes and the necessity for more research into this disease, from both clinical and basic research perspective. Of note, the patients included in our study were not all Americans, but Caucasians of Turkish, European, American and Dutch descent.

Florence A Aeschlimann,[1,2] Ronald M Laxer[1,2,3]

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Contributors RML and FAA drafted, revised and approved the response to the eLetter. The co-authors of the initial publication' A20 haploinsufficiency (HA20): clinical phenotypes and disease course of patients with a newly recognised NF-kB-mediated autoinflammatory disease' by Aeschlimann *et al* (ARD 2018) all approved the submitted response to the eLetter.

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Postpartum breastfeeding status

According to the abstract,[1] it does not appear that the postpartum woman's lactation status was taken into account. So I am wondering, couldn't their status alter the findings, given some of the major hormonal, immune function, and metabolic differences between woman that exclusively breast feed vs formula feed?

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REFERENCE

1 Eudy AM, Siega-Riz AM, Engel SM, *et al*. Effect of pregnancy on disease flares in patients with systemic lupus erythematosus. *Ann Rheum Dis* 2018;77:855–60.

Response to: 'Postpartum breastfeeding status' by Betzold

Thank you for your question[1] regarding our article 'Effect of pregnancy on disease flares in patients with systemic lupus erythematosus'.[2] We did not have data available on lactation status for women during the postpartum period, and we were unable to account for this in our analysis. We agree that it could be an important factor that may influence disease activity during this time period. Previous studies have found a positive association between plasma/serum prolactin levels and disease activity among patients with lupus.[3 4] It is possible that the natural increased levels of prolactin during pregnancy and while breast feeding[5 6] may help explain our finding of increased disease activity during pregnancy and a 3-month postpartum period. Even though we were unable to fully explore this hypothesis, our results did indicate that use of hydroxychloroquine may help reduce the risk of flare during pregnancy and post partum. Future studies could explore the effect lactation has on postpartum flare for hydroxychloroquine users and non-users, as well as analyse how prolactin levels may explain the increased risk of flare during pregnancy and post partum.

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'Evaluation of the impact of concomitant fibromyalgia on TNF alpha blockers' effectiveness in axial spondyloarthritis: results of a prospective, multicentre study' by Moltó *et al*: still a long way to go in the assessment of patients with spondyloarthritis and concomitant fibromyalgia?

We read with great interest the study published by Moltó *et al* on the impact of concomitant fibromyalgia (FM) on tumor necrosis factor (TNF) alpha blockers' effectiveness in axial spondyloarthritis (axSpA).[1] Indeed, this is a challenging problem in daily practice, especially considering the difficulties in differentiating enthesitis and FM symptoms.[2] Therefore, we would like to raise some issues that need clarification in order to better understand the relevance of the study.

In the published paper, data on the history of antidepressant, third-ladder analgesic and nonsteroidal anti-inflammatory drug (NSAID) intake in patients enrolled are extensively reported. The results indicate that the use of antidepressants was significantly greater in patients with FM according to FiRST,[3] American College of Rheumatology (ACR) 1990 criteria[4] and sustained FiRST. However, no data are reported regarding the outcome of these therapies on FM symptoms, which could be evaluated by using the symptom severity score.[5] We believe that this issue could significantly impact the patient-reported outcomes.

We also consider of great relevance the stratification of patients with axSpA in terms of presence or absence of chronic damage. In the paper, the authors report data on X-ray and MRI sacroiliitis. This classification implies the inclusion in the study of different subgroups of patients with axSpA, since those with X-ray sacroiliitis are likely to be patients with ankylosing spondylitis (AS) with a longer disease duration, while patients with MRI sacroiliitis might have been affected by non-radiographic axSpA. This observation deserves attention based on the finding that patients with established AS may fulfil FM criteria more often than patients with non-radiographic axSpA, probably due to the severity and duration of chronic pain.[6] This aspect should be considered when evaluating the response to TNF alpha blockers, being able to affect patient-reported outcomes.

Finally, the results of the study show a higher percentage of the history of peripheral enthesitis in patients with FM. However, the authors do not specify how the enthesitis was diagnosed (ie, by clinical evaluation or imaging tools) and in which site.[7] Again, this is a crucial point since patients with FM experience widespread pain and have tender points that could simulate enthesitis symptoms if detected only on the basis of clinical examination in the absence of an instrumental assessment[8] (see online supplementary table S1).

In conclusion, we appreciate the issue addressed by the authors in their paper, which provides precious information for a more aware treatment of patients with axSpA and concomitant FM. We believe that an answer to our comments would help readers better understand the relevance of this study. Certainly, it is important to face the challenge of a correct interpretation of disease activity indexes including patient-reported outcomes in patients with axSpA and concomitant FM in order to avoid unwarranted use of medications.

Alessio Altobelli, Rossana Scrivo, Guido Valesini

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Response to: '"Evaluation of the impact of concomitant fibromyalgia on TNF alpha blockers' effectiveness in axial spondyloarthritis: results of a prospective, multicentre study" by Moltó *et al*: still a long way to go in the assessment of patients with spondyloarthritis and concomitant fibromyalgia?' by Altobelli *et al*

We would like to thank Altobelli *et al*[1] for the interest they have expressed in our recently published article 'Evaluation of the impact of concomitant fibromyalgia on tumor necrosis factor (TNF) alpha blockers' effectiveness in axial spondyloarthritis (axSpA) : results of a prospective multicentre study'[2] and the ARD editorial team to give us the opportunity to address their comments in this present letter.

First, we would like to emphasise that the purpose of this study was not to address the effectiveness of non-steroidal anti-inflammatory drugs (NSAIDs,) antidepressants or third-ladder analgesics, but to evaluate the effectiveness of TNF alpha blockers in an axSpA population and the potential impact of a concomitant fibromyalgia on such effectiveness. We agree that the evaluation of these other coprescription in patients with concomitant fibromyalgia would be of great interest, but this was not the aim of this study. Furthermore, the symptom severity score was not collected in this study.

Second, regarding their comment on stratification based on disease characteristics (in particular on radiographic and MRI sacroiliitis) when evaluating treatment effect, we would like to thank our colleagues for this comment. Indeed, this is exactly what was performed when analysing treatment effect on the multivariable analysis. First, we explored whether patients with/without concomitant fibromyalgia presented with different disease characteristics, and as reported in table 1 of the manuscript, we did not find any differences with regard to radiographic or MRI sacroiliitis when fibromyalgia was defined by the Fibromyalgia Rapid Screening Test (FiRST). However, some differences were observed for the ACR 1990 criteria and the Sust-FiRST definitions. Disease duration was not different across groups, regardless the definition. Nevertheless, since radiographic and MRI sacroiliitis have been consistently reported across studies as factors associated with treatment response, we included these variables in the multivariable model to assess the impact of fibromyalgia on the TNF blockers treatment effect, that is, the reported results of treatment effect are indeed adjusted by the presence/absence of both radiographic and MRI sacroiliitis, along with other factors (summarised in figure 2 of the manuscript) known to be associated with treatment response (ie, age, male gender, HLAB27+, smoking, elevated C-reactive protein (CRP), TNF blocker previous exposure).

Finally, we would like to thank our colleagues for their comment regarding on how enthesitis was assessed. Indeed, the potential overlap of axSpA-related pain at the entheseal sites and positive trigger points for fibromyalgia is a real concern in clinical practice. In the manuscript, as described in methods, we reported the history of peripheral enthesitis collected by clinical history during a face-to-face interview at the study visit by rheumatologists with an expertise in axSpA evaluation. Precisely, during the study visit, rheumatologists were asked to perform a physical examination to determine the presence/absence of tenderness on examination of 31 points. It was (on purpose) not clearly separated in the case report form that these 31 points included both the axSpA enthesitis points (13, according to the MASES index[3]) and the classic fibromyalgia trigger points (18 points).[4] The evaluation of the agreement/overlap of both assessments is the objective of an ongoing ancillary analysis.

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Reference level of serum urate for clinically evident incident gout

I read the interesting study entitled 'Relationship between serum urate concentration and clinically evident incident gout: an individual participant data analysis' conducted by Dalbeth and colleagues.[1] Their study showed the cumulative incidence of gout was increased with the serum urate levels and the cumulative years. The reference level of serum urate below which the risk of damage is low has not been completely clarified. The benchmark dose (BMD) method, first described by Crump in 1984,[2] has been widely used in the field of health risk assessment. BMD is defined as the exposure level corresponding to a predetermined increase in the probability of an adverse response (eg, 1%–10%) above the background level.[3] The BMD method uses all dose-response data from a study.[4] The BMDL (lower confidence limit of BMD) has an advantage compared with the no observed adverse effect level or low observed adverse effect level.[3 5] Dalbeth and colleagues[1] have shown the cumulative incidence of clinically evident incident gout was increased with the increase of serum urate. We roughly calculated the BMDL in men (table 1) by using gamma model (benchmark response=1%) based on the data in table 2 (the doses of baseline serum urate were set as 5, 6.5, 7.5, 8.5, 9.5 and 11 mg/dL). The reference levels of serum urate were 7.16 mg/dL by 3 years, 6.86 by 5 years, 6.02 by 10 years and 5.49 mg/dL by 15 years, respectively. For those subjects with serum urate <7.16 mg/dL, their risk of gout was low 3 years later. However, we did not have the exact data of serum urate. It would be very interesting if they can calculate the BMDL of serum urate in men and women at different cumulative years, which may be helpful in the management of gout.

Xiao Chen,[1,2] Xiaoqiang Ding[1]

Table 1 The benchmark dose (BMD) and lower confidence limit of BMD (BMDL) of serum urate in men at different cumulative years

	3 years	5 years	10 years	15 years
BMD (mg/dL)	7.37	7.03	6.28	5.81
BMDL (mg/dL)	7.16	6.86	6.02	5.49

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Patient consent Detail has been removed from this case description/these case descriptions to ensure anonymity. The editors and reviewers have seen the detailed information available and are satisfied that the information backs up the case the authors are making.

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Response to: 'The reference levels of serum urate for clinically evident incident gout' by Chen and Ding

We thank Drs Chen and Ding[1] for their interest in our recent paper describing the relationship between serum urate concentrations and risk of developing incident gout.[2] They suggest that we calculate benchmark dose estimates for serum urate and gout risk. Benchmark dose estimates are used in the field of occupational epidemiology to evaluate the minimal levels of exposure to an environmental toxin needed to cause a prespecified increase in an adverse event.

We are not aware of this approach being applied to clinical parameters that are not external exposures and are uncertain about validity of such an approach for serum urate, noting that all humans have some 'exposure' to urate, which is a circulating biochemical analyte. Estimation of the benchmark dose also requires a predetermined increase in risk (eg, 10% extra risk or change in the mean equal to one SD). At present, the clinically meaningful increase in gout risk is unknown. For these reasons, we have not provided benchmark dose estimates.

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Response to: 'Statins in systemic lupus erythematosus' by Abud-Mendoza

It was with great interest that we read the correspondence of Abud-Mendoza[1] on our recent paper in which we described a decreased risk of developing systemic lupus erythematosus (SLE) in statin users who continued their therapy for >1 year.[2]

We agree that prevention of cardiovascular disease in rheumatic diseases is of great importance.[3] Whether statins decrease disease activity in SLE is, however, controversial since a recent meta-analysis of five controlled trials did not suggest any significant effect of statin therapy on Systemic Lupus Erythematosus Disease Activity Index.[4]

Unfortunately, in the UK's Clinical Practice Research Datalink (CPRD)—an ongoing primary care database of anonymised medical records from general practitioners that was used in our study—no measurements for SLE activity before or after initiating statin therapy are available.[2] We, however, do not think that statin therapy is superior to hydroxychloroquine (HCQ) as therapy to reduce relapses and thrombotic events in SLE. HCQ does not only prevent relapses in SLE but also has anti-atherogenic effects and is, in contrast to statins, associated with a reduced risk of development of diabetes mellitus.[5–7]

Abud-Mendoza wondered whether inclusion of patients <40 years changed our findings.[1] When we included these patients and excluded patients with SLE before the index date, we identified 539 431 statin users and 539 431 non-users after using a matched random sampling approach (1:1). The index date ('baseline') was defined as the date of the first prescription of a statin; that is, 'statin user'. Each statin user was matched to one control ('non-user') based on age, sex and general practice at index date, with the index date of the control being the same as that of the statin user. The characteristics at baseline are presented in table 1 and are in line with the characteristics that have been shown in Table 1 in our paper.[2] Statin users and non-users had similar distributions of age (statin users: mean age, 62.7 years; and non-users: 61.9 years) and sex (statin users and non-users: 47.7% women). In our study population aged ≥16 years, the incidence rate was the same as the incidence rate in our recent study,[2] 0.7 cases per 10 000 person-years.

Compared with our previous findings, we found similar associations between statin use and the risk of SLE, only slightly attenuated. Among patients aged ≥16 years, current statin users had a risk of developing SLE which was comparable to that of non-users ($HR_{adjusted}$, 0.81; 95% CI 0.57 to 1.15). Moreover, current statin users who continued therapy for >1 year had a 34% decreased risk of developing SLE ($HR_{adjusted}$, 0.66; 95% CI 0.44 to 0.98) (table 2).

Finally, Abud-Mendoza wondered whether we had information regarding adverse events related to statins.[1] Since our study objective was to assess the association between the statin use and the risk of SLE, we had no access to other study outcomes than SLE. However, several population-based studies using CPRD data have found adverse events of statins such as rhabdomyolysis and cataract.[8 9]

We conclude that statins are probably safe in SLE but that more research is needed to assess the benefit/risk profile of statins in other autoimmune rheumatic diseases such as polymyalgia rheumatica.[10]

Table 1 Baseline characteristics of statin users and non-statin users aged ≥16 years

Baseline characteristics	Statin users (n=539 431)	Non-users (n=539 431)
Duration of follow-up (years)		
Mean (SD)	4.5 (3.4)	4.1 (2.6)
Sex, n (%)		
Female	257 202 (47.7)	257 202 (47.7)
Age (years)		
Mean (SD)	62.7 (12.7)	61.9 (13.5)
Age by category, years (%)		
≤59	238 092 (44.1)	252 672 (46.8)
60–79	242 331 (44.9)	221 013 (41.0)
80+	59 008 (11.0)	65 746 (12.2)
BMI (kg/m^2)		
Mean (SD)	27.3 (7.8)	21.0 (11.6)
Unknown BMI	29 566 (5.5)	111 025 (20.6)
Smoking status, n (%)		
Non-smoker	224 945 (41.7)	242 946 (45.0)
Ex-smoker	168 229 (31.2)	113 898 (21.1)
Smoker	122 289 (22.7)	106 473 (19.8)
Unknown smoking status	23 968 (4.4)	76 114 (14.1)
Drinking status, n (%)		
Non-drinker	68 056 (12.6)	56 286 (10.4)
Ex-drinker	33 857 (6.3)	21 352 (4.0)
Drinker	370 711 (68.7)	333 313 (61.8)
Unknown drinking status	66 807 (12.4)	128 480 (23.8)
Drug use within previous six months, n (%)		
Antihypertensive agents	329 228 (61.0)	124 612 (23.1)
Fibrates	8960 (1.7)	903 (0.2)
Ezetimibe	2077 (0.4)	133 (0.02)
Antidiabetic agents	129 816 (24.1)	18 793 (3.5)
Aspirin	146 641 (27.2)	36 973 (6.9)
Anti-arrhythmic agents	20 961 (3.9)	11 436 (2.1)
NSAIDs	205 971 (38.2)	89 882 (16.7)
Proton pump inhibitors	87 041 (16.1)	48 796 (9.1)
Hormone replacement therapy or oral contraceptives	21 958 (4.1)	21 150 (3.9)
Oral corticosteroids	18 098 (3.4)	15 701 (2.9)
Antibiotics	49 306 (9.1)	37 394 (6.9)
Anticonvulsants	11 401 (2.1)	8282 (1.5)
Antipsychotics	5896 (1.1)	6291 (1.2)
Antidepressants	120 425 (22.3)	98 630 (18.3)
History of disease ever before, n (%)		
Hypertension*	329 257 (61.0)	124 621 (23.1)
Hyperlipidaemia	160 221 (29.7)	12 839 (2.4)
Diabetes†	130 198 (24.1)	18 962 (3.5)
Cardiovascular diseases	176 908 (32.8)	47 839 (8.9)
Cerebrovascular disease	60 552 (11.2)	17 110 (3.2)
Cancer	35 380 (6.6)	40 220 (7.5)
Psoriasis	20 821 (3.9)	17 095 (3.2)
Inflammatory bowel disease	5298 (1.0)	5297 (1.0)
COPD	21 165 (3.9)	20 866 (3.9)
Asthma	64 470 (12.0)	55 677 (10.3)
Dementia	5079 (0.9)	8611 (1.6)
Depression	75 507 (14.0)	50 671 (9.4)

*Diagnosis of hypertension or use of antihypertensive agents.
†Diagnosis of diabetes mellitus or use of antidiabetic therapy.
BMI, body mass index; COPD, chronic obstructive pulmonary disease; NSAIDs, non-steroidal anti-inflammatory drugs.

Table 2 Risk of systemic lupus erythematosus (SLE) in statin users compared with non-statin users aged ≥16 years

	SLE (n)	IR*	Age and sex-adjusted HR (95% CI)	Fully adjusted HR (95% CI)†
No statin use	98	0.6	1.00	1.00
Past statin use	24	1.0	1.70 (1.08 to 2.66)	1.39 (0.86 to 2.23)
Recent statin use	21	1.1	1.66 (0.99 to 2.78)	1.32 (0.76 to 2.28)
Current statin use	124	0.6	1.04 (0.78 to 1.38)	0.81 (0.57 to 1.15)
≤1 year	70	2.0	1.43 (0.97 to 2.10)	1.12 (0.73 to 1.72)
>1 year	54	0.3	0.86 (0.62 to 1.21)	0.66 (0.44 to 0.98)

*Incidence rate is calculated for each recency of statin use by dividing the number of events by the person time within each given recency of use.
†Adjusted for age, sex, practice, smoking, cardiovascular diseases, hyperlipidaemia, hypertension, diabetes and use of non-steroid anti-inflammatory drugs.
IR, incidence rate (per 10 000 person-years).

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Inconsistency between Danish incidence and prevalence data about psoriatic arthritis (PsA)

We are grateful to Egeberg and Kristensen for presenting the detailed data about the prevalence and incidence of psoriatic arthritis (PsA).[1] Based on these detailed information, we tried to estimate the excess mortality of people with diagnosed PsA by uising a mathematical relation between incidence, prevalence and mortality.[2 3] During analysis of the incidence and prevalence data, we have made the following observation: if we assume that—on population average—people with PsA do not have a better survival than those without PsA, we can compute a lower bound for the incidence rate from the prevalence data (the details for the derivation of the lower bound can be found in the Appendix). We calculated this mathematical lower bound based on the prevalence data and compared the lower bound with the incidence data given in Ref 1. We found that in less than 50% of the strata where incidence data were given, the corresponding mathematical lower bounds have been reached (or exceeded). For instance, the lower bound for the incidence rate in the age group 40–49 in 2009 is 43.3 per 100 000 person-years (both sexes). The observed incidence rate in this stratum is only 29.8 per 100 000 person-years—a deviation of more than 30%. More than half of the reported incidence rates stratified by age and year are implausibly small given the observed prevalence values. Unfortunately, we do not have an explanation for the inconsistencies between the incidence and prevalence data. Possibly, in estimating the age-specific prevalence, some double counting of cases has occurred.

Appendix: Deriving a lower bound for the age-specific incidence rate

Mathematically, it can be shown that

$$\partial p = (1 - p) \times \{i - p \times (m1 - m0)\}$$

where ∂p is the temporal change of the age-specific prevalence p with respect to time and age.[1 2] The rates i, m0 and m1 are the age-specific incidence and mortality rates of the people with (m1) and without diagnosed PsA (m0).

A straightforward calculation yields that

$$\partial p/(1 - p) + p \times (m1 - m0) = i.$$

With the assumption that the mortality rate of the people without PsA is not higher than the mortality of the people with PsA, that is, $m0 \leq m1$, this equation implies

$$\partial p/(1 - p) \leq i. \quad (1)$$

This means the incidence rate (i) is always greater than the temporal change (∂p) of the prevalence over 1 minus the age-specific prevalence. Thus, we have a lower bound for the incidence rate.

The question arises, if the assumption $m0 \leq m1$ is reasonable on the population level (here: Denmark). The main reason for this assumption being true (on the population level) is that PsA often is a severe disease coming along with severe side effects and disease-specific complications. Hence, it appears reasonable to us that equation (1) yields a lower bound for the incidence rate.

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Detection of myositis-specific antibodies: additional notes

With interest we read the recent article by Vulsteke *et al*[1] showing data derived from an evaluation of three immunoassay systems for the detection of autoantibodies associated with autoimmune inflammatory myopathies (AIM). As stated by the authors, careful evaluation of autoantibody assays for the detection of myositis-specific (MSA) and myositis-associated (MAA) antibodies is of utmost importance since some of these are included or being considered for the AIM classification criteria.[2–4] The biomarkers are also relevant for establishing the diagnosis and stratification into specific disease subsets.

The authors compared the performance of three test systems and used primarily clinical diagnoses and features as comparators. In the interests of assay evaluation and standardisation, it is valuable to also provide data showing a more comprehensive statistics-based approach for method comparison. However, this might be linked to the small number of AIM patients tested (n=144) and the small number of positive cases for many of the markers, which represents a limitation of this evaluation and most other studies on MSA and MAA. Although some clinical associations yield statistical significance using P values, verifying significance might be relevant by using Benjamini-Hochberg or Bonferroni correction.

When performing clinical evaluations on AIM, two important aspects to consider are the relatively low prevalence of most MSA and the composition of the control population. Although the differential diagnosis of other systemic autoimmune rheumatic diseases (SARD) is important, there are some challenges. When considering patients with SARD as controls, it is important to rule out overlap syndromes.[5] One example is the association of AIM with interstitial lung disease, which can occur in myositis and in other SARD and especially systemic sclerosis.[6] The differences observed for anti-Jo-1 antibodies are surprising and concerning since those antibodies have been measured for many years,[5] and proficiency testing programmes have shown mostly consistent results (eg, https://www.immqas.org.uk).

Historically, most of the clinical associations of MSA and MAA have been established using immunoprecipitation (IP). Consequently, it is important to also compare newer technologies such as line immunoassays (LIA) and dot blots (DB) with IP, as also stated by Lundberg *et al*.[3] Of relevance, in a recent study comparing LIA and IP, poor agreement was found for several MSAs.[7] This observation does not imply that IP is correct in all instances or that IP should be regarded as the 'gold standard', however, such inter-technology comparative data are invaluable.

To address the significant subjectivity of interpreting LIA and DB assays, automated scanning systems have been developed and introduced for LIA and DB.[8 9] A 'semi-quantitative' approach using scanning systems allows for the analysis of discrepant results considering the antibody levels (titres). One significant limitation of LIA and DB is the lack of analyte specific controls and proper calibration. Consequently, studies of run-to-run and also lot-to-lot variability are required to assess the reliability of the assays and to exclude inter-manufacturer variability that may be attributed to limited precision and reproducibility. Ideally, those studies should contain sufficient samples around the cut-off and follow Clinical and Laboratory Standards Institute guidelines (https://clsi.org/). Along those lines, a close collaboration between patient groups, research networks and kit manufacturers is mandatory to make serum samples available for calibration and quality control. An alternative approach is the generation of human or humanised monoclonal antibodies that can be used in a similar manner. In conclusion, we thank the authors for conducting this study and encourage future studies with larger patient cohorts (such as the MyoNet or EuroMyositis) that will eventually provide sufficient evidence to include more MSA into the classification criteria.

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