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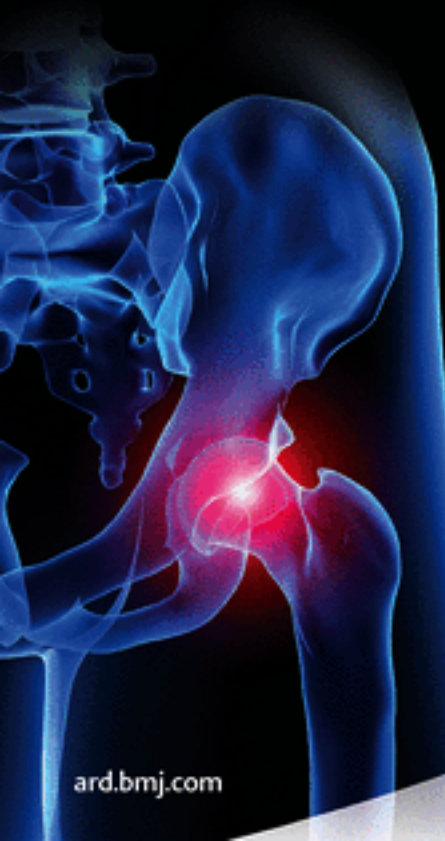

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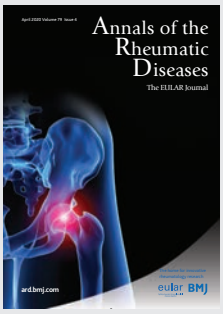

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Heroes and pillars of rheumatology

Viewpoint

Rheumatoid arthritis

Psoriatic arthritis

MORE CONTENTS ►

Systemic sclerosis

Sjögren's syndrome

Osteoarthritis

Crystal arthropathies

Letters

Electronic pages

Gerald Weissmann: Inflammation in rheumatic disease

Bruce N Cronstein,[1] Jill P Buyon,[2] Steven B Abramson [3]

Handling editor Josef S Smolen

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Gerald Weissmann (1930–2019), former President of the American College of Rheumatology (ACR), was at the forefront of a generation of rheumatologists/scientists who transformed the study of the rheumatic diseases from a largely descriptive effort to a hypothesis-driven scientific endeavour. Born in Vienna, his family left Austria in 1938, during the rise of Nazism in Europe, and settled in New York City, where his father resumed his practice of medicine. Through his remarkable scientific career, Gerry Weissmann carved an indelible mark on the field of rheumatology serving as chief of the Division of NYU's Langone Medical Center (1973–2000), training scores of physicians and scientists. To all, he was an exemplar of scientific rigour and unbounded enthusiasm for scholarship, whose legacy continues to influence numerous disciples throughout the world. For all of these attributes, Gerald Weissmann is an academic 'hero'.

Over his 50-year scientific career students, clinicians and scientists sought out Dr Weissmann as a mentor. With his scientific rigour, creativity and, on occasion, acerbic wit, Dr Weissmann introduced a generation of students to the practice of experimental science. He was an inspiration to many aspiring rheumatologists; the excitement of his scientific approach and the changes and developments in the clinic attracted a large number of able trainees. He influenced both at bench and bedside, the latter enforcing on medical rounds the relevance of ear cartilage resilience to general health. Many of us fondly recall Dr Weissmann's trademark purple magic marker which repeatedly circled all the nouns we mistakenly used as adjectives. Surely as his mentees we will always be indebted to him for teaching us to deliver a cogent lecture and to make the message clear to all willing to listen. Colour on his black background slides was the pointer that never shakes. This was a precious gift he handed down.

In choosing rheumatology fellows for his programme, his trademark was to query the last book the applicant read. While we often found this quirky, it was quintessential Weissmann, listening and gathering information on us as people and potential future leaders. In illustrating Dr Weissmann's honest and probing nature, we share an anecdote handed down over the years. In 1982, an applicant sat in front of him during the fellowship interview, in hopes of being chosen one of the lucky three among over 100 applicants. Imagine how it felt when the candidate was boldly told that her essay was wonderful and it was great to be studying complement and lupus and that she had incredibly legible handwriting (no computers then). Dr Weissmann then sat back with his special grin, and said, oh by the way only my compliments to you are spelled with an 'i'. With measured horror, it became clear that the bullets of immunological defence and autoimmune injury had been misspelled, the 'e' substituted. Despite this rather jarring interview, Dr Weissmann must have trusted his instincts, as he picked her—and she never forgot the lesson of the experience: details count, even to a single letter.

As a scientist, Dr Weissmann focused on understanding neutrophil physiology and pharmacology and the role of neutrophils in the rheumatic diseases. Dr Weissmann's scientific work on neutrophils led to the hypothesis, and demonstration, that immune complexes play an important role in the pathogenesis of the rheumatic diseases and rheumatoid arthritis in particular.[1] He postulated that much of the tissue injury that occurred during inflammation resulted from the release of reactive oxygen species, proteolytic enzymes and other reactants after neutrophils and other cells engulfed otherwise insoluble inflammatory stimuli such as crystals and immune complexes; he termed this process 'regurgitation on feeding'.[2] At a basic level, Dr Weissmann also conceived the general hypothesis that cAMP suppressed the inflammatory actions of neutrophils and other cells and later showed, in animal models, that, although prostaglandins are critical mediators of inflammation, some prostaglandins suppress inflammation in vitro and in vivo[3] and that at a cellular level this is mediated by stimulation of cAMP accumulation.[4] By inhibiting cAMP hydrolysis, phosphodiesterase inhibitors diminish inflammation and these observations ultimately led to the development of phosphodiesterase inhibitors, for example, apremilast, for the treatment of rheumatic diseases. Dr Weissmaann also authored many pioneering studies on leucocyte activation and the role of salicylates and corticosteroids in regulating cell signalling responses. In our works with Dr Weissmann, we explored the mechanism by which monosodium urate crystals incited neutrophil inflammation[5] and colchicine treated and prevented neutrophil-mediated inflammation[6] as well as the effect of hormones on neutrophil function.[7]

The study of inflammation and the rheumatic diseases was not the only area of research to which Dr Weissmann contributed. In the early 1960s, while on sabbatical at Cambridge, Dr Weissmann worked with multilamellar lipid vesicles as a model of cellular plasma and lysosomal membranes. Dr Weissmann named these vesicles 'liposomes',[8] a term that remains in use today and for which he is acknowledged in the Oxford English Dictionary.

Figure 1 Dr Weissmann sharing awards with Lewis Thomas and Nobel Prize winners, John Vane and Bengt Samuelsson, at inflammation conference in Bologna Italy, c1985 (photo left to right: Lewis Thomas, Gerald Weissmann, John Vane, Bengt Samuelsson).

More importantly, he recognised that liposomes have great potential as a drug-delivery vehicle. He and his colleagues founded the Liposome Company which commercialised liposomal preparations of amphotericin B and daunorubicin for the treatment of fungal diseases and cancer, respectively, that retained their efficacy while dramatically reducing their toxicity. There are now a number of other liposomal drugs in the clinic as well.

For these works, Dr Weissmann received numerous distinctions and awards. He was a fellow of the American Association for the Advancement of Science and the Royal Society of Medicine. He was elected in 2002 to Galileo's Accademia Nazionale dei Lincei of Rome, the world's oldest scholarly society. He also received the ACR's highest honours: the Distinguished Basic Investigator Award, the Presidential Gold Medal and the Lifetime Achievement Award.

Dr Weissmann had notable accomplishments outside of medicine and the scientific study of rheumatic diseases. At the age of 19, his artwork was displayed in a prominent New York City gallery and was favourably reviewed in the New York Times (December 17, 1949). He was convinced to leave the artworld for a life in medicine after attending a lecture on the use of corticosteroids to treat Rheumatoid Arthritis but maintained a strong interest in the world around him and he turned his observations on life, politics and biology into learnt essays. Starting with The Woods Hole Cantata, published in 1985, and his final work The Fevers of Reason (2018), he published 11 volumes of essays which were widely praised for their style and the breadth of their culture.

Conferences, laboratory meetings and conversations with Dr Weissmann could often be extraordinary experiences; long cherished hypotheses were discarded when the experimental data did not support them. When he was present in the audience, one could always anticipate a challenging interchange regardless of the topic. Most lecturers left the podium with an idea they had not previously considered. Indeed, Dr Weissmann was always the most creative person in the room in his ability to propose new hypotheses and the experiments required to test their validity. We as his direct disciples will always carry with us the imprint of his rigorous thinking—as will many colleagues worldwide who were impacted by his intellect. And in turn as we influence the next generation of rheumatologists, there is no doubt that Dr Weissmann's wisdom will be continuously passed on maintaining a life of its own.

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What is the real role of ultrasound in the management of juvenile idiopathic arthritis?

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The use of musculoskeletal ultrasonography (US) in the management of rheumatoid arthritis (RA) has drawn attention to paediatric rheumatologists, and this has become an important part of the clinical care of patients with juvenile idiopathic arthritis (JIA) as well. However, several points have to be addressed before drawing conclusions on the usefulness in the everyday clinical management of JIA.

First, even in RA a similar viewpoint[1] argued against the use of US for directing treatment decisions. In fact, many pitfalls have been highlighted by the authors, including the meaning of grey scale and/or power Doppler signal as sign of active inflammation versus past synovitis, the presence of abnormal US findings in a sizeable percentage of healthy subjects, the technical differences between different machines and the lack of standardisation. Indeed, the authors pointed out that in prospective therapeutic clinical trials with a Treat to Target (T2T) strategy following patients with US was not needed.

If we go to square one, it needs to be emphasised that in a growing skeleton the imaging results can be quite different than in adults. In fact, in children physiological US findings might be misinterpreted as pathologic. Skeletal maturation has a profound effect on imaging results, since bone, cartilage and adjacent structures undergo continuous modifications with growth. Normal US appearance of paediatric joints should be the starting point for any subsequent definition of pathology, and in particular for studies in JIA.

In order to standardise the use of US in paediatric rheumatology, definitions for US findings in the different parts of the normal paediatric joint have been developed and validated through Delphi process in different publications.[2 3] A systematic standardised examination method was proposed, and an atlas of images of normal joint appearance at different ages has accordingly been created. Subsequently, the Outcome Measures in Rheumatology (OMERACT) task force for paediatric US described the vascularity in normal joints and amended the B-mode definition when using Doppler technique.[4] Importantly, physiological vascularity can be detected by power Doppler in healthy children at any age during growth. The sonographer, therefore, has to be experienced in order to distinguish physiological from pathological findings; for example, feeding vessels can be recognised by their direct trajectory into the bone/cartilage. The OMERACT subtask group analysed the ability of experienced international ultrasonographers to detect normal physiological vascularisation in 12 healthy children of different ages. They identified physiological vascularisation in all children and in all US scans, finding physiological vessels at the physis or epiphysis of long bones, in the intracartilaginous regions of the small bones and patella, and in the fat pad. Moreover, they developed a semiquantitative scoring system for assessing the grade of maturation of ossification nuclei in healthy children.[5] The creation of image acquisition protocols for normal paediatric hip,[6] knee,[7] elbow[8] and wrist[9] has helped in performing US scannings in children. Other factors influencing the correct interpretation of results in children include small joint size with slow blood flow, restless children and effect of transducer pressure in small paediatric joints. However, all these potential pitfalls can be overcome by skilled and trained examiners.

In our opinion, a very important point is also the relative contribution of US in the diagnosis of synovitis, especially if compared with physical examination. There have been numerous studies evaluating this issue,[10–20] and many claimed that US is superior to physical examination. However, when examining these studies in detail, many difficulties arise. First of all, numbers are always very small. Second, US was performed by rheumatologists, radiologists and paediatric rheumatologists alike, with very different experiences. Moreover, as recently pointed, the US definition of synovitis cannot rely only on the presence of Doppler signals, since these need to be detected within an area of abnormal synovium,[21] and flow can be detected within a joint but outside the synovium, giving rise to false positive results.

But above all, it is not clear who would judge if clinical examination or US might be more precise in detecting joint inflammation. Indeed, confidence and competence in musculoskeletal examination is sometimes poor, and the deficiency in these skills is most apparent in the paediatric setting.[22–26] Of note, previous experience demonstrated a lack of concordance in joint counts even among very experienced paediatric rheumatologists.

A systematic literature review on the assessment of synovitis in JIA has been published.[27] Only 20 studies could be included, and the sample size was always small (mean 32 patients per article). MRI was included as a comparator only in four of these studies, and technical aspects (ie, machine brands, probe position descriptions and frequency of linear transducers) were variable. Moreover, less than half included a control group, which as previously described is an essential requirement especially in the paediatric age. None of the articles examined criterion validity, reliability was variable and quality assessment in most articles did not achieve a sufficient score.

The detection of synovitis is particularly important also considering the fact that current

International League of Associations for Rheumatology (ILAR) classification still includes the number of active joints as discriminator between JIA categories, and misclassifications can, therefore, occur. We remind that American College of Rheumatology (ACR) paediatric core set criteria are also based on number of active joints; therapeutic trials for second line drugs mostly include polyarthritis, and just having one more joint counted as active (from 4 to 5) would change a patient from having oligoarthritis to polyarthritis, and be therefore potentially enrolled in such a trial. Finally, Food and Drug Administration (FDA) and European Medicines Agency (EMA) authorisations for expensive biotherapies limit their use to such disease categories, again making the number of affected joints an important factor in health expenditures in a paediatric rheumatology setting.

Another very relevant point is the possibility that US could be used for treatment decisions. One way to approach this problem is to see whether US findings in patients in remission or with inactive disease would be correlated to subsequent flares. Few studies dealt with this subject. Magni-Manzoni *et al*[28] followed 39 consecutive children with clinically defined inactive disease for a minimum of 3 months with US assessment of 52 joints. Patients were then followed clinically for up to 2 years until a flare of synovitis occurred in one or more joints, or until the 2-year visit if the disease remained in clinical remission. During the follow-up, 15 patients (38.5%) had a flare of synovitis, after a median of 10.6 months (range 6.3–13.7 months). Only 17 of the 45 flared joints had US abnormalities at study entry. Therefore, the presence of US pathology did not predict an early flare of synovitis in the affected joints. Subsequently, in another study Zhao *et al* followed 40 JIA children with inactive disease, this time with two US scans (at baseline and after 1 year).[29] Of note, images were scored independently by two paediatric musculoskeletal radiologists and specific cautions were taken into the interpretation of possible physiological findings. Baseline US results had a very poor sensitivity (15%) and an even worse positive predictive value (12%). The predictive value of the second US was even lower than the first one, with regard to prediction of flare at 2 years. Another study, coming from our own centre, has also addressed the issue of possible flares in patients on remission who had a positive US at baseline.[30] Standard scans were based on OMERACT guidelines and on paediatric data available at study onset. Eighty-eight consecutive patients with inactive disease were included. Patients were followed clinically for 4 years and, although the presence of an abnormal US increased the risk of flare, this was true only at patient and not at joint level; we cannot explain why joints where the disease relapsed were different than those with abnormal baseline US. Despite the fact that all necessary precautions were taken in order to minimise the pitfalls and caveats detailed in this viewpoint (indeed, the incidence of 'subclinical synovitis' was much lower than in other published series), we cannot exclude one or more bias in our cohort as well.

Despite its limitations, we acknowledge the various aspects that make US a suitable imaging technique in the field of paediatric rheumatology, such as the lack of ionising radiation or contrast agents, the ease of use and transportability and the lack of need for sedation. We also think that US can be valuable in the assessment of response to local treatments (see online supplementary table 1), in the differentiation of extra-articular from intra-articular swelling, in the detection of enthesitis, and in the evaluation of the integrity of cartilage (see online supplementary table 2). It also offers unique advantages in joint injection guidance, especially in young children and in joints with complex anatomy or more difficult to evaluate clinically.

In conclusion, while the role of US in selected situations is unquestionable, such as the precise localisation of a joint injection or the differentiation of true arthritis from tenosynovitis or soft-tissues swelling in specific joints (eg, tibiotalar or subtalar), it remains for the moment quite difficult to clearly identify its role in routine clinical practice. This will be the focus of future high quality studies. Moreover, we think that its use should be reserved for physicians who have been trained extensively in performing US in JIA patients but also in healthy children. And finally, what is the gold standard in the detection of synovitis still remains an open question, and comparison with other modalities (eg, MRI) is certainly needed and should be a focus of future research (see online supplementary text in the Research agenda). Infact, unfortunately the invasive procedure of a synovial biopsy is rarely if ever needed in the diagnostic workup of a child or adolescent with suspected synovitis, and therefore, the comparison of imaging versus histology will be very difficult to obtain. In the meantime, we should also go back to the past and improve clinical skills of young paediatric rheumatologists, since physical examination is less and less taught but still remains the cornerstone of our diagnostic armamentarium.

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EPIDEMIOLOGICAL SCIENCE

Is the epidemiology of rheumatoid arthritis changing? Results from a population-based incidence study, 1985–2014

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ABSTRACT

Objectives To examine trends in the incidence of rheumatoid arthritis (RA) from 2005 to 2014 overall and by serological status as compared with 1995–2004 and 1985–1994.

Methods We evaluated RA incidence trends in a population-based inception cohort of individuals aged ≧18 years who first fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA between 1 January 1985 and 31 December 2014. Incidence rates were estimated and were age-adjusted and sex-adjusted to the white population in the USA in 2010. Trends in incidence were examined using Poisson regression methods.

Results The 2005–2014 incidence cohort comprised 427 patients: mean age 55.4 years, 68% female, 51% rheumatoid factor (RF) positive and 50% anti-cyclic citrullinated peptide antibody positive. The overall age-adjusted and sex-adjusted annual RA incidence in 2005–2014 was 41/100 000 population (age-adjusted incidence: 53/100 000 in women and 29/100 000 in men). While these estimates were similar to the 1995–2004 decade, there was a decline in the incidence of RF-positive RA in 2005–2014 compared with the previous two decades (p=0.004), with a corresponding increase in RF-negative cases (p<0.001). Smoking rates declined and obesity rates increased from earlier decades to more recent years.

Conclusions Significant increase in incidence of RF-negative RA and decrease in RF-positive RA in 2005–2014 compared with previous decades was found using 1987 ACR criteria. The incidence of RA overall during this period remained similar to the previous decade. The changing prevalence of environmental factors, such as smoking, obesity and others, may have contributed to these trends. Whether these trends represent a changing serological profile of RA requires further investigation.

Key messages

What is already known about this subject?
- The overall age-standardised incidence rates of rheumatoid arthritis (RA) have been increasing globally since 1990.
- A decline in the incidence of rheumatoid factor positive RA has been reported in a European population in 1980–2000.

What does this study add?
- Our study shows decreasing incidence of rheumatoid factor (RF)-positive rheumatoid arthritis (RA) and increasing incidence of RF-negative RA in 2005–2014 as compared with the previous decades.
- In aggregate, the incidence of RA overall was stable during 2005–2014 compared with the previous decade.

How might this impact on clinical practice or future developments?
- Rising incidence of rheumatoid factor (RF)-negative rheumatoid arthritis (RA) suggests the need for increased awareness and timely recognition of RF-negative RA by physicians.
- The changing prevalence of environmental factors, such as smoking, obesity and others, may have contributed to decreasing incidence of RF-positive RA and increasing incidence of RF-negative RA in 2005–2014.

INTRODUCTION

Rheumatoid arthritis (RA) is a major public health problem, associated with a substantial burden of functional disability. Globally, the overall age-standardised prevalence and incidence rates of RA have been increasing since 1990.[1] In the USA, RA affects over 1.3 million adults, representing 0.6%–1% of the population.[2 3] The incidence of RA shows temporal and geographic variability, likely influenced by genetic and environmental factors. Despite variable incidence estimates in different populations, declines in RA occurrence have been reported in several populations in the USA (including the population of Rochester, Minnesota), Western Europe and Japan during the second half of the 20th century.[4–7] An increase in the incidence of RA in the late 1990s to early 2000s, particularly in females, has been reported in Olmsted County, Minnesota and in Denmark.[3 8] More recent trends in RA occurrence, particularly, recent trends in RA incidence by serological status, have not been widely studied. A decline in the incidence of rheumatoid factor (RF) positive RA has been reported in Finland in 1980–2000, primarily among patients born after the mid-1940s compared with earlier birth cohorts.[9] A decline in the prevalence of RF-positive RA has been reported in the Pima Indian population, also in younger birth cohorts.[10] These findings have been suggested to reflect a potential decline in RA severity in association with advancements in RA treatment over time. However, no changes in the incidence or prevalence of RA by

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serological status have been reported thus far in the US population, including the population of Olmsted County, Minnesota, where the proportion of RF-positive and RF-negative RA cases remained largely unchanged since 1955.[3 11]

Understanding the epidemiology of RA by serological phenotype may provide insights into the pathophysiology of RA with implications for the course of the disease and choice of treatments, as well as healthcare use and planning. We aimed to examine trends in the incidence of RA from 2005 to 2014 as compared with the previous decades, and to separately assess trends in the incidence of RF-positive and RF-negative RA.

METHODS

The population of Olmsted County, Minnesota is uniquely suitable for an investigation of RA epidemiology due to availability of comprehensive medical records for all residents seeking medical care for more than half a century. The population-based data resources of the Rochester Epidemiology Project (REP) medical record linkage system provide essentially complete ascertainment of all individuals in the community regardless of age, sex, race/ethnicity, insurance status or care delivery setting (inpatient and outpatient).[12] The REP enables complete, decades-long follow-up for each patient across all care providers, including the Mayo Clinic, the Olmsted Medical Centre and their affiliated hospitals, local nursing homes and the few private practitioners. This system offers a unique opportunity to study the key epidemiological characteristics of morbidity, including incidence.[13]

Using the resources of the REP, we have assembled and continue to update the only population-based, longitudinal RA inception cohort in the USA. In order to identify all potential incident cases of RA in this population during the 2005–2014 period, the computerised diagnostic index was searched for any diagnosis of arthritis (excluding degenerative arthritis or osteoarthritis) made between 1 January 2005 and 31 December 2014 among Olmsted County residents who were 18 years of age and older. All persons in the community who qualified during the defined period, regardless of race, ethnicity or socioeconomic status, were included. The complete inpatient and outpatient medical records for each potential case were reviewed by an experienced nurse abstractor, using a pretested data collection form. All questionable cases were additionally reviewed by coinvestigators supervised by the principal investigator. Confirmation or rejection of RA diagnosis was accomplished based on the 1987 American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria for the classification of RA.[14] The incidence date was defined as the earliest date when the patient fulfilled at least four of the 1987 ACR criteria for RA. Subjects were allowed to accumulate the criteria over time until fulfilment of the fourth criterion.[15] This inception cohort of patients in whom RA was diagnosed during the time period from 2005 through 2014 augmented the previously assembled cohort of residents with incident RA from 1985 through 2004.

Information on the following parameters was collected at RA incidence: age, sex, race/ethnicity (white, American Indian/Alaska Native, Asian, Black or African–American, Native Hawaiian or other Pacific Islander, more than one race or race unknown), smoking status (current, former and never), body mass index (BMI) and obesity (BMI $\geq 30\,kg/m^2$). Data on positivity for RF and/or anti-cyclic citrullinated peptide (anti-CCP) antibody and joint erosions/destructive changes during the first year after RA incidence were also gathered from the medical records.

Both RF and anti-CCP antibody were considered for criteria fulfilment when available. However, anti-CCP testing was not widely available until the 2000s, thus incidence rates were calculated for RF positive versus negative without inclusion of anti-CCP to allow fair comparison of incidence rates over three decades (ie, 1985–2014).

Descriptive statistics (percentages, means, etc) were used to summarise patient characteristics in each cohort. Comparison of patient characteristics between cohorts was performed using χ^2 and rank-sum tests. Age-specific and sex-specific incidence rates were calculated using the number of incident cases as the numerator and population counts from the REP census as the denominator.[12] Overall incidence rates were age-adjusted and/or sex-adjusted to the population of white persons living in the USA in 2010. In order to compute 95% CIs for incidence rates, it was assumed that the number of incident cases followed a Poisson distribution. Trends in incidence rates were examined using Poisson regression models. Statistical analyses were performed using SAS version V.9.4 (SAS Institute, Cary, North Carolina, USA) and R V.3.6.1 (R Foundation for Statistical Computing, Vienna, Austria). This study was approved by institutional review boards of Mayo Clinic (IRB #17-002593) and Olmsted Medical Centre (IRB #017-OMC-17).

Table 1 Patient characteristics by decade of RA incidence

Characteristics*	Decade of RA incidence 1985–1994 (n=240)	1995–2004 (n=344)	2005–2014 (n=427)	P value
Age at RA incidence (years)	56.6 (16.6)	56.0 (15.5)	55.4 (15.4)	0.73
Female sex	160 (67%)	240 (70%)	291 (68%)	0.73
Race				
White	225 (94%)	321 (93%)	377 (88%)	
American Indian/Alaska Native	1 (0%)	2 (1%)	2 (0%)	0.08
Asian	8 (3%)	8 (2%)	2 (0%)	
Black or African–American	0 (0%)	3 (1%)	17 (4%)	
Native Hawaiian/ Other Pacific Islander	1 (0%)	1 (0%)	3 (1%)	
More than one race	3 (1%)	5 (1%)	9 (2%)	
Unknown	2 (1%)	4 (1%)	3 (1%)	
Smoking at RA incidence				
Never smoker	97 (40%)	161 (47%)	242 (57%)	
Current smoker	51 (21%)	62 (18%)	64 (15%)	
Former smoker	91 (38%)	121 (35%)	121 (28%)	
BMI at RA incidence (kg/m^2)	27.0 (5.5)	28.1 (6.1)	29.6 (6.8)	<0.001
Obesity (BMI $\geq 30\,kg/m^2$) at RA incidence	57 (24%)	114 (33%)	175 (41%)	<0.001
History of obesity at or before RA incidence	77 (32%)	147 (43%)	210 (49%)	<0.001
RF positive	166 (69%)	238 (69%)	216 (51%)	<0.001
Anti-CCP positive Not tested	33 (73%) 195	86 (49%) 170	197 (50%) 30	0.009
Erosion in the first year after RA incidence	33 (17%)	65 (21%)	96 (25%)	0.048
RF positive	27 (19%)	50 (23%)	49 (25%)	0.47
RF negative	6 (10%)	15 (16%)	47 (25%)	0.017
No radiograph	41	27	46	
Patients who underwent radiographic examination in the first year after RA incidence	199 (83%)	317 (92%)	381 (89%)	0.002
RF positive	139 (84%)	221 (93%)	195 (90%)	0.012
RF negative	60 (81%)	96 (91%)	186 (88%)	0.15

*Values in the table are mean (±SD) for continuous characteristics and N (%) for discrete characteristics.
anti-CCP, anti-cyclic citrullinated peptide; BMI, body mass index; RA, rheumatoid arthritis; RF, rheumatoid factor.

Table 2 Incidence rates of RA by 1987 ACR criteria per 100 000 population (95% CI)

Group	Decade of RA incidence	Female	Male	Total
Overall	1985–1994	48 (41 to 56)	32 (25 to 40)	40 (35 to 46)
	1995–2004	55 (48 to 63)	30 (24 to 36)	43 (38 to 48)
	2005–2014	53 (47 to 59)	29 (24 to 34)	41 (37 to 45)
RF positive	1985–1994	33 (27 to 40)	23 (17 to 30)	28 (24 to 33)
	1995–2004	39 (33 to 45)	19 (15 to 24)	30 (26 to 33)
	2005–2014	26 (22 to 30)	15 (12 to 19)	21 (18 to 24)
RF negative	1985–1994	15 (11 to 19)	9 (5 to 12)	12 (9 to 15)
	1995–2004	16 (13 to 20)	10 (7 to 14)	13 (11 to 16)
	2005–2014	26 (22 to 31)	14 (11 to 18)	20 (18 to 23)

ACR, American College of Rheumatology; RA, rheumatoid arthritis; RF, rheumatoid factor.

RESULTS

The incidence cohort for 2005–2014 consists of 427 patients. The mean age at incidence of RA was 55.4 years, and 291 (68%) of the patients were female. Table 1 shows patients' characteristics for the 2005–2014 incidence cohort as compared with the 1985–1994 and 1995–2004 cohorts. There were no statistically significant differences in age, sex or race at RA incidence between the 2005–2014, 1995–2004 or 1985–1994 cohorts. Smoking rates declined and obesity rates increased substantially from earlier decades to more recent years.

All patients were tested for RF. In the 2005–2014 cohort, 216 (51%) patients were positive for RF compared with 69% of patients with incident RA in the 1985–1994 and 1995–2004 cohorts ($p<0.001$, table 1).

The proportion of patients positive for anti-CCP antibody was 50% in the 2005–2014 cohort compared with 49% in the 1995–2004 incidence cohort, where only 174 out of 344 patients were tested due to the lack of test availability during most of this time period. Definite radiographic changes (erosions) during the first year after RA incidence were more frequent in patients with RA incident in 2005–2014 (96 patients, 25%) as compared with the 1985–1994 (33 patients, 17%) and 1995–2004 cohorts (65 patients, 21%; $p=0.048$). The proportion of subjects who underwent radiographic examination in 2005–2014 was similar to the previous decade (89% vs 92%), and both were higher than in the 1985–1994 cohort (83%). When the prevalence of erosive disease was compared by RF status, patients with RF-positive RA in 2005–2014 did not differ from those in the previous decades (table 1). However, the proportion of erosive disease in patients with RF-negative RA has increased in the 2005–2014 cohort compared with the prior decades.

Table 2 shows RA incidence rates per 100 000 population for the three most recent decades and by RF status. The overall age-adjusted and sex-adjusted annual RA incidence in 2005–2014 was 41/100 000 population with age-adjusted incidence in women 53/100 000 population and 29/100 000 population in men. These estimates were similar to the previous decades ($p=0.26$). There was a significant decline in the incidence of RF-positive RA in 2005–2014 compared with the previous two decades ($p=0.004$), with a corresponding increase in RF-negative cases ($p<0.001$, table 2). This decline affected both sexes and most age groups. Online supplementary figures S1–S3 show RA incidence rates by age group, sex, time period and RF positivity.

DISCUSSION

The epidemiology of RA is dynamic. Previous studies, including ours, have shown that the incidence of RA varies between geographic areas and over time.[3–8] This retrospective population-based cohort study reports on the recent trends in incidence of RA in Olmsted County, Minnesota in 2005–2014 as compared with the previous decades. The major finding of this study is decreasing incidence of RF-positive RA and increasing incidence of RF-negative RA in 2005–2014 as compared with the previous decades. This decline in incidence of RF-positive and rise in RF-negative RA resulted in a stable incidence of RA overall in 2005–2014 versus 1995–2004. Correspondingly, the proportion of patients in the overall cohort who were RF-positive was only 51%. This is in contrast to our earlier studies showing persistent predominance of patients with RF-positive RA exceeding 65% of all cases over past decades (1955–2004).[3 11]

Several recent studies examined trends in RA incidence after 2000 with inconsistent results. A recent nationwide population-based cohort study from the UK showed decline in the annual incidence of RA by 1.6% between 1990 and 2015, using a code-based definition of RA.[16] The estimates were slightly lower than in our study with an estimate for overall RA incidence of 38.1/100 000, consistent with the lower estimates for Europe and the UK versus the USA in previous studies.[17 18] A nationwide register-based study from Finland reported a decrease in incidence of seronegative RA cases and stable incidence of seropositive RA from 2000 to 2014 based on the International Classification of Diseases version 10 (ICD-10) codes.[19] Variability in the incidence of RA based on ICD-9 and ICD-10 codes has been described in a Canadian province during the 2001–2014 period (age-adjusted and sex-adjusted incidence rates were highest at 73.1 cases/100 000 in 2013 and lowest at 33.7 cases/100 000 in 2015), but no consistent trend towards decrease or increase in RA incidence was detected.[20] The incidence of RA overall and by sex in 2005–2014 in this study was similar to the estimates from the recent nation-wide, register-based study of RA incidence in Sweden in 2006-2008,[21] which may be due to common genetic background, since many Minnesota residents have Northern European ancestry.

What are the potential reasons for the observed increase in incidence of seronegative RA? As incidence estimates vary depending on case definition, the change in classification criteria for RA from 1987 ACR criteria to 2010 ACR/European League Against Rheumatism (EULAR) criteria may have influenced the results of studies using code-based definitions of RA, reflecting differences between coding of inflammatory arthritis diagnoses in practice and classification criteria performance. While 2010 ACR/EULAR criteria were designed to facilitate recognition of early RA, low sensitivity of these criteria to seronegative RA has been reported[22–24] and can account for some variability in identification of seronegative RA cases in population-based studies using different criteria sets.[19]

Changes in environmental exposures may affect the risk of developing RA. RF and/or anti-CCP-positive and RF and/or anti-CCP-negative RA are increasingly recognised as aetiologically distinct subtypes of RA disease, and different risk factors have been shown to be selectively associated with seropositive or seronegative subtype.[25]

Cigarette smoking is an established risk factor for seropositive RA. The link between smoking and anti-CCP is primarily present in patients with RA who have shared epitope (SE) for human leukocyte antigen (HLA) DRB1 gene, the major genetic risk factor for RA.[26] Findings from two recent large

population-based studies in European and Asian populations suggest that the association of cigarette smoking with anti-CCP may be driven by the presence of the SE, while the association of smoking with RF-positivity may be independent of the presence of the SE.[27 28] Smoking cessation has been associated with decreased risk of anti-CCP and RF positivity.[28–30] A decline in incidence of RA, particularly seropositive RA, has been reported alongside the decline in cigarette smoking in European populations in the past several decades.[9 16] Concordantly, the decline in incidence of RF-positive RA in our study coincided with a significant decrease in current or former smoking and an increase in rates of never smokers in Olmsted County, Minnesota.

Obesity is a significant risk factor for RA, even after adjusting for smoking status.[31] Growing evidence from population-based studies shows an association of increased body mass index (BMI) and obesity with anti-CCP-negative RA, particularly in women, while an inverse association between BMI and anti-CCP-positive RA has been shown in men.[25 32] Less is known about the association of obesity with the presence of RF. Given the recognised correlation between seropositivity for RF and anti-CCP,[27 33] it could be hypothesised that the growing obesity rates in the population of Olmsted County, Minnesota can be contributing to the observed increase in RF-negative RA. However, more studies are needed to further understand the effects of increased BMI and obesity on the risk of RA by serological status.

Several other environmental and lifestyle factors have been evaluated for their association with the risk of RA in prior studies, including periodontitis,[34] vitamin D deficiency[35] breast feeding and oral contraceptive use.[36 37] While this study focused on trends in incidence of RA, investigation of time trends in environmental and lifestyle factors and its association with RA incidence is a subject for future research.

Seronegative RA is frequently thought of as a milder disease. However, growing evidence suggests delayed diagnosis, more severe disease at presentation and challenges in remission induction in patients with seronegative RA.[38 39] In our study, erosions in the first year were more frequent in patients with RA in 2005–2014, and this trend was driven by RF-negative RA that was more prevalent in this recent decade than in the previous decades. In fact, radiographic erosions within the first year of RA disease onset in the 2005–2014 cohort were as common in RF-negative as in patients with RF-positive RA. Taken together with our findings of a rising incidence of RF-negative RA, these data suggest a need for increased awareness and timely vigilant management of RF-negative RA.

Strengths of our study include its longitudinal population-based design and the use of a systematic and standardised approach to case identification over several decades. RA was defined based on 1987 ACR classification criteria rather than current 2010 ACR/EULAR criteria, ensuring comparability of the estimates with earlier decades and minimising the possibility of misclassification due to changes in criteria sets. In addition, prior studies have indicated low sensitivity of 2010 criteria in patients with seronegative RA, suggesting that use of this criteria set may not be ideal in population-based studies of RA epidemiology.[22] Incidence rates were calculated for RF positive versus RF negative without inclusion of anti-CCP-antibody to allow fair comparison of incidence rates over three decades (ie, 1985–2014) when anti-CCP testing was on the rise.

There is a possibility of under-ascertainment of RA cases in studies involving medical record review. However, the comprehensive and standardised approach to case ascertainment in this study makes this unlikely. While there is a possibility for misclassification of cases, particularly those with seronegative RA, patients included in this study met at least four 1987 ACR criteria for RA and had no alternative diagnosis for their inflammatory arthritis in the medical records. While the increasing recognition and the need for early treatment of RA disease may affect diagnostic code-based estimates of RA incidence, we believe that defining RA onset based on accumulation of at least four 1987 ACR classification criteria rather than a physician diagnosis may have minimised potential bias associated with variable awareness of RA disease among individual rheumatology providers. The rates of radiographic testing have increased from earlier decades to more recent decades and may have contributed to increase in identification of erosions. However, this increase would be expected to affect patients with RA regardless of RF status, and would not explain increased rates of erosions in RF-negative but not in RF-positive patients. Radiographs were interpreted by certified Mayo Clinic radiologists blinded to the study hypothesis, per routine protocol; thus radiographic interpretation is unlikely to bias the study results. Finally, the population of Olmsted County, Minnesota is ~90% white, suggesting that the results of our study may not be generalisable to other, more racially diverse populations. The rates of RF-positivity may vary across racial and ethnic groups. There was a marginal decrease in the proportion of white individuals in the 2005–2014 cohort in keeping with increasing diversity within the Olmsted County population. White patients have been previously noted to have lower percentages of RF-positive RA,[40] thus the borderline decline in the proportion of white individuals in Olmsted country would not be expected to explain the decline in incidence of RF-positive RA or otherwise influence the observed epidemiological trends.

In summary, there has been an increase in RF-negative RA and decrease in RF-positive RA in recent years. In aggregate, the incidence of RA overall was stable during 2005–2014 compared with the previous decade. The changing prevalence of environmental factors, such as smoking, obesity and others, may have contributed to these trends in seropositive disease in this population.

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CLINICAL SCIENCE

Testing different thresholds for patient global assessment in defining remission for rheumatoid arthritis: are the current ACR/EULAR Boolean criteria optimal?

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ABSTRACT

Objectives This study aimed to evaluate different patient global assessment (PGA) cut-offs required in the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) Boolean remission definition for their utility in rheumatoid arthritis (RA).

Methods We used data from six randomised controlled trials in early and established RA. We increased the threshold for the 0–10 score for PGA gradually from 1 to 3 in steps of 0.5 (Boolean1.5 to Boolean3.0) and omitted PGA completely (BooleanX) at 6 and 12 months. Agreement with the index-based (Simplified Disease Activity Index (SDAI)) remission definition was analysed using kappa, recursive partitioning (classification and regression tree (CART)) and receiver operating characteristics. The impact of achieving each definition on functional and radiographic outcomes after 1 year was explored.

Results Data from 1680 patients with early RA and 920 patients with established RA were included. The proportion of patients achieving Boolean remission increased with higher thresholds for PGA from 12.4% to 19.7% in early and 5.9% to 12.3% in established RA at 6 months. Best agreement with SDAI remission occurred at PGA cut-offs of 1.5 and 2.0, while agreement decreased with higher PGA (CART: optimal agreement at PGA≤1.6 cm; sensitivity of PGA≤1.5 95%). Changing PGA thresholds at 6 months did not affect radiographic progression at 12 months (mean ΔsmTSS for Boolean, 1.5, 2.0, 2.5, 3.0, BooleanX: 0.35±5.4, 0.38±5.14, 0.41±5.1, 0.37±4.9, 0.34±4.9, 0.27±4.7). However, the proportion attaining HAQ≤0.5 was 90.2%, 87.9%, 85.2%, 81.1%, 80.7% and 73.1% for the respective Boolean definitions.

Conclusion Increasing the PGA cut-off to 1.5 cm would provide high consistency between Boolean with the index-based remission; the integer cut-off of 2.0 cm performed similarly.

Key messages

What is already known about this subject?

- The patient global assessment (PGA) is the most common reason in patients with rheumatoid arthritis (RA) for not reaching American College of Rheumatology/European League Against Rheumatism Boolean remission.
- The PGA has been criticised to not adequately reflect disease activity of RA.

What does this study add?

- A PGA cut-off of 2 (on a 0–10 scale) coincides with a better agreement between Boolean and Simplified Disease Activity Index remission.
- Patients in Boolean remission definition using ≤2 (on a 0–10 scale) on a PGA show good long-term functional and radiologic outcomes.

How might this impact on clinical practice or future developments?

- Using the adapted cut-off in clinical trials and practice will improve evaluation of remission in RA.

Disease activity in rheumatoid arthritis (RA) has been found best reflected in a number of so-called core set variables defined many years ago by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR).[1 2] Irrespective of the use of individual core set variables, composite measures of disease activity comprising several components have better validity than individual components based on the heterogeneity of the disease presentations between and within individual patients[3–5]; in addition, they correlate better with structural and functional outcomes in RA.[3 6]

When the core set variables were defined, remission was more an aspirational than a realistic goal.[7] Today, remission is achievable in a significant proportion of patients and has become a major therapeutic target.[8–10] A clinical definition of remission for RA should reflect no, or at most only minimal, disease activity in terms of inflammation, such as swollen joints or acute phase reactant (APR) levels to prevent structural progression and functional deterioration.[10] The remission definition of the Disease Activity Score using 28 joint counts (DAS28) allows for a significant number of residual swollen joints,[11–13] which cannot be overcome by lowering the cut-off for remission on its scale.[11 14] DAS28 also overweighs the acute phase response,[15] making results from drugs that target interleukin (IL)-6—and thus the APR directly—less comparable with those attained with other compounds.[16 17]

ACR and EULAR provided remission definitions almost one decade ago.[11] Despite the proven validity of the ACR/EULAR remission criteria, the definition of remission is still in discussion and alternative definitions are still frequently used in clinical trials and practice. To attain an ACR/EULAR Boolean remission, a patient must have, among other criteria, a patient global assessment (PGA) score ≤1 (0–10 scale), and this definition has been criticised because patients who have no active joints and a normal C reactive protein (CRP) often have PGA scores exceeding the cut-off of 1.[18] PGA has been incorporated into composite scores and remission definitions to include the patient's perspective in the assessment of disease activity, and it is also recommended for evaluation in clinical trials.[19] Further, the committee developing the remission definition showed that inclusion of PGA improved the discriminant ability of remission criteria to separate effective RA treatments from placebo, suggesting that it represents elements of disease activity missed by other outcome measures. In other words, inclusion of PGA in remission criteria makes it more likely that efficacy of different treatments can be discriminated. However, the PGA sometimes not only reflects symptoms based on inflammatory disease activity but also other factors such as depressive symptoms or functional limitations due to pre-existing joint damage or even comorbidities.[20 21]

The ACR/EULAR index-based Simplified Disease Activity Index (SDAI) remission criteria are slightly less stringent than Boolean remission, given that the sum of several components permits one of them to be slightly elevated (eg, a PGA above 1) if compensated by a lower score of others.[22] Both remission definitions are associated with optimal clinical, functional and structural outcomes[11] and are widely used in clinical trials, where a substantial number of patients today achieve this stringent outcome.[23] To this end, studies have shown that some patients meeting SDAI remission do not meet the more stringent Boolean definition of remission primarily due to the requirement for a PGA of ≤1.[18 24] Since both, the Boolean and the SDAI remission, are recommended by ACR and EULAR, they ideally should be consistent and identify the same patients.

We therefore aimed to determine whether an increase of the PGA threshold in the ACR/EULAR Boolean-based criteria might increase its agreement with the ACR/EULAR index-based remission by SDAI without jeopardising good clinical, functional and structural implications, associated with the state of remission.

METHODS

Patients

RA patient data were retrieved from six clinical trials testing the efficacy of tumour necrosis factor inhibitors (TNFi) versus placebo or placebo+methotrexate (MTX) with an observation period between 1 and 2 years (ASPIRE, ATTRACT, PREMIER, DE019, Go Before and Go Forward). The individual trials have been previously reported[25–30] and so has the use of pooled data of these trials obtained from the trial sponsors.[22 31 32] These trials included patients with RA with varying disease durations and treatment histories representing a large spectrum of the disease. ASPIRE (infliximab), Go Before (golimumab) and PREMIER (adalimumab) were trials in MTX-naïve patients with early RA (mean disease duration of the pooled population at baseline 1.5±3.0 years), while ATTRACT (infliximab), DE019 (adalimumab) and Go Forward (golimumab) were performed in MTX-insufficient responders with a mean disease duration of the pooled patients at baseline of 9.7±8.4 years. In all six clinical trials, the patients were asked to provide the assessment of the activity of their RA using a 100 mm visual analogue scale (VAS).[25–30]

Definitions of remission and their modifications

The Boolean definition includes swollen joint counts (SJC), tender joint counts (TJC), PGA (in cm) and CRP levels (in mg/dL) and for a patient to meet remission criteria, all of these must have scores of 1 or less. The SDAI index-based definition of remission sums the scores for the components used in the Boolean definition plus evaluator/physician global assessment, and patients meet this definition if the score is ≤3.3.[11]

We evaluated an expansion of the current Boolean definition of remission by increasing the cut-off of the PGA criterion stepwise (using a 0–10 cm VAS) by 0.5 cm increments from 1 cm to 1.5, 2.0, 2.5 and 3.0 cm. We will refer to them as Boolean1.5, Boolean2.0, Boolean2.5 and Boolean3.0, respectively. Additionally, we omitted the PGA criterion completely from the Boolean definition, labelling this definition as BooleanX; in this definition, only CRP, TJC and SJC need to score ≤1 to attain remission, independent of the PGA value.[33]

Analyses

We assessed agreement of modified Boolean remission rates at 6 and 12 months with the SDAI definition of remission using McNemar's test for agreement. We tested which PGA cut-off in the Boolean remission criteria yielded the best agreement with SDAI remission.[11]

As a next step, we explored the impact of using the modified Boolean remission definitions assessed at 6 months on outcomes at 1 year. Differences in mean radiographic progression (based on the change in modified total Sharp score (mTSS) between baseline and 1 year), number of patients without progression (change in score ≤0), mean functional scores (Health Assessssment Questionnaire (HAQ) scores, physical component scores of the Short Form 36 (SF-36)) and patients with normal function (HAQ ≤0.5 at 1 year), were assessed. The distribution of 1-year outcomes was depicted in cumulative frequency plots, separately for patients attaining the various 'modified' remission definitions at 6 months. These analyses were then repeated separately for patients with early and late RA.

To obtain a more sensitive assessment of differences in structural and functional outcomes, we looked at these outcomes for the non-overlapping modified Boolean definition groups (ie, Boolean20 would not include Boolean15 or lower; and analogously for the other definitions). We compared differences in distribution of mTSS changes, HAQ and SF-36 physical component scores at 1 year between discrete modified Boolean definitions (ie, Boolean1.5 only those with PGA of 1.1–1.5, and so on) at the 6-month time point. We used data from patients with early RA only, since numbers of patients with established RA were too few for this analysis.

Furthermore, we conducted a classification and regression tree (CART) analysis to predict SDAI remission in early and established RA based on PGA at weeks 22 and 54 (R rpart package; https://cran.r-project.org/web/packages/rpart/index.html) to determine the PGA cut-off in patients fulfilling the other three Boolean criteria, which shows the highest likelihood of fulfilling the SDAI definition of remission. We then performed receiver operating curve analyses (ROC) to test sensitivity and specificity of all PGA cut-offs between 1 and 2 cm.

Patient and public involvement statement

The place and interpretation of the PGA in defining remission in RA from a patient perspective have repeatedly raised concerns of

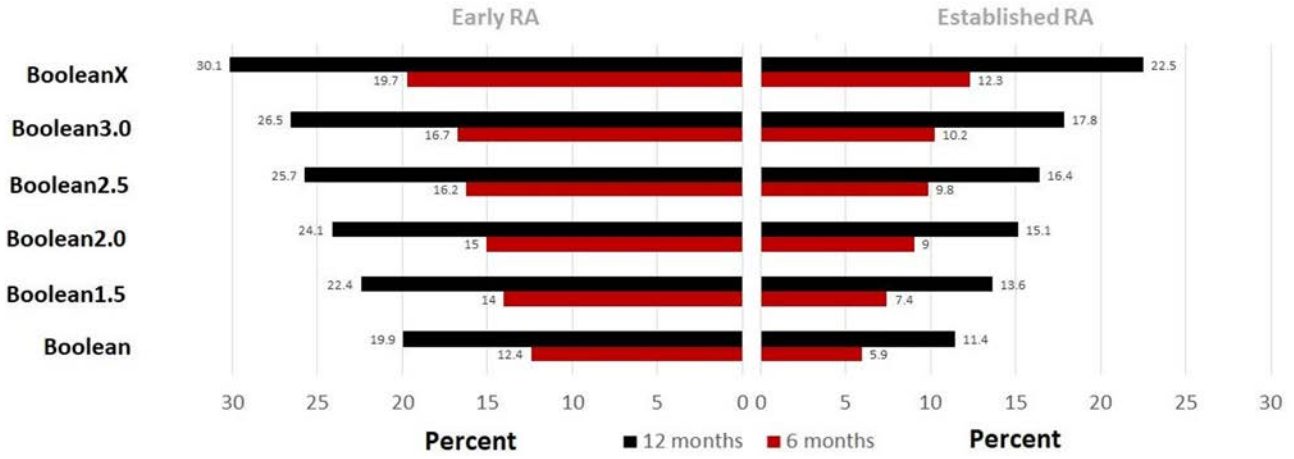

Figure 1 Rates of remission by modified Boolean classifications, using a patient global assessment (PGA) cut-off of 1.0 ('Boolean'), 1.5, 2.0, 2.5, 3.0 cm, or omitting the PGA completely (BooleanX). Rates in % of total, separately depicted at 6 (red bars) and 12 months (black bars) time points; on the left for those in early rheumatoid arthritis (RA) and on the right for established RA.

physicians and patients.[34] For this reason, we included an experienced patient research partner (PRP) in this study (MdW). The PRP was involved throughout the research process and provided critical feedback during all stages of analysis. Face-to-face meetings with the PRP took place in conjunction with EULAR meetings and the PRP will disseminate findings in relevant patient communities.

RESULTS

Data from 2600 trial patients, 1680 with early RA (mean disease duration: 1.5±3.0 years) and 920 with established RA (mean disease duration: 9.7±8.4 years) were included. As expected, the rates of patients achieving modified Boolean remission increased with an increase in the PGA cut-off from 12.4% (n=208) to 19.7% (n=331) in early RA and 5.9% (n=54) to 12.3% (n=113) in established RA at 6 months and 19.9% (n=335) to 30.1% (n=506) and 11.4% (n=105) to 22.5% (n=207), respectively, at 1 year (figure 1). For both early and late RA, the increase in remission rates was already pronounced when moving the PGA cut-off of from 1.0 to 2.0 cm (+44 patients (+21%) at 6 months) and less when moving the cut-off from 2.0 to 3.0 cm (+29 patients (+14%) at 6 months); however, omitting the PGA criterion completely (BooleanX definition) led to an even larger increase in remission rates compared with the Boolean3.0 category (+50 patients at 6 months; see also online supplementary table 1).

Concordance of modified Boolean remission with SDAI remission

When evaluating the best cut-off for concordance of SDAI and Boolean remission, we found that by increasing the PGA cut-off to 1.5 or 2.0 cm, higher concordance rates between the two definitions were achieved, leading to fewer patients who only fulfilled SDAI remission without fulfilling the respective Boolean remission. The percentage of Boolean remitters (within the SDAI remitters) increased from 74% to 85% when using the Boolean2.0 definition at 6 months, and from 79% to 89% at the 1-year visit. At the same time, however, there was a slight increase in patients fulfilling the Boolean criteria only within the SDAI non-remitter group (from 1.3% to 3.0% at 6 months and from 1.5% to 4.1% at 1 year). Overall, kappa values with SDAI remission were almost identical for the Boolean2.0 definition compared with the traditional Boolean definition (at 6 months: 0.80; 95% CI 0.76 to 0.83, vs 0.78; 0.74 to 0.81; at 1 year: 0.83; 0.80 to 0.86 vs 0.82; 0.80 to 0.85).

When exploring this separately for patients with early and established RA, we found that the concordance between Boolean and SDAI definitions (by means of kappa) was lower in patients with established RA in particular at 6 months, with similar values to early RA at 1 year (figure 2). Regardless of population (early vs late) or time point during the trial (6 months vs 12 months), agreements between the two remission definitions were better when using the Boolean1.5 and 2.0 definition (as seen in the overall data). A further increase in the PGA cut-off beyond 2 cm led to a decrease in concordance; this drop in congruence was very clear when omitting the PGA (lower kappa values than for the traditional Boolean remission). In summary, the increase of the cut point from 1.0 to 2.0 increased the number of patients in remission with a similar overall agreement with the SDAI definition.

Additionally, using CART analyses revealed in patients with SJC, TJC and CRP all <1, that depending on the population (early vs established) or time point of analysis (6 months vs 12 months), the PGA cut-off with the highest likelihood of concurrent SDAI remission ranged between ≤1.1 and ≤1.6 cm (table 1,

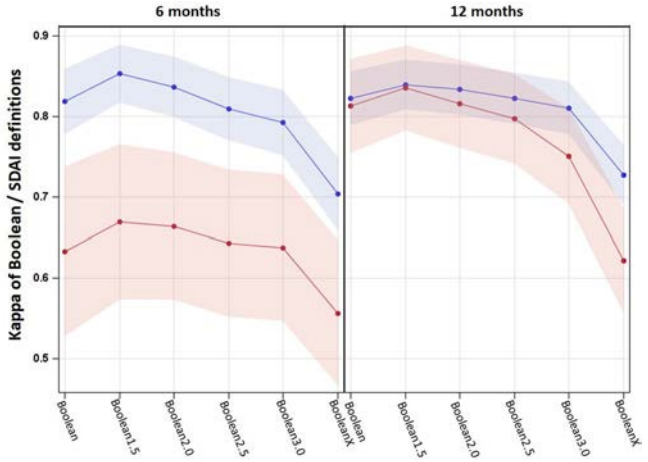

Figure 2 Kappa with CIs between modified Boolean remission categories and Simplified Disease Activity Index (SDAI) remission, separately for early rheumatoid arthritis (RA) (blue line) and established RA (red line) at 6 months and at 12 months.

Table 1 Calculated sensitivity, specificity, positive likelihood ratio (+LR), negative likelihood ratio (–LR) of PGA cut-offs using coinciding SDAI remission as outcome, in patients fulfilling SJC28, TJC28 and CRP all ≤1

6 months						12 months					
Definition	**Cut-off**	**Sensitivity**	**Specificity**	**+LR**	**–LR**	**Definition**	**Cut-off**	**Sensitivity**	**Specificity**	**+LR**	**–LR**
Early	1	88.1 (83.1–92.1)	87.4 (79.7 to 92.9)	7.0 (4.3–11.4)	0.14 (0.09–0.2)	Early	1	87.2 (83.2–90.4)	85.6 (78.9 to 90.9)	6.1 (4.1–9.0)	0.2 (0.1–0.2)
	1.1	90 (85.2–93.6)	84.7 (76.6 to 90.8)	5.9 (3.8–9.1)	0.1 (0.08–0.2)		1.1*	89.1 (85.4–92.1)	84.3 (77.3 to 89.7)	5.7 (3.9–8.2)	0.1 (0.10–0.2)
	1.2	92.7 (88.4–95.8)	82.9 (74.6 to 89.4)	5.4 (3.6–8.2)	0.1 (0.05–0.1)		1.2	89.9 (86.4–92.9)	81.5 (74.2 to 87.4)	4.9 (3.5–6.8)	0.1 (0.09–0.2)
	1.3	94.1 (90.1–96.8)	82.9 (74.6 to 89.4)	5.5 (3.6–8.3)	0.1 (0.04–0.1)		1.3	91.9 (88.6–94.5)	78.8 (71.2 to 85.1)	4.3 (3.2–5.9)	0.1 (0.07–0.1)
	1.4	95.9 (92.3–98.1)	81.1 (72.5 to 87.9)	5.1 (3.4–7.5)	0.1 (0.03–0.10)		1.4	93.6 (90.5–95.9)	76.7 (69.0 to 83.3)	4 (3.0–5.4)	0.1 (0.06–0.1)
	1.5	96.8 (93.5–98.7)	80.2 (71.5 to 87.1)	4.9 (3.4–7.1)	0.04 (0.02–0.08)		1.5	93.9 (90.8–96.1)	73.3 (65.3 to 80.3)	3.5 (2.7–4.6)	0.1 (0.06–0.1)
	1.6*	97.3 (94.1–99.0)	79.3 (70.5 to 86.4)	4.7 (3.3–6.8)	0.04 (0.02–0.08)		1.6	95 (92.2–97.0)	71.2 (63.2 to 78.4)	3.3 (2.6–4.3)	0.1 (0.04–0.1)
	1.7	98.2 (95.4–99.5)	77.5 (68.6 to 84.9)	4.4 (3.1–6.2)	0.02 (0.009–0.06)		1.7	96.1 (93.5–97.8)	70.6 (62.4 to 77.8)	3.3 (2.5–4.2)	0.1 (0.03–0.09)
	1.8	98.2 (95.4–99.5)	73.9 (64.7 to 81.8)	3.8 (2.7–5.1)	0.03 (0.009–0.07)		1.8	96.4 (93.9–98.1)	69.2 (61.0 to 76.5)	3.1 (2.5–4.0)	0.1 (0.03–0.09)
	1.9	98.6 (96.0–99.7)	71.2 (61.8 to 79.4)	3.4 (2.6–4.6)	0.02 (0.006–0.06)		1.9	96.7 (94.2–98.3)	67.1 (58.9 to 74.7)	2.9 (2.3–3.7)	0.1 (0.03–0.09)
	2	98.6 (96.0–99.7)	68.5 (59.0 to 77.0)	3.1 (2.4–4.1)	0.02 (0.01–0.06)		2	96.9 (94.6–98.5)	61.6 (53.2 to 69.6)	2.5 (2.1–3.1)	0.1 (0.03–0.09)
Established	1	73.1 (59.0–84.4)	73.8 (60.9 to 84.2)	2.8 (1.8–4.4)	0.4 (0.2–0.6)	Established	1	83.9 (75.8–90.2)	90.3 (82.4 to 95.5)	8.7 (4.6–16.2)	0.2 (0.1–0.3)
	1.1	75 (61.1–86.0)	73.8 (60.9 to 84.2)	2.9 (1.8–4.5)	0.3 (0.2–0.6)		1.1	83.9 (75.8–90.2)	89.3 (81.1 to 94.7)	7.8 (4.3–14.1)	0.2 (0.1–0.3)
	1.2	76.9 (63.2–87.5)	72.1 (59.2 to 82.9)	2.8 (1.8–4.2)	0.3 (0.2–0.5)		1.2	85.7 (77.8–91.6)	87.1 (78.5 to 93.2)	6.6 (3.9–11.3)	0.2 (0.1–0.3)
	1.3*	84.6 (71.9–93.1)	68.9 (55.7 to 80.1)	2.7 (1.8–4.0)	0.2 (0.1–0.4)		1.3	88.4 (81.0–93.7)	85 (76.0 to 91.5)	5.9 (3.6–9.6)	0.1 (0.08–0.2)
	1.4	84.6 (71.9–93.1)	67.2 (54.0 to 78.7)	2.6 (1.8–3.8)	0.2 (0.1–0.4)		1.4	91.1 (84.2–95.6)	83.9 (74.8 to 90.7)	5.7 (3.5–9.0)	0.1 (0.06–0.2)
	1.5	86.5 (74.2–94.4)	62.3 (49.0 to 74.4)	2.3 (1.6–3.2)	0.2 (0.1–0.4)		1.5*	93.8 (87.5–97.5)	80.7 (71.1 to 88.1)	4.8 (3.2–7.4)	0.1 (0.04–0.2)
	1.6	88.5 (76.6–95.6)	59 (45.7 to 71.4)	2.2 (1.6–3.0)	0.2 (0.09–0.4)		1.6	93.8 (87.5–97.5)	79.6 (69.9 to 87.2)	4.6 (3.1–6.9)	0.1 (0.04–0.2)
	1.7	90.4 (79.0–96.8)	54.1 (40.8 to 66.9)	2 (1.5–2.6)	0.2 (0.07–0.4)		1.7	95.5 (89.9–98.5)	76.3 (66.4 to 84.5)	4 (2.8–5.8)	0.1 (0.02–0.1)
	1.8	92.3 (81.5–97.9)	52.5 (39.3 to 65.4)	1.9 (1.5–2.6)	0.2 (0.06–0.4)		1.8	96.4 (91.1–99.0)	74.2 (64.1 to 82.7)	3.7 (2.6–5.3)	0.05 (0.02–0.1)
	1.9	94.2 (84.1–98.8)	47.5 (34.6 to 60.7)	1.8 (1.4–2.3)	0.1 (0.04–0.4)		1.9	96.4 (91.1–99.0)	73.1 (62.9 to 81.8)	3.6 (2.6–5.0)	0.05 (0.02–0.1)
	2	96.2 (86.8–99.5)	45.9 (33.1 to 59.2)	1.8 (1.4–2.3)	0.1 (0.02–0.3)		2	97.3 (92.4–99.4)	69.9 (59.5 to 79.0)	3.2 (2.4–4.4)	0.04 (0.01–0.1)
All	1	85.2 (80.4–89.2)	82.6 (76.0 to 87.9)	4.9 (3.5–6.8)	0.2 (0.1–0.2)	All	1	86.4 (82.9–89.4)	87.5 (82.6 to 91.4)	6.9 (4.9–9.6)	0.2 (0.1–0.2)
	1.1	87.1 (82.5–90.8)	80.8 (74.1 to 86.4)	4.5 (3.3–6.2)	0.2 (0.1–0.2)		1.1*	88.1 (84.8–90.9)	85.4 (80.2 to 89.6)	6 (4.4–8.2)	0.1 (0.1–0.2)
	1.2	87.5 (82.9–91.2)	80.2 (73.5 to 85.9)	4.4 (3.3–6.0)	0.2 (0.1–0.2)		1.2	88.3 (85.0–91.1)	85.4 (80.2 to 89.6)	6 (4.4–8.2)	0.1 (0.1–0.2)
	1.3	92.3 (88.4–95.1)	77.9 (71.0 to 83.9)	4.2 (3.1–5.5)	0.1 (0.07–0.2)		1.3	89.6 (86.5–92.2)	82.9 (77.5 to 87.4)	5.2 (3.9–6.9)	0.1 (0.1–0.2)
	1.4	87.5 (82.9–91.2)	80.2 (73.5 to 85.9)	4.4 (3.3–6.0)	0.2 (0.1–0.2)		1.4	91.7 (88.8–94.0)	80.8 (75.2 to 85.6)	4.8 (3.7–6.2)	0.1 (0.08–0.1)
	1.5	94.8 (91.5–97.1)	73.8 (66.6 to 80.2)	3.6 (2.8–4.7)	0.1 (0.04–0.1)		1.5	93.8 (91.3–95.8)	76.2 (70.2 to 81.4)	3.9 (3.1–4.9)	0.1 (0.06–0.1)
	1.6*	95.6 (92.4–97.7)	72.1 (64.8 to 78.7)	3.4 (2.7–4.4)	0.1 (0.04–0.1)		1.6	94.7 (92.2–96.5)	74.5 (68.5 to 79.9)	3.7 (3.0–4.6)	0.1 (0.05–0.1)
	1.7	96.7 (93.8–98.5)	69.2 (61.7 to 76.0)	3.1 (2.5–3.9)	0.05 (0.03–0.09)		1.7	94.9 (92.5–96.7)	74.5 (68.5 to 79.9)	3.7 (3.0–4.6)	0.1 (0.05–0.1)
	1.8	97.1 (94.3–98.7)	66.3 (58.7 to 73.3)	2.9 (2.3–3.6)	0.05 (0.02–0.09)		1.8	96.4 (94.3–97.9)	71.1 (64.9 to 76.8)	3.3 (2.7–4.1)	0.1 (0.03–0.08)
	1.9	97.8 (95.2–99.2)	62.8 (55.1 to 70.0)	2.6 (2.2–3.2)	0.04 (0.02–0.08)		1.9	96.4 (94.3–97.9)	70.7 (64.5 to 76.4)	3.3 (2.7–4.0)	0.1 (0.03–0.08)
	2	98.2 (95.7–99.4)	60.5 (52.7 to 67.8)	2.5 (2.1–3.0)	0.03 (0.01–0.07)		2	97 (95.1–98.4)	64.9 (58.4 to 70.9)	2.8 (2.3–3.3)	0.05 (0.03–0.08)

Cut-offs (to be interpreted as ≤) marked with * were proposed in the classification and regression tree (CART) analyses.
CRP, C reactive protein; PGA, patient global assessment; SDAI, Simplified Disease Activity Index; SJC, swollen joint counts; TJC, tender joint counts .

'*' marks). In ROC analyses, sensitivity and specificity characteristics of PGA cut-offs in 0.1 cm increments from 1.0 to 2.0 are outlined in table 1, supporting cut-offs of CART analyses. The retrieved lower sensitivity and specificity of PGA at 6 months in patients with established RA compared with early RA is in line with the general worse agreement (lower kappa) of Boolean definitions and SDAI definitions in this population. When aiming for high sensitivity of the PGA criterion in modified Boolean definition to coincide with the SDAI definition for all patients with RA, 1.5 seems to be an appropriate cut-off, resulting in similar sensitivity at both time points (95% at 6 months, and 94% at 12 months).

Structural and functional implications of remission definitions

We studied the distribution of HAQ scores at 1 year and of X-ray progression (ΔmTSS) separately for patients in the different Boolean definitions. The radiographic outcomes were independent of the PGA cut-off, and score changes were similar between different definitions (mean ΔmTSS for Boolean1.5, 2.0, 2.5, 3.0 and BooleanX were: 0.38±5.14, 0.41±5.1, 0.37±4.9, 0.34±4.9 and 0.27±4.7). These Boolean definitions also led to similar fraction of patients progressing during the first year (defined as ΔmTSS>0; for Boolean1.0, 1.5, 2.0, 2.5, 3.0 and BooleanX: 39.3%, 39.4%, 38.6%, 38.1%, 37.5% and 37.3%).

In contrast to the expected radiographic data, higher PGA thresholds were accompanied by higher HAQ scores, with BooleanX showing the highest level of functional impairment. The proportion achieving a good functional outcome defined as HAQ≤0.5 was 90.2%, 87.9%, 85.2%, 81.1%, 80.7% and 73.1% for Boolean, 1.5, 2.0, 2.5, 3.0 and BooleanX, respectively; mean HAQ scores were 0.15±0.31, 0.19±0.37, 0.22±0.39, 0.26±0.42, 0.27±0.43 and 0.37±0.52, respectively. Scores in established RA were generally worse than for early RA but the distribution over different Boolean classifications remained similar (figure 3 depicts results for early RA; and online supplementary figure 1 oneestablished RA). The SF-36 physical component scores were distributed like the HAQ scores and were worse when the PGA was completely omitted (green line). The distribution of scores was likewise similar in established RA but appears generally worse than in early.

We have also explored these distribution plots in non-overlapping groups of modified Boolean remitters, so that every patient is attributed to only one definition (eg, Boolean2.0 remitters would not include Boolean1.0 or Boolean1.5 remitters in this analysis). We found distinct distributions of scores on HAQ and SF-36 physical components (online supplementary figure 2). The rate of progressors in mTSS was not different between Boolean and BooleanX patients (40.6% vs 32.0%; p=0.264). However, as the remission threshold for PGA increased, the proportion with good functional outcomes (defined as HAQ≤0.5) decreased and this proportion dropped further when PGA was completely removed (HAQ≤0.5 (n) in Boolean, 1.5, 2.0, 2.5, 3.0 and BooleanX: 92.3% (193), 75.9% (22), 47.1% (8), 33.3% (7), 77.8% (7), 37.3% (19)).

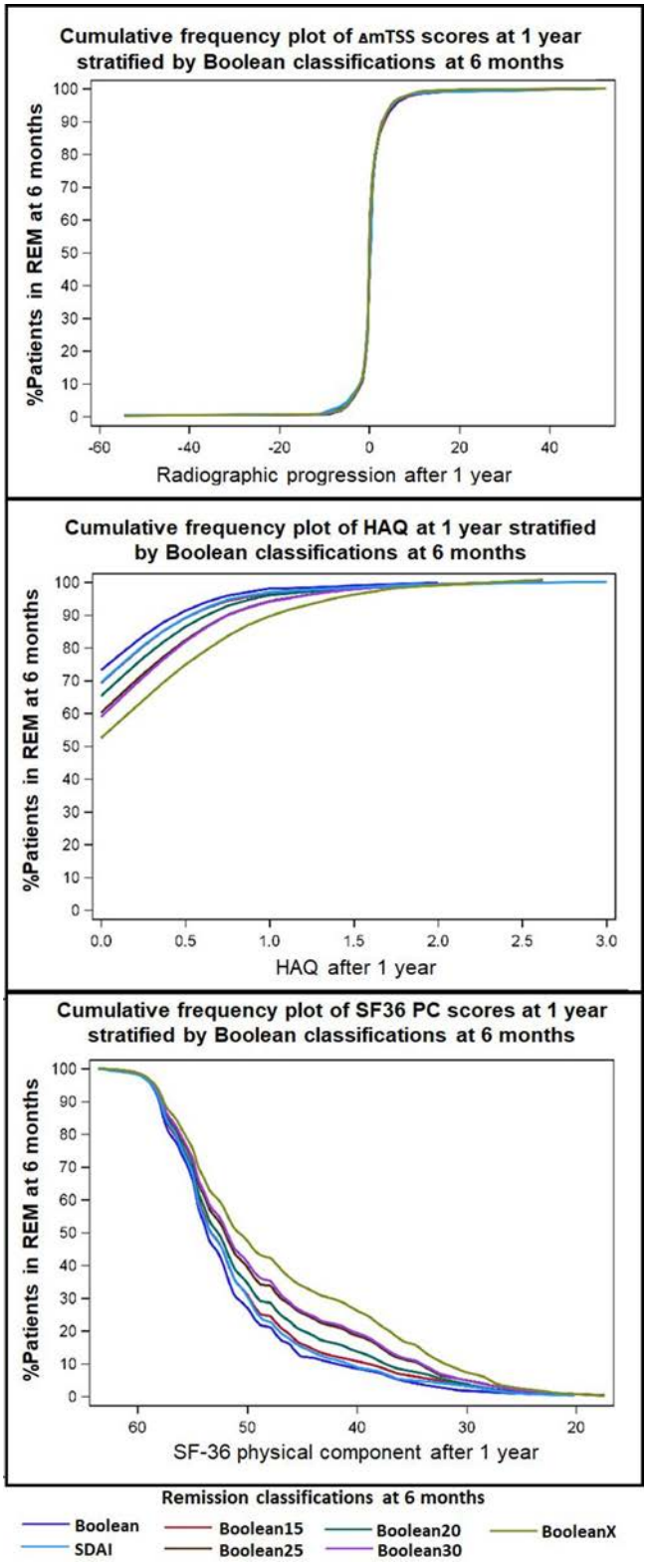

Figure 3 Cumulative frequency distribution of X-ray change (mTSS), HAQ and SF-36 physical component scores in patients with early rheumatoid arthritis (RA), separately by categorisation in modified Boolean remission definitions and Simplified Disease Activity Index (SDAI) remission (overlapping groups).

DISCUSSION

Pooling six different large clinical trials, we evaluated the role of PGA, or its cut-off, in the Boolean remission definition, as well as its impact on outcomes. We used the SDAI remission definition, which is the ACR/EULAR index-based remission criterion, as the comparator in our analyses. Maintaining SJC, TJC and CRP at their maximum cut-point of 1, we tested different levels of PGA as the fourth component of the Boolean criteria to see if higher PGA scores would change overall outcomes.

Generally, in our population, around 40% of the patients showed radiographic progression, in accordance with other

studies. Nevertheless, the mean ᴀmTSS was low, in line with observations of a secular trend of lower progression rates.[35] The observed somewhat high rates of progression in remission can be explained by the latency (or carry-over) effect of disease activity on radiographic progression.[36] Furthermore, since SJC and CRP are associated with joint damage,[13 37] we did not expect to see differences in damage progression rates when higher PGA scores were a component of the Boolean remission criteria, and this was observed in our analyses. In contrast, physical function as assessed by the HAQ, but also by SF-36, deteriorated with increasing the threshold for PGA. However, the difference in good functional outcomes was small when comparing 1, 1.5 and 2 cm ratings of the PGA (about 5% difference in proportions of normative HAQ), while this difference was much larger when PGA was completely excluded. Since remission ought to encompass clinical, structural and functional remission,[11] the omission of the PGA from Boolean criteria is not in line with an optimal understanding of remission. On the other hand, many more patients (+20% in early RA at 6 months) can be classified as in remission by Boolean criteria when the threshold for the PGA is increased from 1 to 2 cm, without a major loss of good outcomes. Still, one may ask if the PGA should be included at all in a definition of remission of inflammation, since functional outcomes, for whatever reason are worse, independent of differences in radiographic progression. Other studies have, however, shown that the HAQ has only a minor influence on PGA score, suggesting there is little reverse causation, whereas pain is the greatest driver of PGA.[20 38] This integration of patient-derived factors and more objective markers provides a robust overall assessment of disease activity. An exclusion would constitute a step back in disease activity assessment. In addition, studies informing the work developing the definition of improvement[1] showed that PGA was usually the outcome measure that best discriminated disease modifying antirheumatic drugs (DMARDs) from placebo, suggesting that PGA provides information on inflammation and its response to treatment. Omitting PGA would compromise the ability to detect treatment efficacy.

One goal of this study was to increase the concordance between two equally applicable definitions of remission. While this may seem to be circular, it can also be seen as a strength, since both definitions have been confirmed to coincide with high predictive validity for the inhibition of bad outcome.[11 39] This constitutes a main reason for targeting remission in the treatment of patients with RA. All trials included in this study have been conducted in the last decade and investigated MTX and TNFi, although nowadays many other DMARD classes are available. In particular, Jak inhibitors have shown fast response; however, Jak-inhibitor trials of the last years outlined 6-month and 12-month Boolean remission rates between 7% and 23%,[40–43] similar to our patients with early RA (12%–20%).

Based on the comprehensive interpretation of the results from the kappa, CART and ROC analyses, increasing the PGA cut-off to 1.5 cm would provide the highest consistency between Boolean and index-based remission, while the integer cut-off of 2 cm (or 2/10) would also allow the use of an integer-based numerical rating scales. We acknowledge that a 2 cm cut-off, instead of 1.5 cm, harbours the risk of lower specificity for remission. However, when considering that in patients, who score a PGA≤1 cm, a smallest detectable difference for the PGA ranging between 1.3 and 1.8 cm has been reported.[44] Another study outlined even a smallest detectable difference of 2.3 cm in the PGA.[45] This suggested new cut-off would discount the stringency of the PGA in the remission context, while keeping the patient perspective as a core element of RA disease activity evaluation, without compromising long-term structural outcomes.

A cut-off beyond 2 cm would not only jeopardise agreement with the index definition and be associated with poorer long-term function but also require other factors to be considered. While mostly pain and partly fatigue influence PGA irrespective of disease activity,[38] pain and fatigue may also reflect active inflammation and thus disease activity in many patients.[46]

Although to a much smaller extent than PGA, it needs to be noted that also joint swelling and CRP levels may not always be accurate: joint swelling may often be doubtful, observer-dependent or related to concomitant diseases, such as osteoarthritis, and increased CRP may be caused by other concomitant diseases, such as undetected infection.[47–49] Analogously, SJC and CRP levels may be elevated even though a patient is in RA remission.[18] Furthermore, certain drugs, such as IL-6- and Jak inhibitors, may normalise CRP irrespective of clinical improvement[50] (and, thus, lead to potential undertreatment with the consequence of joint damage progression and irreversible disability). This may be even more misleading than a high patient global which still necessitates a physician's attention. Its relation to inflammation can be well differentiated from a relation to non-inflammatory abnormalities by most rheumatologists using a patient-centred approach.

This patient-centred approach needs to accompany any clinical consultation and should address the background to situations, where the PGA may indeed be unduly high.[51] The fact that fatigue, pain, anxiety and function influence the variance of the PGA in a state of near remission[33 52 53] also shows that the score represents factors, that would not be covered otherwise and may be influenced by inflammation. Some lack of specificity may be caused by the question phrasing (eg, DAS used to include a PGA on global health, not specifying arthritis-related symptoms). When the PGA does not specify arthritis-related symptoms, it may lead to a misimpression that RA is active while in reality other factors may explain a patient's score.

Other factors that influence the outlined remission criteria need to be considered on application to the respective patient. This has been clearly stated in the treat-to-target recommendations, where recommendation 5 states: "The choice of the (composite) measure of disease activity and the target value should be influenced by comorbidities, patient factors and drug-related risks" and certain comorbidities, such as fibromyalgia, are explicitly mentioned.

Our findings suggest that modifying the cut-off for PGA in the Boolean criteria for remission to 2 (on a scale of 0–10) results in better agreement with the SDAI-based ACR/EULAR definition of remission than when using the current PGA definition of 1. This change should be strongly considered.

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CLINICAL SCIENCE

Is treat-to-target really working in rheumatoid arthritis? a longitudinal analysis of a cohort of patients treated in daily practice (RA BIODAM)

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ABSTRACT

Objectives To investigate whether following a treat-to-target (T2T)-strategy in daily clinical practice leads to more patients with rheumatoid arthritis (RA) meeting the remission target.

Methods RA patients from 10 countries starting/changing conventional synthetic or biological disease-modifying anti-rheumatic drugs were assessed for disease activity every 3 months for 2 years (RA BIODAM (BIOmarkers of joint DAMage) cohort). Per visit was decided whether a patient was treated according to a T2T-strategy with 44-joint disease activity score (DAS44) remission (DAS44 <1.6) as the target. Sustained T2T was defined as T2T followed in ≥2 consecutive visits. The main outcome was the achievement of DAS44 remission at the subsequent 3-month visit. Other outcomes were remission according to 28-joint disease activity score-erythrocyte sedimentation rate (DAS28-ESR), Clinical Disease Activity Index (CDAI), Simplified Disease Activity Index (SDAI) and American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) Boolean definitions. The association between T2T and remission was tested in generalised estimating equations models.

Results In total 4356 visits of 571 patients (mean (SD) age: 56 (13) years, 78% female) were included. Appropriate application of T2T was found in 59% of the visits. T2T (vs no T2T) did not yield a higher likelihood of DAS44 remission 3 months later (OR (95% CI): 1.03 (0.92 to 1.16)), but sustained T2T resulted in an increased likelihood of achieving DAS44 remission (OR: 1.19 (1.03 to 1.39)). Similar results were seen with DAS28-ESR remission. For more stringent definitions (CDAI, SDAI and ACR/EULAR Boolean remission), T2T was consistently positively associated with remission (OR range: 1.16 to 1.29), and sustained T2T had a more pronounced effect on remission (OR range: 1.49 to 1.52).

Conclusion In daily clinical practice, the correct application of a T2T-strategy (especially sustained T2T) in patients with RA leads to higher rates of remission.

Key messages

What is already known about this subject?

- Randomised controlled trials have demonstrated the efficacy of treat-to-target approaches in rheumatoid arthritis. Real life data from cohorts are still needed to support the widespread implementation of treat-to-target (T2T) in clinical practice.

What does this study add?

- In daily clinical practice, the correct application of a T2T-strategy in patients with rheumatoid arthritis (RA) leads to higher rates of remission as compared with not following it.
- Not only in early RA, but also in established RA, following a T2T-strategy leads to higher remission rates.

How might this impact on clinical practice or future developments?

- Rheumatologists should be encouraged to follow a T2T-strategy to contribute to the achievement of higher rates of remission for their patients.

INTRODUCTION

Early diagnosis, prompt commencement of disease modifying anti-rheumatic drug (DMARD) treatment and applying treat-to-target (T2T) strategies are now engrained in rheumatoid arthritis (RA) treatment paradigms. These approaches have substantially improved the outcomes of patients with RA.[1] Remission has been defined and agreed on as the optimal target when managing a patient with RA.[2 3] Reaching the state of remission is associated with reduced radiographic progression and improved functional ability.[4]

Thoroughly monitoring disease activity, adjusting treatment according to a fixed protocol and aiming

at a predefined treatment goal, the so-called T2T-strategy, has advantages over usual care.[5 6] Several strategy studies provide the basis of this evidence, namely the TICORA (Tight Control of RA study)[7] and CAMERA (Computer Assisted Management in Early RA)[8] studies. Subsequently, several strategy studies have incorporated a T2T-strategy in their treatment algorithm in the formal comparison of specific therapies, such as was done in the BeSt (Behandel Strategiëen) study.[9] However, such evidence was gathered in the setting of randomised controlled trials (RCTs), with strict inclusion and exclusion criteria, following strict protocols and all particularities of RCTs. These studies provide the best evidence for the efficacy of T2T as an intervention, but to some extent compromise the generalisability of the findings, when one wants to consider applying them more broadly.

Having formally demonstrated the efficacy of T2T in RCTs, it is important to assess whether this strategy also improves outcomes in unselected patients from daily clinical practice. The first cohort studies focussed on patients with very early disease and confirmed that following a standardised intensive treatment led to improved achievement of remission.[10] Subsequently, some cohort studies have shown that tight-control treatment leads to more rapid remission and higher remission achievement after 1 or 2 years than usual care.[11 12] Nevertheless, the conclusions from these two studies were based on an indirect comparison between two different cohorts (one with T2T applied and another with usual care), with different patient characteristics, and focussed on the remission achievement at 1 or 2 years in the two cohorts. Such a comparison should ideally be made within the same cohort of patients, wherein some patients receive a T2T-strategy while others receive usual care. Real life data from cohorts without strict protocol specifications regarding choice of treatment are still needed to support the widespread implementation of T2T in clinical practice. Furthermore, previous studies have focussed on the achievement of remission at a given time point, for example, 1 or 2 years, ignoring whether or not the remission outcome was achieved in each of the visits throughout the follow-up (eg, three monthly visits, per T2T recommendations). A true longitudinal analysis taking all observations over time into account, both in terms of following T2T or not, and achieving remission or not, reflecting daily clinical practice, has not yet been conducted. Additionally, T2T has not yet been investigated in patients with established RA.

The aim of the present study was to investigate whether following a T2T-strategy leads to more patients with RA meeting the treatment target (remission) in daily clinical practice.

METHODS

Study population

Patients from RA BIODAM (BIOmarkers of joint DAMage), which has been previously described, were included.[13] In brief, RA BIODAM is a 2-year multinational prospective observational study, including patients with a clinical diagnosis of RA and also fulfilling the 2010 RA Classification Criteria,[14] recruited in daily practice from 10 countries from October 2011 to April 2015. To be eligible, patients presented with active disease (44-joint disease activity score, DAS44 >2.4)[15] and were to be started on or changing DMARD treatment, including conventional synthetic DMARDs (csDMARDs) and a first tumour necrosis factor inhibitor (TNFi); patients who had prior biological DMARD (bDMARD) experience were excluded. All patients were included in this analysis. The database used for this analysis was locked in April 2017. The study fulfilled Good Clinical Practice Guidelines and all patients provided informed consent.

Remission

Remission was the outcome of interest. According to the study protocol, patients were monitored every 3 months using DAS44 calculated with the erythrocyte sedimentation rate (ESR).[15] DAS44 remission, that is, DAS44 <1.6[16] was therefore chosen as the main outcome for this analysis. Alternative definitions of remission were also used, namely the 28-joint disease activity score[17] (DAS28-ESR) remission (ie, DAS28-ESR <2.6),[18] the Clinical Disease Activity Index (CDAI) remission (ie, CDAI ≤2.8),[19] the Simplified Disease Activity Index (SDAI) remission (SDAI ≤3.3)[20] and the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) Boolean remission (ie, tender joint count (TJC) ≤1, swollen joint count (SJC) ≤1, C-reactive protein (CRP) ≤1 mg/dL and patient global assessment (PGA) (0 to 10) ≤1).[2] All definitions of remission were binary (yes/no).

Treat-to-target

Participating rheumatologists were required by protocol to follow a T2T-strategy with DAS44 remission (DAS44 <1.6) as benchmark. In order to define whether a T2T-strategy was appropriately followed or not, every visit was checked according to predefined criteria. T2T was considered appropriate: (i) if a patient had already a disease activity score below the target (DAS <1.6) and treatment was not intensified; or (ii) if treatment was intensified on a DAS ≥1.6. Treatment intensification was defined as increasing dosage or adding a drug from the following categories: csDMARDs, bDMARDs or corticosteroids. T2T was considered incorrectly applied if: (i) the target was met and treatment was nevertheless intensified; or (ii) the target was not met and treatment was not intensified.

Additional definitions for T2T were also considered for sensitivity analyses: (i) T2T without corticosteroids, that is, without considering corticosteroids as a treatment intensification; (ii) T2T less strict, that is, considering T2T as adequate as long as the target, DAS44 remission, is met, regardless of whether treatment is nevertheless intensified or not; (iii) T2T-low disease activity (T2T-LDA) using LDA (ie, DAS <2.4)[21] instead of remission as the benchmark.

Furthermore, 'sustained T2T' strategy was defined as following T2T in at least two consecutive visits.

Other relevant clinical information

Age, gender, disease duration, rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) status (positive/negative) and being DMARD-naïve (yes vs no), all collected at baseline, were considered in this analysis as potential effect modifiers or confounders of the relationship of interest. Country of residence was also considered as a potential confounder.

Statistical analysis

The relationship between following T2T at a given visit and meeting the target of remission at the subsequent visit 3 months later was investigated using time-lagged generalised estimating equations (GEE) models. GEE is a suitable technique to make use of all available observations from each patient while adjusting for inherent within-subject correlations of the repeated measurements. Models were time-lagged to allow investigation of the effect of the main predictor of interest (ie, following T2T) on the outcome (ie, remission) with a lag of 3 months; in other words, with the outcome occurring 3 months later. The same analyses were conducted to investigate the effect of sustained T2T on meeting the target of remission. The 'exchangeable' working correlation structure, demonstrating the best fit to the data, was used.

Table 1 Baseline characteristics

	n=571 mean (SD) or n (%)
Age, years	55.7 (12.9)
Female gender	434 (76.0%)
Disease duration, years	6.5 (8.0)
Education, years	12.6 (3.8)
Number of comorbidities	1.2 (1.3)
Rheumatoid factor positivity	370 (68.0%)
Anti-CCP positivity	388 (69.3%)
RF and/or anti-CCP positivity	431 (77.7%)
DAS44 (0–10)	3.8 (1.0)
DAS28-ESR (0–10)	5.2 (1.2)
CDAI (0–76)	26.9 (11.6)
SDAI (0–86)	28.5 (12.4)
Patient global (0–10)	5.7 (2.3)
HAQ (0–3)	1.1 (0.7)
SJC (0–44)	8.4 (6.1)
TJC (0–53)	13.6 (9.1)
ESR (mm/h)	28.7 (22.2)
CRP (mg/dL)	1.5 (2.3)
Number of prior DMARDs	0.9 (1.1)
DMARD naïve	274 (48.0%)
Smoking status	
Never smoker	282 (49.4%)
Current smoker	161 (28.2%)
Ex-smoker	128 (22.4%)
Treatment csDMARD/TNFi started at baseline	
Both	196 (34.6%)
csDMARD only	334 (58.9%)
TNFi only	36 (6.3%)
None	1 (0.2%)
Treatment with oral corticosteroids started at baseline	255 (45%)

anti-CCP, anti-citrullinated protein; CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; csDMARD, conventional synthetic DMARD; DAS44, 44-joint disease activity score; DAS28-ESR, 28-joint disease activity score (with ESR); DMARD, disease modifying anti-rheumatic drug; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; RF, rheumatoid factor; SDAI, Simple Disease Activity Index; SJC, swollen joint count; TJC, tender joint count; TNFi, tumour necrosis factor inhibitor.

As treatment intensification has a central role in T2T, we sought to investigate the extent to which the components of the disease activity scores contributed to it. We therefore investigated the effect of TJC >1, SJC >1, PGA >1 and CRP >1 mg/dL on treatment intensification (yes/no). This analysis was also conducted with GEE, including all above-mentioned disease activity components in one multivariable model.

For each model, interactions between the T2T variable and age, gender, disease duration and RF/ACPA positivity were tested, and if significant ($p<0.15$) and clinically relevant the model was fitted in each subgroup. If these proved to be not relevant, final models were adjusted for potential confounders selected a priori: age, gender, disease duration and country of residence. Stata/SE V.12 was used.

RESULTS

In total, 571 patients were included with a mean age of 56 (SD 13) years, 78% females and a mean disease duration of 6.5 (8.0) years, 37% with a disease duration up to 2 years (table 1). In total, 78% of the patients were RF and/or ACPA positive, and 48% were DMARD-naive at baseline (mean disease duration of 3.6 (5.6), 50% with ≤2 year disease duration). At the end of the baseline visit, almost 60% of the patients were on treatment with csDMARDs only, 35% of the patients on a TNFi with a csDMARD and only 6% on TNFi monotherapy. Almost half of the patients were on corticosteroids after the baseline visit.

T2T was appropriately applied in 59% of 4356 visits. This included 31% of patient visits where DAS44 remission was met and treatment was not intensified, and 28% of visits where treatment was appropriately escalated. In 3% of visits (9% of those with treatment intensification), treatment intensification took place even though DAS44 remission was met (making a total of 31% of the visits with treatment intensification). In the remaining 38% of visits T2T was not being followed as there was no treatment intensification despite active disease (DAS44 ≥1.6) (figure 1).

Throughout the 2-year follow-up period an increasing proportion of patients met remission definitions. At 3 months 24% of the patients were in DAS44 and DAS28-ESR remission, and 8% in ACR/EULAR Boolean remission. At 24 months 52% of the patients were in DAS44 remission, also 52% in DAS28-ESR remission and 27% in ACR/EULAR Boolean remission (figure 2).

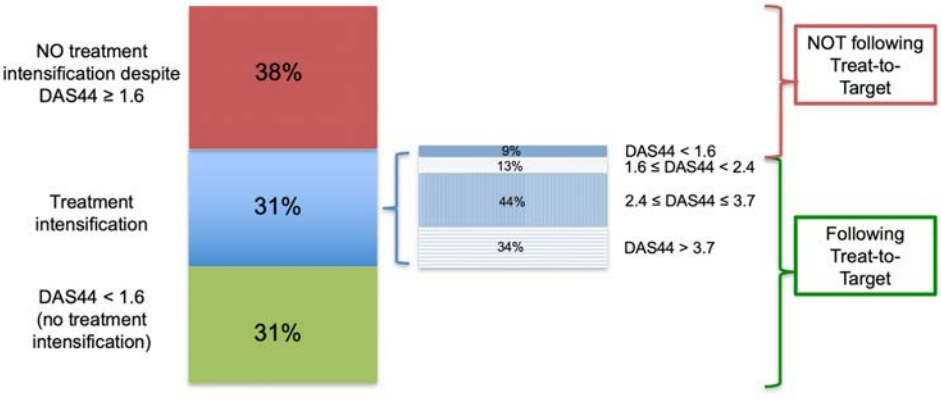

Figure 1 Proportion of the visits (n=4356) in which treat-to-target strategy (with DAS44 <1.6 as benchmark) is followed versus not and the details regarding the proportion of visits with target achievement and/or treatment intensification. Treatment intensification was defined as start or dosage increase of a conventional synthetic or biological disease modifying anti-rheumatic drug or of a corticosteroid. DAS44: 44-joint disease activity score.

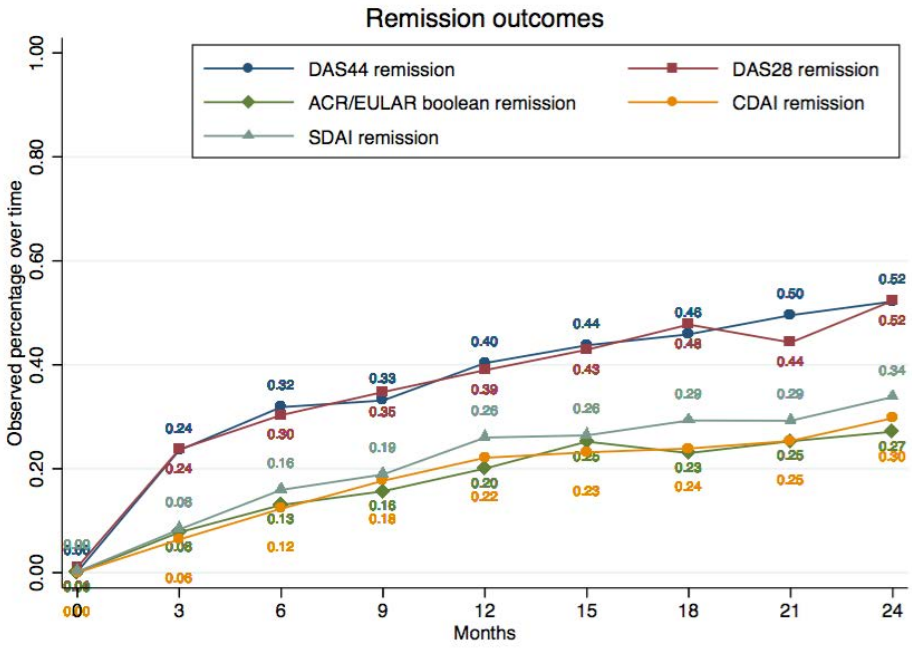

Figure 2 Proportion of achievement of the different remission outcomes throughout the 2-year follow-up. ACR,American College of Rheumatology; CDAI, ClinicalDisease Activity Index; DAS28, 28-joint disease activity score; DAS44, 44-jointdisease activity score; EULAR, European League Against Rheumatism; Simplified Disease Activity Index (SDAI).

T2T on remission outcomes

Following a T2T-strategy, as compared with not following it, was not significantly associated with a DAS44 or DAS28-ESR remission 3 months later (OR (95% CI) 1.03 (0.92 to 1.16) and 1.03 (0.91 to 1.16), respectively), but was significantly associated with ACR/EULAR Boolean remission (OR 1.16 (1.01 to 1.34)) and also with CDAI remission (OR 1.29 (1.12 to 1.49)) and SDAI remission (OR 1.24 (1.08 to 1.41)) (table 2). Results of the sensitivity analyses were similar, except for a slightly stronger association between T2T and remission (REM) outcomes for both 'T2T without corticosteroids' and 'T2T-REM less strict'. With T2T-LDA, with LDA as the benchmark, there was a significant association between T2T and all remission outcomes (OR between 1.3 and 1.4). None of the tested effect modifiers, namely age, gender, disease duration, seropositivity or DMARD naïve (vs not), modified the relationships of interest.

Sustained T2T on remission outcomes

Following a sustained T2T-strategy compared with not following it was associated with remission 3 months later according to all definitions, for example, DAS44 remission OR 1.19 (1.03 to 1.39) or ACR/EULAR Boolean remission (OR 1.49 (1.24 to 1.81)) (table 3).

Relationship between disease activity components and treatment intensification

All disease activity components were significantly associated with treatment intensification, with SJC and TJC showing the strongest associations, also in a multivariable model including all the components: OR 'SJC >1' 3.42 (2.89 to 4.05), OR 'TJC >1' 3.35 (2.72 to 4.11), OR 'PGA >1' 2.14 (1.71 to 2.68) and OR 'CRP >1' 2.00 (1.66 to 2.42).

Table 2 Effect of following treat-to-target strategies on remission outcomes 3 months later*

	DAS44 remission (OR (95% CI))	DAS28-ESR remission (OR (95% CI))	ACR/EULAR Boolean remission (OR (95% CI))	CDAI remission (OR (95% CI))	SDAI remission (OR (95% CI))
T2T	1.03 (0.92 to 1.16)	1.03 (0.91 to 1.16)	**1.16 (1.01 to 1.34)**	**1.29 (1.12 to 1.49)**	**1.24 (1.08 to 1.41)**
T2T without corticosteroids	1.07 (0.95 to 1.20)	1.11 (0.98 to 1.26)	**1.23 (1.06 to 1.42)**	**1.37 (1.18 to 1.59)**	**1.34 (1.17 to 1.53)**
T2T-REM less strict	1.06 (0.94 to 1.19)	1.07 (0.95 to 1.21)	**1.32 (1.13 to 1.53)**	**1.43 (1.22 to 1.67)**	**1.34 (1.17 to 1.54)**
T2T-LDA	**1.26 (1.10 to 1.44)**	**1.36 (1.17 to 1.56)**	**1.27 (1.09 to 1.47)**	**1.39 (1.18 to 1.64)**	**1.36 (1.17 to 1.59)**

T2T without corticosteroids: without considering corticosteroids in treatment intensification. T2T-REM less strict: considering T2T as adequate as long as the target, DAS44 remission, is met, regardless of whether treatment nevertheless intensified or not.
*All models adjusted for age, gender, disease duration and country. T2T was considered being followed: (i) if a patient had already a disease activity score below the target (DAS <1.6; DAS <2.4 for LDA definition) and treatment was correctly not intensified; or (ii) if treatment was intensified on a DAS ≥1.6 (or DAS ≥2.4 for LDA definition).
ACR, American College of Rheumatology; CDAI, Clinical Disease Activity Index; DAS44, 44-joint disease activity score; DAS28-ESR, 28-joint disease activity score (with ESR); ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; SDAI, Simple Disease Activity Index; T2T, treat-to-target; T2T-LDA, T2T-low disease activity; T2T-REM, T2T-remission.

Table 3 Effect of following a sustained treat-to-target strategy on remission outcomes 3 months later*

	DAS44 sustained remission (OR (95% CI))	DAS28-ESR sustained remission (OR (95% CI))	ACR/EULAR Boolean sustained remission (OR (95% CI))	CDAI sustained remission (OR (95% CI))	SDAI sustained remission (OR (95% CI))
Sustained T2T	**1.19 (1.03 to 1.39)**	**1.23 (1.06 to 1.44)**	**1.49 (1.24 to 1.81)**	**1.45 (1.19 to 1.77)**	**1.52 (1.27 to 1.82)**

*All models adjusted for age, gender, disease duration and country. Sustained treat-to-target was considered followed if T2T was followed in ≥2 subsequent visits. T2T was considered being followed: (i) if a patient had already a disease activity score below the target (DAS <1.6; DAS <2.4 for LDA definition) and treatment was correctly not intensified; or (ii) if treatment was intensified on a DAS ≥1.6 (or DAS ≥2.4 for LDA definition).
ACR, American College of Rheumatology; CDAI, Clinical Disease Activity Index; DAS44, 44-joint disease activity score; DAS28-ESR, 28-joint disease activity score; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; LDA, low disease activity; SDAI, Simple Disease Activity Index; T2T, treat-to-target.

DISCUSSION

In the present study we have shown that following a T2T-strategy, and particularly sustained T2T, in daily clinical practice leads to more patients with RA meeting the most stringent remission criteria over time. This is the first comprehensive analysis that considers all available visits of unselected patients who were followed by protocol for a period of 2 years. The results of the analysis provide direct evidence that following T2T, and particularly sustained T2T, immediately results in a higher likelihood of remission at the next visit, 3 months later (the longitudinal interpretation of a T2T-strategy). Moreover, we have for the first time shown that following T2T is also efficacious in patients with established RA, while previous studies focussed on the effect of T2T in patients with early RA.

The strictly temporal relationship between following a T2T-strategy and meeting remission was statistically significant for almost all remission outcomes and for the different T2T definitions used. The exceptions were the DAS44 and DAS28-ESR remission definitions with an interval of 3 months only, while for sustained T2T the relationship with all remission outcomes was statistically significant. The explanation is rather technical: the independent variable (T2T with DAS44 as benchmark) and the outcome (ie, DAS44 remission) include exactly the same disease activity score, which implies that the model becomes inherently auto-regressive. Such a scenario effectively removes the variability in the data necessary to demonstrate efficacy of an intervention. The other definitions of remission are slightly different from the benchmark definition and allow more statistical separation. An alternative explanation is that DAS44 and DAS28-ESR definitions are more lenient in comparison to ACR/EULAR Boolean, CDAI and SDAI remission and are more frequently met even if T2T is not applied.[2] Nevertheless, the signal that a T2T-strategy, and particularly sustained T2T-strategy, increases the likelihood of stringent remission is clear and consistent. Also, these findings became even more evident throughout the follow-up of this study. The proportion of patients achieving remission, regardless of its definition, increased substantially through follow-up (figure 2). Even after 2 years, a plateau has not yet been reached, reassuring clinicians that if we measure disease activity and treat patients effectively over time, high remission rates can be achieved.

These findings come from a population of patients with an average of 6.5 years of disease duration. One may speculate that the effect of following T2T could be even better in early disease. In this study, we have not found any differences between patients DMARD naïve versus not and also according to disease duration, but a lack of statistical power cannot be excluded. Additionally, even patients who were DMARD naïve had a relatively long disease duration (average of 3.6 years), not being the most representative DMARD naïve patients.

If T2T is so clearly associated with clinical remission, as shown here and in the literature,[5 6] why, then, is a T2T-strategy not always followed in clinical practice? Even in this study, with a protocol requiring implementation of T2T, this strategy was 'only' followed in less than two-thirds of the visits. Also within the RA BIODAM cohort, we have shown that, among other factors, the absence of objective signs of inflammation (eg, swollen joints) implied a lower likelihood to follow T2T.[22] Also, in the 10-year follow-up of the BeSt trial, non-adherence to the protocol has been assigned to disagreement with how DAS reflects disease activity (felt to overestimate the real disease activity) and disagreement with the subsequently required step in the protocol.[23] Many clinicians find regularly measuring disease activity too time consuming endeavour and consider it an additional barrier to implementation of T2T.[24 25]

In order to launch new strategies or interventions in clinical practice, the formulation of recommendations, like the T2T recommendations,[26] does not suffice and implementation should actively be promoted. Studies like ours may further corroborate the message that T2T leads to more stringent remission and may help implementation in clinical practice. Appropriate education may also help. The intervention of the TRACTION trial included one educational face-to-face meeting and monthly webinars on the principles and practical advice on implementation of T2T. A substantial improvement in the adherence to T2T was demonstrated with improvement of 46% in the arm following the training programme compared with 14% in the control arm.[27] Still, rheumatologists may report compliance with recommendations but in practice do not always follow them.[28]

Some limitations of this study need to be considered. First, it is designed as an observational study reflecting daily clinical practice with unselected patients contrasting with the reality of RCTs from which most evidence on T2T originates to date. However, one may question how close to daily clinical practice the RA BIODAM cohort really is, with participation from only a few centres per country, several being tertiary referral centres, and with rheumatologists mandated to follow a strict T2T protocol. As in principle, rheumatologists were required to follow T2T per protocol, we have in this study in essence compared the visits in which the protocol was followed to others in which protocol was violated. One can therefore not exclude a bias intrinsic to this comparison. Additionally, detailed reasons for not following T2T have not been adequately registered precluding additional analysis of adherence to T2T versus taking the physician's reasoning into account. Moreover, only patients with active disease were included, and the average baseline disease activity was high. This may preclude the generalisability of the findings to patients with low disease activity, and not answer the question of whether following a T2T-strategy is beneficial in patients

already in low disease activity, given the risks of overtreatment.[29 30] Lastly, when investigating the impact of following a T2T-strategy, one is not only analysing the impact of treatment intensification but implicitly one is evaluating visits in which patients are already in remission, which have accentuated the benefit of T2T. However, it was our aim to investigate the impact of following the T2T-strategy in its whole and not parts of it, as well as to take all disease activity measurements into account as the longitudinal technique chosen properly does. As a main strength, this is a multinational observational study, including unselected patients reflecting daily clinical practice, with the first truly longitudinal analysis addressing the impact of following a (sustained) T2T-strategy.

In conclusion, following a T2T-strategy, and especially sustained T2T, works in daily clinical practice and leads to more patients meeting the target, that is, remission. Rheumatologists should be encouraged to follow a T2T-strategy to contribute to the achievement of higher rates of remission for their patients.

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CLINICAL SCIENCE

Effect on efficacy and safety trial outcomes of also enrolling patients on ongoing glucocorticoid therapy in rheumatoid arthritis clinical trials of tocilizumab or adalimumab or methotrexate monotherapy

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ABSTRACT

Background In rheumatoid arthritis (RA) trials, inclusion of patients on background treatment with glucocorticoids (GCs) might impact efficacy and safety outcomes.

Objectives To determine if inclusion of patients on background GC use influenced efficacy and safety outcomes of RA randomised clinical trials on initiation of tocilizumab (TCZ) or adalimumab (ADA) or methotrexate (MTX) monotherapy.

Methods Data of four double-blind RA randomised controlled trials (AMBITION, ACT-RAY, ADACTA and FUNCTION) with in total four TCZ, one ADA and two MTX monotherapy arms were analysed. Analyses of covariance of changes from baseline to week 24 in efficacy endpoints and radiographic progression up to week 104 were performed, correcting for relevant covariates. Incidence rates of serious adverse events (SAEs) were assessed.

Results No statistically significant differences were found in efficacy parameters between background GC users and non-GC users, except for less radiographic progression associated with GC usage in one MTX arm. SAE rates were not statistically significantly different between GC users and non-GC users in the treatment arms.

Conclusion No effect of including patients on background GC treatment on efficacy and safety trial outcomes was found, with the exception of reduced radiological joint damage in one MTX arm.

Key messages

What is already known about this subject?

- Concomitant treatment with glucocorticoids (GCs) is common among patients with rheumatoid arthritis (RA) in daily practice, as well as in clinical trials.
- Inclusion of patients on background GCs might impact efficacy and safety outcomes of clinical drug trials.

What does this study add?

- No significant differences in severe adverse rates and efficacy parameters between background GC users and non-GC users were found in tocilizumab, adalimumab and methotrexate trial arms, except for less radiographic progression in GC users in one methotrexate trial arm.

How might this impact on clinical practice or future developments?

- Researchers do not have to regard inclusion of patients with RA on background GC use as a major bias in clinical trials when investigating efficacy and safety of tocilizumab, adalimumab or methotrexate.
- Rheumatologists may anticipate the known efficacy and safety of recently initiated tocilizumab, adalimumab and methotrexate, irrespective of background GC use.

INTRODUCTION

The efficacy and safety of low to moderate dose glucocorticoids (GCs) have been established in numerous randomised controlled trials (RCTs) in early rheumatoid arthritis (RA).[1–5] GC use in early RA is endorsed by current European League against Rheumatism recommendations; low-dose GC treatment is generally applied in many patients with active RA despite treatment with disease-modifying antirheumatic drugs (DMARDs).[6] For RCTs, patients on a stable background low-dose GC therapy are generally not excluded: of RA patients included in RCTs, 38%–64% used GC at baseline when initiating infliximab (IFX) or tocilizumab (TCZ).[7] Patients with RA on background GC use had reduced radiographic progression of joint damage in placebo arms of IFX trials.[7] Inclusion of patients with RA on background GC use may improve efficacy outcomes of trials, because GCs reduce RA signs and symptoms.[1–5] However, patients on stable background GC treatment may have more refractory RA and thus may show less clinical improvement in a trial. Background GC treatment might negatively affect the safety in DMARD trials.[8]

The potential effects of patients with RA on GC background use in RA studies including the use of biologicals so far has only been reported in an open-label trial programme with TCZ[9] but has not yet been evaluated in the context of rigorously

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controlled RCTs. Therefore, the aim of this study was to establish whether inclusion of patients with RA on stable background oral GC use influenced efficacy and safety outcomes in RCTs on initiation of TCZ, adalimumab (ADA) or methotrexate (MTX) monotherapy for RA in a rigorously controlled RCT setting.

METHODS

In this post hoc study, we analysed data of individual patients with RA from four double-blind RCTs on initiation of TCZ, ADA and/or MTX monotherapy: AMBITION, ACT-RAY, ADACTA and FUNCTION.[10–13] Study participants were MTX naïve,[10 13] or MTX intolerant,[12] or had an inadequate response to MTX.[11 12] Furthermore, patients were all biological DMARD (bDMARD) naive or, in the case of AMBITION, were either bDMARD naive or had discontinued bDMARDs but were not bDMARD nor MTX irresponsive. FUNCTION excluded patients with an RA duration >2 years. Other selection criteria of these RCTs were similar. GC use at inclusion (background GC use) was allowed,

Table 1 Efficacy outcome measures of included GC users versus non-GC users per initiated monotherapy

	AMBITION				FUNCTION			
	TCZ mono		MTX mono		TCZ mono		MTX mono	
	GC users (n=137)	Non-GC users (n=149)	GC users (n=133)	Non-GC users (n=151)	GC users (n=118)	Non-GC users (n=174)	GC users (n=109)	Non-GC users (n=178)
CDAI change*								
LSM	−26.5	−25.1	−21.8	−20.9	−26.7	−27.5	−24.1	−22.3
LSM difference (95% CI)	−1.4 (−4.8 to 2.1)		−0.9 (−5.5 to 3.6)		0.8 (−2.5 to 4.1)		−1.7 (−6.0 to 2.5)	
CDAI remission†								
n (%)	18 (13.1)	14 (9.4)	9 (6.8)	7 (4.6)	20 (16.9)	40 (23.0)	16 (14.7)	22 (12.4)
OR (95% CI)	1.2 (0.5 to 2.6)		1.4 (0.5 to 4.2)		0.7 (0.4 to 1.3)		1.31 (0.6 to 2.7)	
ACR50†								
n (%)	60 (43.8)	66 (44.3)	45 (33.8)	50 (33.1)	52 (44.1)	87 (50.0)	44 (40.4)	80 (44.9)
OR (95% CI)	0.8 (0.5 to 1.3)		0.9 (0.5 to 1.5)		0.8 (0.5 to 1.3)		0.8 (0.5 to 1.3)	
FACIT-F score*								
LSM	9.4	9.7	7.7	6.9	9.7	9.3	6.5	10.3
LSM difference (95% CI)	−0.3 (−2.5 to 1.9)		0.9 (−1.6 to 3.4)		0.3 (−2.2 to 2.9)		−3.8 (−6.3 to −1.2)	
PCS of SF-36*								
LSM	10.3	10.7	9.1	8.3	10.3	12.1	8.7	9.7
LSM difference (95% CI)	−0.4 (−2.6 to 1.8)		0.8 (−1.2 to 2.8)		−1.8 (−4.0 to 0.4)		−0.9 (−3.3 to 1.4)	
MCS of SF−36*								
LSM	7.5	8.1	5.5	5.8	9.3	9.7	4.0	6.2
LSM difference (95% CI)	−0.6 (−4.0 to 2.8)		−0.3 (−2.8 to 2.3)		−0.3 (−3.1 to 2.5)		−2.2 (−4.9 to 0.6)	

	ADACTA				ACT-RAY	
	TCZ mono		ADA mono		TCZ mono	
	GC users (n=89)	Non-GC users (n=74)	GC users (n=92)	Non-GC users (n=70)	GC users (n=140)	Non-GC users (n=136)
CDAI change*						
LSM	−26.3	−22.1	−19.1	−23.5	−25.4	−26.5
LSM difference (95% CI)	−4.2 (−9.7 to 1.4)		4.3 (−4.0 to 12.6)		1.2 (−4.0 to 6.3)	
CDAI remission†						
n (%)	18 (20.2)	10 (13.5)	9 (9.8)	6 (8.6)	11 (7.9)	10 (7.4)
OR (95% CI)	1.6 (0.7 to 4.3)		3.1 (0.5 to 19.0)		1.0 (0.4 to 2.6)	
ACR50†						
n (%)	48 (53.9)	29 (39.2)	25 (27.2)	20 (28.6)	59 (42.1)	52 (38.2)
OR (95% CI)	1.8 (0.9 to 3.7)		1.1 (0.5 to 2.2)		1.3 (0.8 to 2.1)	
FACIT-F score*						
LSM	11.8	11.0	10.0	11.3	–	–
LSM difference (95% CI)	0.7 (−3.7 to 5.2)		−1.3 (−4.9 to 2.3)		–	–
PCS of SF-36*						
LSM	10.5	11.2	8.8	8.2	–	–
LSM difference (95% CI)	−0.8 (−4.0 to 2.4)		0.6 (−2.4 to 3.5)		–	–
MCS of SF-36*						
LSM	10.5	10.3	5.2	7.4	–	–
LSM difference (95% CI)	0.2 (−4.4 to 4.8)		−2.3 (−6.0 to 1.5)		–	–

The results shown are changes from baseline to week 24 (for CDAI/disease activity score, assessing 28 joints (DAS28)/FACIT-F score/PCS/MCS) or scores at week 24 (CDAI remission/ACR50).
*Analyses of covariance models for CDAI and DAS28 all included baseline GC use (yes/no), and baseline covariates CDAI/DAS28, region, sex and RA duration. For some models, age, race (Asian, black, white, Native American/Pacific Islander and other) and rheumatoid factor (RF) positivity (ADA arm only) are included. Most of the models included interaction terms among these covariates or with GC use. Included interactions with GC use are with region, sex, race and RF positivity.
†Logistic regression models all included baseline GC use (yes/no) and covariate age. All but one included baseline CDAI/DAS28. All but two models included region and gender. Two models included the interaction of age with GC use. Other interactions were age with CDAI, gender with DAS28 and age with RA duration.
‡Analyses of covariance models for patient-reported outcomes for AMBITION/FUNCTION/ADACTA were performed and all included baseline GC use (yes/no) and baseline covariate FACIT/PCS/MCS score. Other covariates included are age, gender, region, C reactive protein (CRP), Health Assessment Questionnaire Disability Index (HAQ-DI), RA duration and baseline DAS28. Most of the models included interaction terms among these covariates or with GC use. Five models included the interaction of baseline score (FACIT/PCS/MCS) with GC use. Other interactions with GC use were with region, age, gender, CRP and HAQ-DI.
ACR50, American College of Rheumatology ≥50% improvement; ADA, adalimumab; CDAI, Clinical Disease Activity Index; FACIT-F, functional assessment of chronic illness therapy fatigue subscale; GC, glucocorticoid; LSM, least squares means; MCS, mental component summary; MTX, methotrexate; PCS, physical component summary; SF-36, 36-item short-form health survey; TCZ, tocilizumab.

if dose was stable for ≥4–6 weeks prior to randomisation and continued unchanged during the first 24 weeks of the trial. We selected as efficacy endpoints Clinical Disease Activity Index (CDAI), a disease activity score assessing 28 joints without acute phase reactant, because of the direct biological effects of TCZ on the reduction of acute-phase reactant levels,[14] American College of Rheumatology 50 (ACR50) response as well as the patient-reported outcomes (PROs) 'functional assessment of chronic illness therapy' fatigue subscale and mental component summary and physical component summary of the '36-item short-form health survey'.

Statistical analyses

Per trial arm, we used analyses of covariance to estimate differences between GC users and non-GC users in changes from baseline to week 24 in efficacy endpoints, CDAI and radiographic progression (modified Total Sharp Score (mTSS) or Genant Modified Sharp Score (GSS)), corrected for relevant covariates (see online supplementary file 1).

In addition, unadjusted CDAI scores over time for GC users versus non-GC users in TCZ, ADA and MTX monotherapy arms were plotted.

Differences in incidence rates of serious adverse events (SAEs) as a group and serious infections by GC use were tested by comparing exact Poisson 95% CI for the rates. P values<0.05 were considered statistically significant. All were intent-to-treat analyses and all statistical tests were two sided and were performed with SAS V.9.4.

RESULTS

Data from a total of 1750 patients with RA were used for analyses, except for radiographic data and PROs, which were only available from FUNCTION and ACT-RAY (n=855) and AMBITION, FUNCTION and ADACTA (n=1474), respectively. The numbers of GC users versus non-GC users were for TCZ arms 484 versus 533, for MTX arms 242 versus 329 and for the ADA arm 92 versus 70, respectively. Baseline characteristics were mostly similar between background GC users and non-GC users in each treatment arm for each study (online supplementary table 1). Baseline mean (SD) GC dosage in mg/day prednisone equivalents was low for all RCTs: 7.4 (2.7) for AMBITION, 7.5 (2.4) for FUNCTION, 6.7 (2.5) for ACT-RAY and 6.4 (2.7) for ADACTA.

Efficacy

The adjusted differences with 95% CIs of CDAI change at week 24 between background GC users and non-GC users in TCZ monotherapy arms of AMBITION, ACT-RAY, ADACTA and FUNCTION were small with values of −1.4 (−4.8 to 2.1), 1.2 (−4.0 to 6.3),–4.2 (−9.7 to 1.4) and 0.8 (−2.5 to 4.1), respectively (table 1). Similarly, differences in CDAI change to 24 weeks were small and 95% CI for the mean differences between GC users and non-GC users in ADA and MTX arms included 0, indicating non-significance. Figure 1 shows the CDAI scores over time for GC users versus non-GC users in TCZ, ADA and MTX monotherapy arms. Differences in CDAI remission rates and ACR50 response rates at 24 weeks between GC users and non-users were also small and 95% CI of ORs included 1 in all arms, indicating non-significance (table 1). Repeated measures analyses up to week 24 showed similar changes in CDAI between GC users and non-GC users in the TCZ arms. Analyses of PROs showed no statistically significant differences between GC users and non-GC users (see table 1).

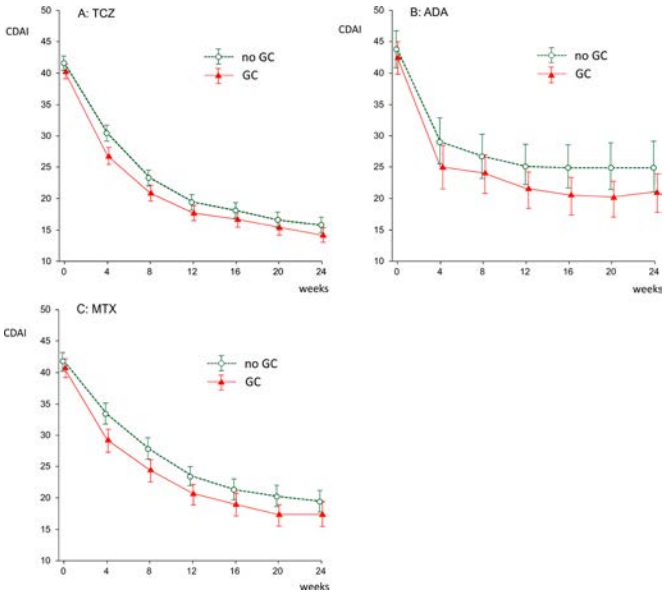

Figure 1 CDAI scores of GC users and non-GC users in TCZ, ADA and MTX monotherapy arms over time. Unadjusted CDAI scores are plotted. CDAI: clinical disease activity index, range 0–76, interpretation 2.9–10 reflects low, 10.1–22 moderate and 22.1–76 high disease activity; mean with 95% CI. For tocilizumab (TCZ) four trial arms, n=533 with no glucocorticoid background use (no GC) and 484 with glucocorticoid background use (GC). For adalimumab (ADA) one trial arm, no GC: n=70, GC: n=92. For methotrexate two trial arms, no GC: n=329, GC: n=242. Intent-to-treat analyses.

Adjusted differences with 95% CI in radiographic change between GC users and non-GC users in the TCZ arm of FUNCTION or ACT-RAY were similar and not statistically significant: in FUNCTION, the adjusted difference in mTSS at week 52 was 0.18 (−0.28 to 0.64), at week 104, 0.32 (−0.73 to 1.36); ACT-RAY: adjusted difference in GSS at week 52 0.5 (−0.0 to 1.1), week 104 0.70 (−0.30 to 1.60). However, in the MTX arm of the FUNCTION trial, adjusted differences in mTSS change from baseline to week 52 and week 104 between GC users and non-GC users were modest but statistically significant: −1.16 (−2.21 to –0.12) and −1.60 (−3.12 to –0.08), respectively, indicating in this trial arm less progression of radiological joint damage in background GC users versus non-GC users.

Safety

The SAE rate among GC users and non-GC users in the TCZ arms was equal (16 vs 16 per 100 patient-years (PYs), as shown in table 2. SAE rate was not statistically significantly different for GC users versus non-GC users in the MTX arms (16 vs 9 per 100 PYs). In the ADA arm, GC users had not statistically significantly different SAE rates nor serious infections rates compared with non-GC users: 37 versus 13 per 100 PYs and 12 versus 5 per 100 PYs, respectively.

DISCUSSION

No effect of including patients with RA on background GC use on efficacy, including PROs, was found for initiating TCZ, ADA and MTX monotherapy, nor for TCZ on radiographic progression. Less radiographic progression was observed for patients on background GC, initiating MTX monotherapy in the FUNCTION trial,[13] an early RA trial conducted among MTX-naïve patients. This finding is in line with results of the Computer Assisted Management in Early Rheumatoid Arthritis trial-II[4] and

Table 2 SAE and serious infection rate of included GC users and non-GC users for initated TCZ, MTX and ADA monotherapy

	TCZ arms		MTX arms		ADA arm	
	GC users n=484	Non-GC users n=533	GC users n=242	Non-GC users n=329	GC users n=92	Non-GC users n=70
All SAEs						
N (%)	30 (6)	34 (6)	13 (5)	9 (3)	12 (13)	4 (6)
Rate per 100 PY (95% CI)	16 (11 to 22)	16 (11 to 21)	16 (10 to 26)	9 (5 to 15)	37 (22 to 59)	13 (4 to 31)
Serious infections						
N (%)	12 (3)	9 (2)	3 (1)	2 (1)	4 (4)	1 (1)
Rate per 100 PY (95% CI)	6 (3 to 10)	4 (2 to 7)	3 (1 to 8)	1 (0 to 5)	12 (5 to 27)	5 (1 to 19)

SAE, serious adverse event; GC, glucocorticoid; TCZ, tocilizumab; MTX, methotrexate; ADA, adalimumab; PY, patient-years.

the Better Anti-Rheumatic PharmacOTherapy study,[15] which showed less radiographic progression in patients with early RA treated with MTX plus GC compared with MTX monotherapy. The finding that there was some progression of radiographic damage in the MTX monotherapy groups in these previous studies[4 15] and our study, as well as in placebo-IFX arms of two pooled IFX trials,[7] but no significant progression in the TCZ groups (data not shown), could explain that no joint sparing effect of GC was found if used concomitantly with TCZ.

Studies on the effect on outcomes of including patients with RA on background GC therapy in bDMARD RCTs are scarce. In an open-label study,[9] efficacy benefits of TCZ were similar between RA patients with and without previous and continued oral GC treatment, with generally similar safety profiles, corroborating our results. In six tofacitinib trials, background GC use did not affect clinical or radiographic efficacy.[16]

In our study, SAE rates and serious infection rates were not statistically significantly different between GC users and non-GC users, initiating TCZ, ADA or MTX monotherapy.

Our study has some limitations. We analysed clinical data a period up to maximally 24 weeks, based on the available trial data. Our research does not answer the question whether initiation of ADA or TCZ together with GC therapy would modify outcome when compared with initiation of ADA or TCZ without GC therapy; this would necessitate randomisation for GC.

In conclusion: no effect of including patients on background GC treatment on efficacy and safety outcomes of trials, initiating TCZ or ADA or MTX monotherapy, was found, with the exception of reduced radiological joint damage in one MTX arm in an early RA population. These findings support inclusion of patients with RA, who are on a low-moderate and stable GC dose, in RCTs, as is common practice.

Contributors MS-K, JWGJ, AP-S and JWJB contributed to the study design. MS-K, AP-S, YL, MDE and JWGJ contributed to data collection. All authors had full access to the study data, contributed to data analysis, data interpretation, writing and review of the manuscript and approved the final version for publication.

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Competing interests MS-K received a student grant from AstraZeneca. AstraZeneca was not involved in this study. AP-S, XT, YL and JD are employees of F Hoffmann-La Roche. MdH is an employee of Novartis. MDE is an employee of Everest Clinical Research. JWJB reported grants and fees form Roche, AbbvVie, Bristol-Myers, Squibb, Merck Sharp & Dohme, Pfizer and UCB. JMvL received fees from Arthrogen, MSD, Pfizer, Eli Lilly and BMS and research grants from Astra Zeneca and Roche-Genentech. JWGJ and PMJW report no competing interests. MJHdH is an employee of Novartis Pharma BV. Novartis Pharma BV was not involved in this study

Patient consent for publication Not required.

Ethics approval Medical ethical approval was not required for this post hoc study.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request at the Roche coauthors.

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TRANSLATIONAL SCIENCE

Pragmatic randomised controlled trial of very early etanercept and MTX versus MTX with delayed etanercept in RA: the VEDERA trial

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ABSTRACT

Objectives We sought to confirm in very early rheumatoid arthritis (ERA) a much greater superiority (30%) of first-line etanercept+methotrexate (ETN+MTX) over treat-to-target MTX (MTX-TT) than previously reported in ERA (14%); and explore whether ETN following initial MTX secures a comparable response to first-line ETN+MTX.

Methods Pragmatic, open-label, randomised controlled trial of treatment-naïve ERA (≤12 months symptom), Disease Activity Score 28 joint (DAS28)-erythrocyte sedimentation rate (ESR) ≥3.2, rheumatoid factor (RF)+/−anticitrullinated peptide antibody (ACPA) positive or ultrasound power Doppler (PD) if RF and ACPA negative. Subjects were randomised 1:1 to ETN+MTX; or MTX-TT, escalated to ETN if week 24 DAS28-ESR ≥2.6 and intramuscular corticosteroid at protocolised time points. Primary endpoint of week 48 DAS28ESR remission with clinical and imaging secondary endpoints.

Results We randomised 120 patients, 60 to each arm (71% female, 73% RF/84% ACPA positive, median (IQR) symptom duration 20.3 (13.1, 30.8) weeks; mean (SD) DAS28 5.1 (1.1)). Remission rates with ETN+MTX and MTX-TT, respectively, were 38% vs 33% at week 24; 52% vs 38% at week 48 (ORs 1.6, 95% CI 0.8 to 3.5, p=0.211). Greater, sustained DAS28-ESR remission observed with ETN+MTX versus MTX-TT (42% and 27%, respectively; p=0.035). PD was fully suppressed by week 48 in over 90% in each arm. Planned exploratory analysis revealed OR 2.84, 95% CI 0.8 to 9.6) of achieving remission after 24 weeks of ETN administered first line compared with administered post-MTX.

Conclusions Compared with remission rates typically reported with first-line tumour necrosis factor inhabitor+MTX versus MTX-TT, we did not demonstrate a larger effect in very ERA. Neither strategy conferred remission in the majority of patients although ultrasound confirmed local inflammation suppression. Poorer ETN response following failure of MTX-TT is also suggested.

Trial registration number
NCT02433184

Key messages

What is already known about this subject?

- In new onset, early rheumatoid arthritis (ERA), biological disease-modifying antirheumatic drug (bDMARD) (with mainly tumour necrosis factor inhibitor (TNFi) tested)+methotrexate (MTX) has not been shown to be superior to MTX+/− additional conventional synthetic DMARD in strategy trials to justify first-line use; although studies to date have not necessarily included all the elements of optimal treat-to-target (TT) strategies. Randomised controlled trial data of targeted synthetic DMARDs (janus kinase (JAK) inhibitors in MTX and bDMARD-inadequate response (IR)), suggest similar pragmatic evaluation is needed to inform on its place.

What does this study add?

- This study did not confirm a large effect size (of 30%) suggested in previous exploratory analysis with first-line TNFi+MTX compared with MTX-TT. This highlights that despite incorporating all the recommended TT strategies in a real-life, treatment-naïve, early(≤12 months symptom) RA cohort, a ceiling effect with both first-line MTX-TT and etanercept-TNFi+MTX exists; that does not appear attributable to ongoing local inflammation (as evidenced by power Doppler ultrasound).
- The data suggest that in a very ERA MTX-TT-IR cohort (compared with longer-duration cohort of previous pivotal MTX-IR trials), a proportion still may not respond to TNFi; implying preceding inflammation and drug exposure may lead to an acquired biology of less TNFi responsiveness.

How might this impact on clinical practice or future developments?

- There is a continued need to understand the basis for this limited response rate and testing of alternative strategies to ensure more complete remission rates are achieved.
- The exploratory observations support research to understand the biology of a very ERA MTX-TT-IR subgroup for future therapeutic opportunities acknowledgements.

INTRODUCTION

Biological disease-modifying antirheumatic drugs (bDMARDs) are established in the treatment of rheumatoid arthritis (RA) but failure of conventional synthetic DMARD (csDMARD), usually methotrexate (MTX), is a minimum hurdle requirement.[1]

Extensive evaluation of first-line csDMARD and bDMARD, mainly tumour necrosis factor inhibitor (TNFi),[2] including pragmatic strategic studies in DMARD-naïve and MTX-naïve cohorts have been contradictory in demonstrating clear benefit of bDMARD.[3–7] Therefore, bDMARDs are still restricted to MTX-inadequate response (IR), which avoids overtreatment.[8] Nevertheless, with first-line bDMARD combination, remission is achieved earlier,[9 10] with benefits for quality of life and jobs,[11] and greater possibility of bDMARD tapering.[12] Exploratory analysis in a previous study suggested a heightened difference in remission rate (of 30%) with first-line bDMARD compared with MTX in very early RA (ERA).[13] None of the treatment strategies achieve remission in the majority and remission rates are virtually always higher when drug is used first-line.[14]

The Very early Etanercept and MTX versus MTX with Delayed Etanercept in RA (VEDERA) study aimed, in a real-life cohort with treat-to-target (TT) strategies, to determine whether initial etanercept (ETN) and MTX compared with MTX-TT, conferred a larger than standard effect (30%) in very ERA and to explore the performance of ETN when administered first-line or following MTX.

PATIENT AND METHODS

VEDERA was a pragmatic investigator-initiated study conducted at Leeds Teaching Hospitals NHS Trust rheumatology outpatient department (full protocol details published[15]). All patients gave their written, informed consent to take part. Independent lay individual from our public and patient advocacy group provided input into study design.

Patients

Eligible patients were ≥18 years, had new-onset ERA fulfilling 2010 American College of Rheumatology/European League against Rheumatism (ACR/EULAR) RA classification criteria[16]; no prior DMARD therapy; ≤12 months symptom duration; disease activity score 28 joint (DAS28)-erythrocyte sedimentation rate (ESR) ≥3.2 with clinical evidence of synovitis; positive anticitrullinated peptide antibody (ACPA) and/or rheumatoid factor (RF), or if RF and/or ACPA negative, evidence on hand ultrasound (US) of power Doppler (PD) defined as grade ≥1 in at least one joint.

Study design

VEDERA is a single-centre, phase IV, open-label, two-arm, randomised controlled trial in patients with ERA. Patients were block randomised 1:1 to first-line ETN+MTX or first-line MTX-TT for a total duration of 48 weeks.

ETN+MTX regimen: intramuscular (IM) depomedrone 120 mg, subcutaneous ETN 50 mg weekly and oral MTX 15 mg weekly, increased to 20 mg and 25 mg weekly at weeks 4 and 8, respectively. MTX-TT protocol: IM depomedrone and oral MTX monotherapy 15 mg weekly, increased to 25 mg weekly at 2 weeks. If not in low disease activity (LDA) (DAS28-ESR ≤3.2) weeks 8, 12, 16 or 20, oral sulfasalazine (SSZ) 1 g two times per day and hydroxychloroquine (HCQ) 200 mg daily were added to MTX. At week 24, if DAS28-ESR ≥2.6, ETN was added to MTX (MTX-TTb), and SSZ and HCQ were discontinued. IM depomedrone 120 mg was administered in both arms at week 12 if DAS28-ESR ≥3.2, weeks 24 and 36 if DAS28-ESR ≥2.6. Subcutaneous MTX was administered with intolerance to oral MTX. All patients received folic acid 5 mg each day (except day of MTX). Stable doses of oral glucocorticoids (≤10 mg/day of prednisone or equivalent) and/or a single non-steroidal anti-inflammatory drug were permitted.

All patients on ETN at week 48, stopped the ETN. Patients were treated as per standard practice with 48-week observational follow-up and established on bDMARD if they fulfilled National Institute for Health and Care Excellence (NICE) criteria (DAS28 >5.1)[17] (with ETN prescribed unless already tried and failed during the trial).

Blinding

Trained research nurses blinded to allocation performed assessments throughout the study. US assessments were performed by an ultrasonographer blinded to allocation.

Outcomes and assessments

The primary endpoint was the proportion who at week 48 achieved DAS28-ESR remission (DAS28-ESR ≤0.6). Multiple secondary endpoints: proportion achieving at weeks 12, 24, 48 and 96 (only to be inferentially compared at week 96): DAS44 remission, DAS28 remission,[18] Simplified Disease Activity Index (SDAI), Clinical Disease Activity Index (CDAI),[19] ACR and EULAR response[20] Boolean remission rates[21]; time to sustained remission (SR; defined as DAS28-ESR (or DAS44, SDAI, CDAI) remission observed at ≥2 consecutive visits within weeks 12, 24, 36 and 48); change from baseline in Health Assessment Questionnaire Disability Index (HAQ)[22] and normalisation of HAQ (to <0.5); change from baseline in Visual Analogue Scales (VASs) for patient pain, and patient and physician global assessments of disease activity, EuroQoL-5 Dimensions-3 Level[23] and Rheumatoid Arthritis Quality of Life[24]; cumulative steroid dose. High-resolution US of dominant hand MCPs 1–5/wrists (or hand with greater evidence of inflammation) at weeks 0, 12, 24 and 48 to assess for synovitis, using semiquantitative (0–3) scores of Grey Scale (GS) and PD, and for presence of erosions.[25] One of two assessors scanned the participants, with a third scanning <10%. Plain radiology of hands and feet to determine change in total van der Heijde modified Sharp score[26] at weeks 48 and 96. The mean of scores by two independent readers who knew the order of the films but were blind to allocation was used and any significant disagreement adjudicated by a third reader. Finally, change in MRI synovitis score at 12, 24, 48 and 96 weeks (to be reported separately).

Safety

Adverse events (AE) and serious AE (SAE) were recorded during the 48-week protocolised treatment strategies.

Statistical analysis

Sample size and power calculation

The Comparison of methotrexate monotherapy with a combination of methotrexate and etanercept in active, early, moderate to severe rheumatoid arthritis (COMET) studyubgroup remission rates of patients with ≤4 months since diagnosis treated with ETN+MTX or MTX monotherapy were 70% vs 35%, respectively.[13] We expected remission rates in VEDERA patients, recruited at <12 months symptom onset rather than diagnosis, would be similar to the COMET very ERA subgroup (of less than 4 months since diagnosis). Therefore, remission rate in ETN+MTX was anticipated at 70% and 40% in MTX-TT (delayed or deferred ETN); at 1-beta=0.8, alpha=0.05 and accounting for 10% drop-out, 49 patients per arm were required, increased to 60 per arm to allow for an exploratory subgroup analysis of ETN+MTX compared with MTX-TTa (csDMARD throughout) and MTX-TTb (delayed ETN). We estimated that 50% of MTX-TT patients would require delayed ETN.

Analysis

Complete plan can be found in the online supplementary material.

The full analysis set (intention to treat (ITT)), for efficacy and safety, included all patients randomised, as randomised, with per protocol (PP) set comprising all ITT patients with primary endpoint data available and no major protocol violations. Two-sided tests were conducted throughout at the 5% level of significance. The Holm correction (modified Bonferroni) to control for multiple comparisons of secondary outcomes set the critical p value for testing significance at the 5% level to $p<0.00088$.

Primary outcome

The primary analysis compared the proportions achieving DAS28ESR <2.6 between the ETN+MTX and MTX-TT arms using Pearson's χ^2 test. The ORs and 95% CI for the odds of achieving DAS28-ESR remission is reported. A number of planned sensitivity analyses of the primary outcome were conducted (detailed in results).

Secondary outcomes

Proportions achieving remission, ACR or EULAR response at 96 weeks were compared between groups using Pearson's χ^2 tests (with descriptive evaluation for the other time points). Changes in continuous variables were analysed using linear multilevel modelling. Baseline values were included as covariates. An exponential autoregressive within-subject covariance pattern was found to be optimal using Akaike information criterion values after inspection of correlations between repeated observations. Severely skewed US and radiographic variables were analysed using quantile regression. Time to SR (as defined in 'outcomes and assessment') was analysed using log-rank tests.

Additional planned analyses

The response in ETN+MTX over the first 24 weeks was compared with the response in MTX-TTb (delayed ETN) over weeks 24–48. Proportions requiring escalation to triple therapy and bDMARD have been presented, as has cumulative IM steroid dose up to week 48.

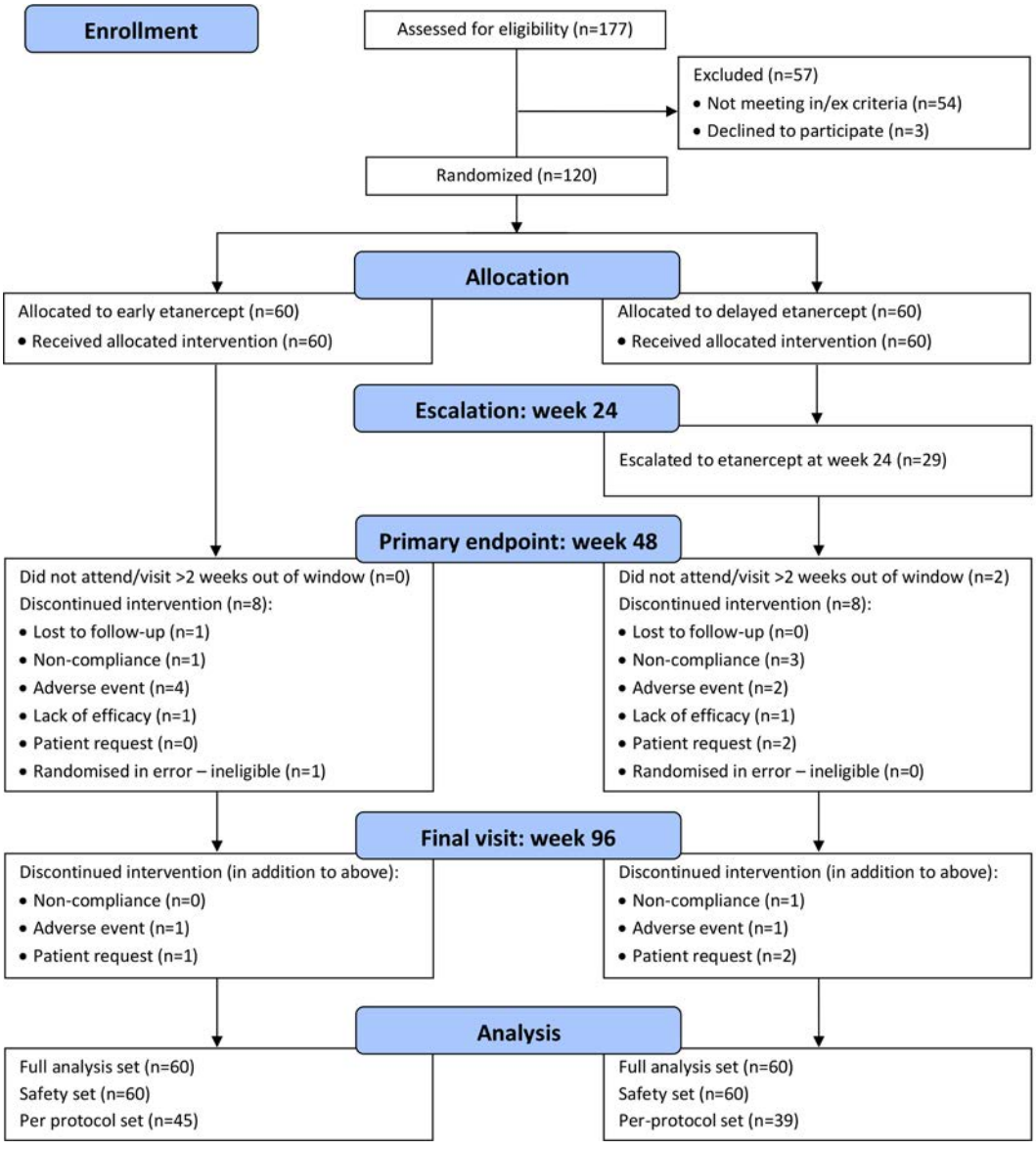

Figure 1 CONSORT flow diagram participant flow diagram up to week 96. CONSORT, Consolidated Standards of Reporting Trials.

Table 1 Baseline demographics and disease profile for the entire group, ETN+MTX and MTX-TT

Variable	All	ETN+MTX	MTX-TT
Demographics			
Age, years Mean (SD)	50.0 (12.8)	49.6 (12.5)	50.3 (13.2)
Female % (n/N)	71 (85)	65 (39)	77 (46)
RA presenting history, % (n/N) (unless otherwise stated)			
Symptom duration, weeks, median (Q1, Q3)	20.3 (13.1 to 30.8)	19.2 (12.5 to 28.1)	20.8 (15.9 to 31.9)
Previous IM steroid	1 (1/120)	0 (0/60)	2 (1/60)
Previous IA steroid	0 (0/120)	0 (0/60)	0 (0/60)
Concomitant oral steroid	3 (3/120)	0 (0/60)	5 (3/60)
Concomitant NSAID	88 (105/120)	92 (55/60)	83 (50/60)
RA disease phenotype, % (n/N)			
RF positive	73 (87/120)	70 (42/60)	75 (45/60)
ACPA positive	84 (101/120)	82 (49/60)	87 (52/60)
ANA positive	15 (18/120)	18 (11/60)	12 (7/60)
RA disease activity components, Median (Q1, Q3) (unless otherwise stated)			
TJC28	11.0 (7.0, 17.0)	11.5 (6.0, 20.0)	10.0 (7.0, 16.0)
SJC28	5.0 (2.0, 9.0)	5.0 (3.0, 10.5)	5.0 (2.0, 9.0)
ESR, mm/hour	31.5 (18.5 to 51.0)	30.5 (17.0 to 51.5)	32.5 (20.5 to 51.0)
CRP, mg/L	8.8 (2.3, 24.0)	10.2 (1.8, 28.0)	8.0 (2.7, 21.5)
Disease activity VAS, mm Mean (SD)	57.1 (22.3)	60.7 (21.6)	53.6 (22.6)
RA disease activity scores, Mean (SD)			
DAS28-ESR	5.7 (1.1)	5.8 (1.1)	5.6 (1.0)
DAS44-ESR	3.7 (0.8)	3.7 (0.9)	3.7 (0.7)
DAS28-CRP	5.1 (1.2)	5.2 (1.2)	4.9 (1.1)
DAS44-CRP	3.4 (0.8)	3.5 (0.9)	3.3 (0.8)
SDAI	31.6 (13.7)	34.2 (14.7)	29.0 (12.3)
CDAI	29.8 (12.7)	32.2 (13.6)	27.3 (11.2)
Patient-reported outcome measures, Mean (SD) (unless otherwise stated)			
Global pain VAS, mm	53.5 (24.5)	59.0 (23.4)	48.1 (24.6)
HAQ-DI	1.2 (0.5)	1.2 (0.5)	1.1 (0.5)
RAQoL	17.3 (7.3)	16.8 (7.4)	17.9 (7.2)
In paid work % (n/N)	73 (88/120)	82 (49/60)	65 (39/60)
EQ-5D-3L index	0.5 (0.3)	0.4 (0.3)	0.5 (0.3)
RAWIS	18.2 (6.6)	19.0 (6.7)	17.3 (6.4)
Ultrasound scores Median (Q1, Q3)			
Total GS score	2.0 (0.0, 5.0)	3.0 (0.5, 5.0)	2.0 (0.0, 5.0)
Total PD score	0.0 (0.0, 2.5)	0.0 (0.0, 3.0)	0.0 (0.0, 2.0)
Total erosion score	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)
Radiographic score median (Q1, Q3)			
Total modified Sharp score	2.5 (0.5, 6.0)	2.0 (0.5, 5.0)	2.5 (0.5, 6.3)

ACPA, anticitrullinated protein antibody; ANA, antinuclear antibody; CDAI, Clinical Disease Activity Index; CRP, C reactive protein; DAS28, Disease Activity Score 28 joint; EQ-5D-3L, EuroQoL-5 Dimensions-3 Level; ESR, erythrocyte sedimentation rate; ETN, etanercept; GS, Grey Scale; HAQ-DI, Health Assessment Questionnaire Disability Index; IA, intra-articular; IM, intramuscular; MTX, methotrexate; NSAID, non-steroidal anti-inflammatory drug; PD, power Doppler; RA, rheumatoid arthritis; RAQoL, Rheumatoid Arthritis Quality of life Questionnaire; RAWIS, Rheumatoid Arthritis Work Instability Scale; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; SJC, swollen joint count; TJC, tender joint count; TT, treat-to-target; VAS, Visual Analogue Scale.

Additional unplanned remission analyses

The ACR/EULAR remission criteria[27] were 'provisional' in 2011; hence not included as an outcome of the trial. Nevertheless, additional, unplanned, descriptive data comparing ACR/EULAR remission between groups at week 48 are presented.

The online supplementary file details handling of missing data.

RESULTS

Patient disposition

Of 177 patients screened between October 2011 and October 2015, 120 patients were recruited and randomly assigned to receive ETN+MTX (n=60) or MTX-TT strategy (n=60) (see online supplementary table S1). One hundred and four (87%) subjects reached week 48 and 98 (82%) week 96 (figure 1). Reasons for exclusion from PP efficacy set, withdrawals, inclusion and exclusion criteria for all screened patients are included in the online supplementary table S1.

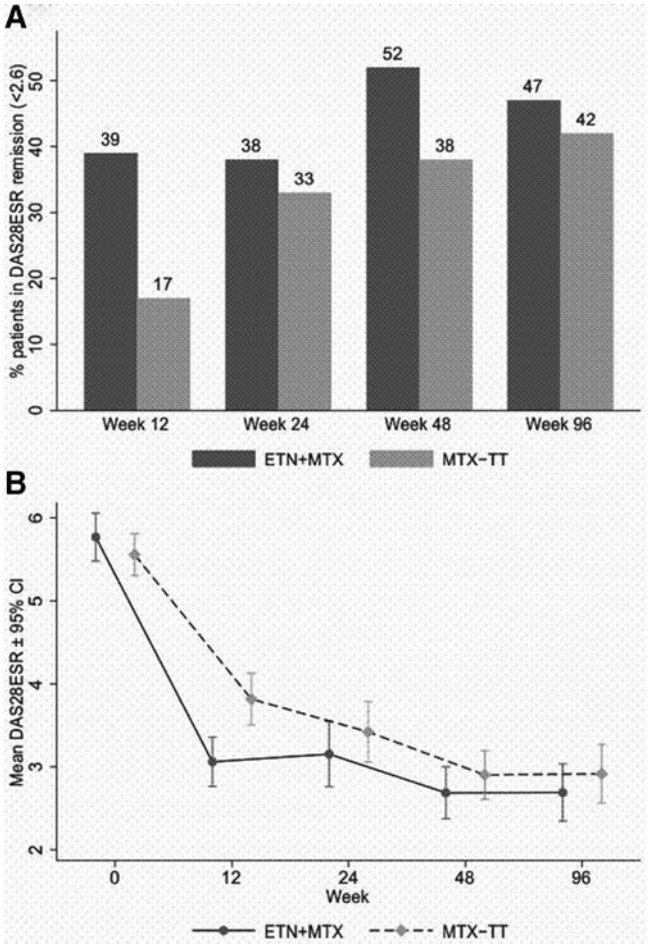

Figure 2 (A) DAS28-ESR remission rates in ETN+MTX (n=60) and MTX-TT (n=60). Percentage patient estimated via multiple imputation. (B) Individual DAS28-ESR scores over time in ETN+MTX (n=60) and MTX-TT (n=60). DAS28, Disease Activity Score 28 joint; ESR, erythrocyte sedimentation rate; ETN, etanercept; MTX, methotrexate; TT, treat-to-target.

Baseline demographics and characteristics

Patient demographics and baseline characteristics (table 1) were comparable between the two arms, and were representative of a new-onset, treatment-naïve, high disease activity ERA population. Seventy-two per cent (81/113) had evidence of erosive disease (any Sharp erosion score >0). Only four patients had any prior steroid exposure reflecting a treatment-naïve inception cohort.

Primary endpoint

Of the full analysis set, 52% ETN+MTX vs 38% MTX-TT achieved DAS28-ESR remission at week 48 (OR 1.73, 95% CI 0.81 to 3.70) p=0.160) (figure 2A; online supplementary table S2). Sensitivity analyses supported this main analysis, except when assuming that those with missing data treated with ETN+MTX responded while those receiving MTX-TT did not (see online supplementary table S3). Only under this assumption did we observe the disproportionately large difference (30%; 63% ETN+MTX vs 33% MTX-TT) expected.

Secondary endpoints

Clinical outcomes

DAS remission rates

Thirty-nine per cent receiving ETN+MTX achieved remission at week 12 vs 17% receiving MTX-TT (OR 3.18, 95% CI 1.35 to 7.50); figure 1B). By week 24, the groups were similar (see online supplementary table S2). Between-group differences in alternative DAS-based remission criteria were descriptively similar to DAS28-ESR remission (see online supplementary tables S4–S6). At week 96, there were no statistically significant differences between the groups in remission rates; continuous DAS scores (unplanned descriptive analysis) were similar across arms (see online supplementary table S7).

Boolean remission and DAS28ESR LDA rates (unplanned analysis)

Differences in the proportions achieving ACR/EULAR Boolean remission and DAS28ESR LDA were consistent with those reported for DAS28ESR remission (see online supplementary tables S8–S9).

Sustained remission

Sustained (DAS28-ESR) remission was achieved earlier in the ETN+MTX group compared with MTX-TT (after 24 vs 36 weeks, in 42% vs 27%, respectively, p=0.035); but at the corrected significance threshold (p<0.0008) this was not statistically significant (see online supplementary table S10).

EULAR and ACR response rates

ETN+MTX arm achieved earlier EULAR and ACR responses compared with MTX-TT; but response rates were comparable by week 48, maintained at week 96 (see online supplementary tables S11–S15).

Planned exploratory analysis of early and delayed ETN + MTX

At week 24, 29 patients in MTX-TT arm had not achieved DAS28-ESR remission, and switched to ETN+MTX. One received only one dose of ETN and was excluded from subgroup analysis. Response to 24 weeks duration ETN+MTX exposure if received early (ETN+MTX) versus delayed (MTX-TTb), (with resetting of 'baseline' DAS28 in MTX-TTb to week 24) revealed an adjusted OR (95% CI) of achieving DAS28-ESR remission of 2.84 (0.84 to 9.60) (figure 3).

MTX-TTb (delayed ETN) was on average, in a moderate disease activity state at week 48 (mean (SD) DAS28-ESR (3.21 (1.12)). MTX-TTa (csDMARD throughout) maintained remission state at week 48 (2.58 (0.97)) (see online supplementary figure S1).

Patient-reported outcome measures

Tests of differences across the course of the trial revealed no statistically significant differences in functional improvement (figure 4A; online supplementary tables S16–S17), overall quality of life (see online supplementary table S18); patient VAS (figure 4B,C; online supplementary tables S19–S20), disease-specific quality of life (figure 4D; online supplementary table S21) or work instability (see online supplementary table S22).

Imaging outcomes

There were no statistically significant differences in total mTSS scores between the treatment arms at weeks 48 and 96, with minimal changes on average in both groups (see online supplementary table S23 and figure S2).

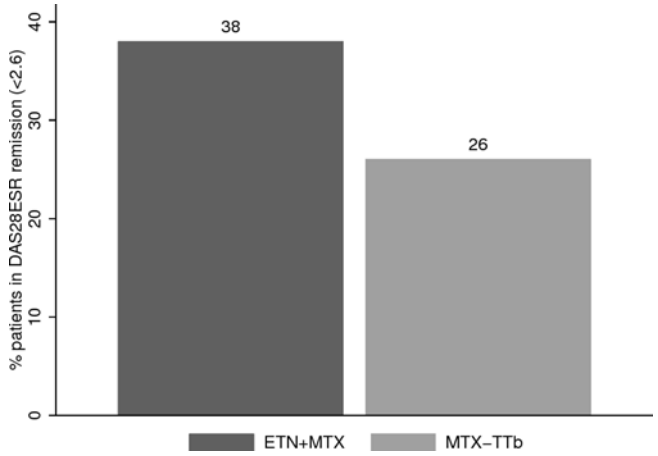

Figure 3 Proportion of patients achieving DAS28-ESR remission following 24 weeks ETN exposure, either received first-line (ETN+MTX) or following failure to achieve remission on MTX-TT (MTX-TTb). Percentage patient estimated via multiple imputation. DAS, Disease Activity Score 28 joint; ESR, erythrocyte sedimentation rate; ETN, etanercept; MTX, methotrexate; TT, treat-to-target.

US GS and PD scores decreased at week 12 in both arms, with no notable differences (table 2; online supplementary figures S3–S4). Comparable proportions in each arm had GS >0 and PD >0 at each time point. Over 50% in each arm scored GS >0 from baseline to week 48. In contrast, the proportion with PD >0 diminished rapidly by week 12%–15% in each arm, maintained to week 48 (table 2). By week 48, the median number of erosions was 0 in both arms and the 90th percentile did not differ between ETN+MTX (0.38) and MTX-TT (0.78) (see online supplementary table S24 and figure S5).

Intervention period DMARD changes

Comparable cumulative IM glucocorticoid doses were administered in each arm (see online supplementary table S25). In MTX-TT, 53/60 (88%) escalated to triple therapy by week 24 (three-quarters by week 12) in line with the 48-week randomised treatment protocol. In all patients receiving ETN at week 48, ETN was stopped (total 91; 60 and 31 in ETN+MTX and MTX-TT, respectively). Four patients (ETN+MTX) withdrawn prior to week 48 consented to continued observational follow-up; all four were escalated to double/triple csDMARD therapy by week 48 (see online supplementary table S26).

Observational period DMARD changes

On cessation of ETN (in ETN+MTX arm) at week 48 25/60 escalated to at least one additional csDMARD, with six on triple therapy by week 96. Three patients in ETN+MTX arm were commenced on a bDMARD (two adalimumab and one abatacept) as per NICE guidelines (DAS28 >5.1) and one patient in MTX-TT (ETN) (see online supplementary table S26).

On withdrawal of ETN in the ETN+MTX arm, DAS28-ESR remission rate from week 48 to week 96 dropped by only 4% (with addition of csDMARD as above; figure 2).

Safety

A number of AEs per 100 patient-years in the ETN+MTX and MTX-TT arms were 413.6 and 509.6, respectively, most frequently infections and gastrointestinal events (numerically higher in MTX-TT). A number of SAEs per 100 patient-years were 10.6 and 5.9 in ETN+MTX and MTX-TT, respectively.

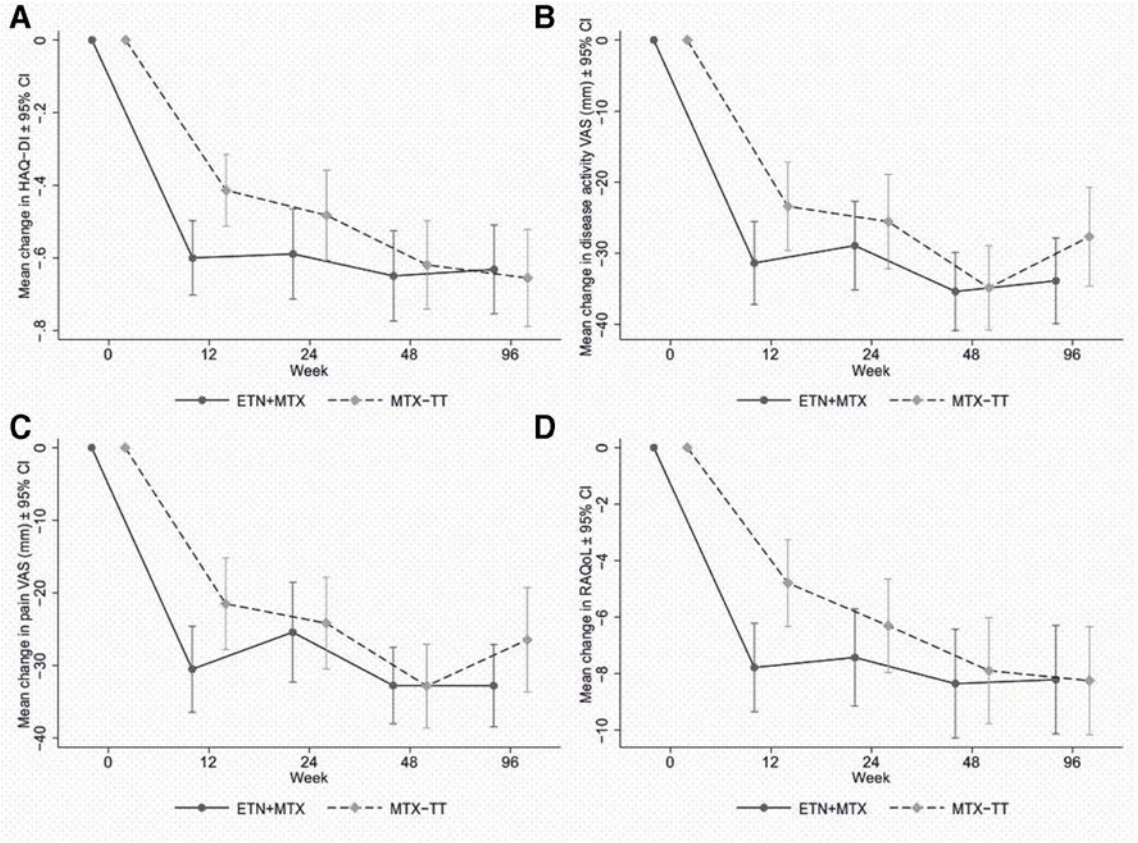

Figure 4 Patient-reported outcomes over time. ESR, erythrocyte sedimentation rate; ETN, etanercept; HAQ-DI, Health Assessment Questionnaire Disability Index; MTX, methotrexate; RAQoL, Rheumatoid Arthritis Quality of Life; TT, treat-to-target; VAS, Visual Analogue Scale.

Withdrawals due to AE/SAE up to week 48 occurred in six subjects (three SAEs, pulmonary embolism (ETN+MTX), pneumonia and acute appendicitis (both MTX-TT); and three AEs, neutropaenia and palmoplantar pustulosis (both ETN+MTX) and general non-specific symptoms (MTX-TT)). Online supplementary table S27 details all AE and SAE.

DISCUSSION

This study was not designed to demonstrate the standard level of superiority with first-line ETN-MTX such as was observed in COMET.[10] We aimed to validate the post hoc analysis of COMET[13] that suggested a much larger effect (30%) of ETN-MTX compared with MTX-TT in patients at the earliest stages of their RA, which we did not confirm in our study. A 14% difference was instead observed, which is consistent with the smaller, but still clinically relevant, effect reported for ERA.[10] Escalation to ETN in those that failed to achieve remission with MTX-TT at 6 months may not secure a comparable response to first-line ETN, possibly suggesting reduced TNFi-responsiveness.

Table 2 Total Grey Scale (GS) and power Doppler (PD) ultrasound scores

Total GS						
	Estimated % GS >0		**Estimated median (95% CI)**			
Visit	**ETN+MTX (n=60)**	**MTX-TT (n=60)**	**ETN+MTX (n=60)**	**MTX-TT (n=60)**	**Difference (95% CI)**	**T value, p value**
Baseline	75	70	3.00 (1.96 to 4.04)	2.00 (0.70 to 3.30)		
Week 12	56	69	1.00 (0.48 to 1.52)	2.00 (1.22 to 2.78)	1.00 (0.14 to 1.86)	t=2.29, p=0.024
Week 24	60	58	1.00 (0.22 to 1.78)	1.18 (0.02 to 2.34)	0.18 (–1.21 to 1.57)	t=0.26, p=0.797
Week 48	53	53	0.96 (0.32 to 1.60)	0.94 (0.21 to 1.67)	–0.02 (–0.98 to 0.94)	t=–0.04, p=0.967
Total PD						
	Estimated % PD >0		**Estimated 90th percentile* (95% CI)**			
Visit	**ETN+MTX (n=60)**	**MTX-TT (n=60)**	**ETN+MTX (n=60)**	**MTX-TT (n=60)**	**Difference (95% CI)**	**T value, P value**
Baseline	47	45	5.00 (1.93 to 8.07)	4.00 (0.93 to 7.07)		
Week 12	15	15	1.00 (–0.80 to 2.80)	3.00 (–0.07 to 6.07)	1.82 (–1.79 to 5.43)	t=1.00, p=0.320
Week 24	13	18	1.02 (–1.19 to 3.23)	1.68 (–0.66 to 4.02)	0.42 (–2.77 to 3.61)	t=0.26, p=0.795
Week 48	8	13	0.06 (–2.62 to 2.74)	0.70 (–1.13 to 2.53)	0.46 (–2.28 to 3.20)	t=0.33, p=0.739

*Median was 0 in both groups at all visits. Unplanned use of 90th percentile instead of median as point of comparison.
ETN, etanercept; MTX, methotrexate; TT, treat-to-target.

While remission is the goal in early, new-onset RA[1] 60% receiving MTX-TT did not achieve this by week 48 (comparable to other studies that report 30%–60%[28–30]; and only 50% in the ETN+MTX arm, lower than predicted.[13 30] A more positive interpretation, namely, 40% and 50% achieving remission, respectively, still highlights what we would consider suboptimal rates for the contemporary era. Our study optimised design features that could contribute to reduced response including ERA defined by symptom as opposed to disease duration and all DMARD-naive not MTX-naive[5 10 28 30]; expedient MTX, csDMARD and bDMARD escalation and adjunctive corticosteroid use.[31] MTX intolerance does not appear to explain the findings, with minimal drop-out in both arms (with n=2 AE and n=3 non-compliance in MTX-TT and n=4 and 1, respectively, ETN+MTX).

Our study eligibility aligned with clinical practice, representing a real-world population. Half the cohort had at least one comorbidity, and 20% at least two. This may have partly driven the generally poorer than expected performance[32 33]; the exact mechanisms for which are unclear.

The suboptimal remission rates did not appear to be driven by joint-related inflammation; as evidenced by US PD suppressed in both arms to <13% with any PD by week 48. GS persisted in over half the cohort (in particularly, the wrist), likely indicating normal background GS in joints and fibrotic change. Radiographic and US erosion scores were comparable. Pain was also effectively suppressed by both strategies. Of note, only 50% of patients had PD at baseline despite a minimum of moderate DAS28 disease activity Discrepant observations between US findings and DAS are well recognised.[34–36] Our clinical and US findings further highlight the complexities of achieving remission (see online supplementary figures S6–S7) plot DAS28 components and different DAS28 definitions for each treatment arm to illustrate some of these issues in this cohort).

Remission rate did not improve appreciably with escalation to bDMARD at week 24 in the MTX-TT cohort[1] (with 60% still failing to achieve remission at week 48), and TNFi escalation unable to move this subgroup even to a low disease activity state. Planned exploratory analysis suggested 24 weeks exposure of ETN following MTX-TT-IR may be associated with a lower remission rate compared with first-line ETN+MTX. In comparison, a post hoc exploratory analysis of the Optimal Protocol for Treatment Initiation with Methotrexate and Adalimumab (OPTIMA) trial[6] demonstrated little advantage to starting with adalimumab and MTX. To definitively confirm our findings would require comparison of strategies in (an as of yet undefined) patient population who were all likely to fail first-line MTX-TT; (where arguably unethical to include MTX-TT as a strategy). Finally, this study did not seek to address how to manage new onset RA and first-line TNFi-IR at week 24.

The open-label nature of this study is a legitimate source of bias (although would be expected to overestimate response). However, we sought to capture real-world practice. The use of blinded assessors ensured key components of the endpoints were free from such bias. Also, the drop-out rate was almost twice that anticipated. US of only the dominant hand may partly explain the discrepancy between suppression of PD in almost all subjects but failure to achieve remission in half the patients. Finally, ETN tapering protocol after week 48 would have been desirable; however, in England, NICE does not reimburse ETN until in high disease activity, forcing immediate cessation. In contrast to PRESERVE,[37] we observed minimal (4%) drop in remission rates, likely attributable to the early, treatment-naïve cohort.

In summary, the VEDERA study did not demonstrate the larger than standard effect size (of 30%), which was proposed to exist in a previous exploratory subgroup analysis with first-line TNFi-MTX in very ERA. These data also highlight a ceiling effect in achieving remission in a real life, comorbid ERA cohort. The suggestion that expedient addition of ETN to MTX-TT-IR may not be as effective in a proportion as in treatment-naïve patients requires validation and further investigation.

Patient and public involvement

Independent lay individual from our public and patient advocacy group provided input into study design.

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Contributors The VEDERA trial was conceived by MHB and PE. EMAH was principal statistician. EMAH and MHB provided overall responsibility for the research methodology and statistical analysis plan. SH was the principal clinical fellow who supported MHB to submit the trial ethics and setup. SH, RBD and KN were the clinical research fellows over the trial duration. DvdH oversaw the scoring and interpretation of X-ray data, and RJW oversaw ultrasound component of the study. MHB drafted the manuscript, with critical input from EMAH and PE. All authors had the opportunity to further revise the manuscript and approved the final version.

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Disclaimer The views expressed are those of the author(s) and not necessarily those of the NIHR or the Department of Health and Social Care. Pfizer did not have any role in the study design, study delivery, statistical analyses, interpretation of data or manuscript preparation. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit the report for publication.

Competing interests PE has undertaken clinical trials and provided expert advice to Pfizer, MSD, Abbvie, BMS, UCB, Roche, Novartis, Samsung, Sandoz and Lilly. PE has received consultant fees from BMS, AbbVie, Pfizer, MSD, Novartis, Roche and UCB. PE has received research grants paid to his employer from AbbVie, BMS, Pfizer, MSD and Roche. MHB has provided expert advice and received consultant fees from AbbVie, Bristol-Myers Squibb, Eli Lilly, EMD Serono, Pfizer, Roche, Sandoz, Sanofi and UCB and has received research grants paid to her employer from Pfizer Bristol-Myers Squibb Ltd, Roche, UCB. DvdH has provided expert advice and received consultant fees from Abbvie, Amgen, Astellas, AstraZeneca, Bristol-Myers Squibb, Boehringer Ingleheim, Celgene, Cyxone, Daichii, Eisai, Eli Lilly, Galapagos, Gilead, GSK, Janssen, Merck, Novartis, Pfizer, Regeneron, Roche, Sanofi, Takeda, UCB Pharma.

Patient consent for publication Not required.

Ethics approval The National Research Ethics Service (Leeds (West) Research Ethics Committee) approved the protocol (reference 10/H1307/138); and its amendments. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Provenance and peer review Not commissioned; externally peer reviewed.

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TRANSLATIONAL SCIENCE

Antibodies and B cells recognising citrullinated proteins display a broad cross-reactivity towards other post-translational modifications

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ABSTRACT

Objective Autoantibodies against antigens carrying distinct post-translational modifications (PTMs), such as citrulline, homocitrulline or acetyllysine, are hallmarks of rheumatoid arthritis (RA). The relation between these anti-modified protein antibody (AMPA)-classes is poorly understood as is the ability of different PTM-antigens to activate B-cell receptors (BCRs) directed against citrullinated proteins (CP). Insights into the nature of PTMs able to activate such B cells are pivotal to understand the 'evolution' of the autoimmune response conceivable underlying the disease. Here, we investigated the cross-reactivity of monoclonal AMPA and the ability of different types of PTM-antigens to activate CP-reactive BCRs.

Methods BCR sequences from B cells isolated using citrullinated or acetylated antigens were used to produce monoclonal antibodies (mAb) followed by a detailed analysis of their cross-reactivity towards PTM-antigens. Ramos B-cell transfectants expressing CP-reactive IgG BCRs were generated and their activation on stimulation with PTM-antigens investigated.

Results Most mAbs were highly cross-reactive towards multiple PTMs, while no reactivity was observed to the unmodified controls. B cells carrying CP-reactive BCRs showed activation on stimulation with various types of PTM-antigens.

Conclusions Our study illustrates that AMPA exhibit a high cross-reactivity towards at least two PTMs indicating that their recognition pattern is not confined to one type of modification. Furthermore, our data show that CP-reactive B cells are not only activated by citrullinated, but also by carbamylated and/or acetylated antigens. These data are vital for the understanding of the breach of B-cell tolerance against PTM-antigens and the possible contribution of these antigens to RA-pathogenesis.

Key messages

What is already known about this subject?

- Autoantibodies in patients with rheumatoid arthritis (RA) target different post-translational modifications (PTMs), such as citrullination (anti-citrullinated protein antibodies (ACPAs)), homocitrullination/carbamylation (anti-carbamylated protein antibodies (ACarPAs)) and acetylation (anti-acetylated protein antibodies (AAPAs)).

What does this study add?

- ACPA, ACarPA and AAPA-IgG show a broad reactivity to various antigenic backbones and are highly cross-reactive towards at least two different PTMs.
- Citrullinated protein-reactive B-cell receptors show activation not only upon stimulation with citrullinated, but also after contacting carbamylated or acetylated antigens indicating a broad cross-reactive nature on the cellular level. These results indicate that B cells directed against a particular PTM can be activated by other PTM-antigens in inflamed tissues or other sites conceivably involved in the breach of B-cell tolerance.
- ACPAs, ACarPAs and AAPAs cannot be separated into three independent autoantibody classes and should be regarded as anti-modified protein antibodies (AMPAs).

How might this impact on clinical practice or future developments?

- AMPA probably reflects a better serological marker and combinatorial ACPA/ACarPA/AAPA immunoassays could improve RA diagnosis and treatment.
- The data further our understanding of the breach of B-cell tolerance in RA.

INTRODUCTION

Autoreactive B cells and their secreted autoantibodies are important players in many autoimmune diseases and often implicated in disease pathogenesis. Rheumatoid arthritis (RA) is hallmarked by the presence of several autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs). The presence of these autoantibody families is routinely tested to aid RA-diagnosis and included into the EULAR/ACR-criteria for RA classification.[1] ACPAs are present in 50%–70% of patients with RA and are known to recognise multiple citrullinated antigens, such as α-enolase, fibrinogen, filaggrin, vimentin and type II collagen.[2–7] Their recognition profile is

generally broad and the serological ACPA-response expands closer to disease-onset (epitope spreading) probably reflecting an escalation in the activation of citrullinated protein (CP)-reactive B cells.[8–10] Recently, autoantibodies recognising other post-translationally modified (PTM)-antigens, such as anti-carbamylated protein antibodies (ACarPAs) and anti-acetylated protein antibodies (AAPAs), were identified.[11–13] ACarPAs are directed against homocitrulline-containing (carbamylated) antigens and present in approximately 45% of patients with RA, while AAPAs target acetylated-lysine residues and are found in 40% of patients with RA.[12 13] So far, it is unclear how these autoantibodies are generated and if their underlying B-cell responses are interrelated. As citrullination targets arginine residues, while carbamylation/acetylation predominantly lysine residues, the 'modified'-epitopes are, by definition, unrelated as they occur at different positions in the protein backbone and hence are surrounded by different flanking regions. Likewise, although homocitrullination and acetylation are both lysine modifications, they are structurally dissimilar. Consequently, ACPAs, ACarPAs and AAPAs are often considered as three independent autoantibody classes.[11] Nevertheless, these autoantibodies often occur concurrently in RA and cross-reactivity has been reported, both on a polyclonal-level and monoclonal-level, within an ELISA setting.[13–17] Hence, it is clearly relevant to understand the (in)dependence of these different autoantibody responses in greater detail and to delineate the possibility that autoreactive B cells expressing a B-cell receptor (BCR) against one particular PTM can be activated by other modifications as well. Such understanding would be relevant for the comprehension of the breach of B-cell tolerance in RA and to uncover the antigens that could drive the expansion of autoreactive B cells conceivably present in the inflamed joint. Likewise, insights into the relations between AAPAs, ACarPAs and ACPAs and their cross-reactivity, could help in understanding RA-initiation and could also lead to more refined serological markers for RA-diagnosis. Therefore, we characterised the properties of monoclonal IgG generated from BCR sequences of citrullinated and acetylated antigen-reactive B cells. Additionally, we generated, for the first time, human B-cell transfectants expressing CP-reactive BCRs to investigate the hypothesis that B cells recognising citrullinated antigens are cross-reactive and can be activated by other PTMs.

MATERIALS AND METHODS

Patient and public involvement

Peripheral blood samples from ACPA+ or ACPA+/AAPA+ patients with RA visiting the outpatient clinic of the Rheumatology Department at the Leiden University Medical Center (LUMC) were included in this study. Additional information on patient characteristics is given in the supplementary section (online supplementary table S1).

Protein modification and peptide synthesis

Experimental procedures for protein modification and peptide synthesis are provided in the supplementary section. Peptide sequences and masses are given in online supplementary tables 2-4. Protein masses are provided in online supplementary table S5.

Production of monoclonal anti-modified protein antibody (AMPA)-IgG based on BCR sequences from patients with RA

Eleven ACPA-IgG sequences were isolated from patients with ACPA+RA. Cyclic-citrullinated-peptide 2 (CCP2) and CArgP2 streptavidin-tetramers were used for the isolation of CP-reactive B cells as previously described.[18] Single sorted cells were cultured on irradiated CD40 L-cells and a cytokine mixture in complex IMDM (Gibco) medium for 10–12 days.[19] RNA isolation, cDNA synthesis, ARTISAN PCR and sequencing were performed as previously described.[20 21] The same methodology using acetylated-vimentin (HC55) and lysine-vimentin (HC56)[22] streptavidin-tetramers was used to isolate two AAPA-IgG sequences. The ACPA-IgG 7E4 sequence was provided by Dr Rispens, Sanquin, The Netherlands.[23] Expression vector cloning, monoclonal antibody (mAb) production and purification procedures are described in the supplementary section.

Generation of human Ramos B-cell transfectants expressing CP-reactive IgG BCRs

7E4, 2G9 and 3F3 ACPA-IgG1 HC and LC containing single vector constructs were created with the In-Fusion HD Cloning Kit (Clontech) using the pMIG-IRES-GFP-2AP vector as a backbone including the IGHG1 transmembrane domain. The lymphoma Ramos cell line expressing the murine cationic amino-acid transporter 1 (*slc7a1*) under blasticidin resistance to be able to infect them with Moloney murine leukemia virus-based retrovirus particles, was provided by Dr Engels, University Göttingen. The MDL-AID (IGHM, IGHD, IGLC and activation-induced cytidin deaminase (AID)) knockout (KO) variant of the *slc7a1* expressing Ramos cells was generated by Dr He, University Freiburg. All inserts were verified by sequencing. Ramos cell lines were cultured in RPMI1640/GlutaMAX/10%FCS/10mMHEPES medium (Thermo Scientific) with 100 units/mL penicillin/streptomycin (P/S) (Lonza). Retroviral transductions in Ramos cells were performed as previously described.[24] Briefly, Phoenix-ECO (ATCC CRL-3212) cells were transfected with PolyJet DNA transfection reagent following the manufacturer's instructions (SignaGen Laboratories). Retrovirus containing supernatants were collected 72 hours after transfection and used for the transduction into MDL-AID KO Ramos cells carrying *slc7a1*.

ELISA, SDS-PAGE and western blot analysis

Experimental procedures used for the analysis of the monoclonal AMPA-IgG (ELISA, SDS-PAGE and western blot) are given in the online supplementary section.

Activation assays of Ramos B cells expressing CP-reactive BCRs

GFP+BCR+ (7E4, 2G9, 2C4) Ramos B-cell lines (1×10^6 cells) were stimulated with C(Arg/Lys/C/Hcit/Ac)P2 streptavidin-tetramers (10 µg/mL)[18] for 5 min at 37°C in stimulation medium (RPMI/GlutaMAX/1%FCS/10mMHEPES/100 units/mL P/S). Additionally, stimulation was performed with unmodified, citrullinated-fibrinogen, carbamylated-fibrinogen and acetylated-fibrinogen proteins (50 µg/mL). Afterwards, cells were fixed (Biolegend Fixation Buffer, 420801) and permeabilized (True-Phos Perm Buffer, 425401). After washing, cells were stained with mouse anti-human pSyk(Y348)-PE mAb (moch1ct, eBioscience) diluted 1:20 in PBS/0.5%BSA/0.02%NaN3. The rate of pSyk expression in Ramos cells was calculated as the percentage and proportion of pSyk+GFP+double positive cells. Gating was based on the MDL-AID KO control cell line stimulated with the citrullinated antigen and on Isotype control staining's using mouse IgG1 kappa Isotype control-PE mAb (P3.6.2.8.1, eBioscience). Stained cells were analysed on a BD LSR-II flow cytometry instrument. Data were analysed with FlowJo_V10.

RESULTS

Isolation and successful production of monoclonal ACPA-IgG and AAPA-IgG from peripheral blood B cells of patients with RA

To characterise the reactivity patterns of various AMPA-IgG, we produced 14 monoclonal IgG1 antibodies from BCR sequences of single cell sorted B cells from patients with ACPA+ and AAPA+RA. Eleven antibodies were obtained from CCP2-reactive B cells, one antibody from citrullinated-fibrinogen (7E4) and two antibodies from acetylated-vimentin (HC55)-reactive B cells (table 1).[23] All mAbs were successfully produced as IgG1 molecules and exhibited the expected apparent molecular weight as determined by SDS-PAGE (figure 1A and online supplementary figure S1). The mAbs were subsequently tested for reactivity towards peptides carrying the same modification as used for the isolation of the B cell from which the mAbs were generated (figure 1B). All 12 ACPA-IgG showed a high reactivity to CCP2 but not to its arginine control variant (CArgP2). Likewise, the AAPA-IgG molecules showed acetylated-vimentin (HC55) reactivity, but no reactivity to the unmodified lysine-vimentin peptide (HC56).

Cross-reactivity of ACPA-IgG and AAPA-IgG towards various PTM-antigens

Having verified the successful production of monoclonal PTM-directed IgGs, we next determined their binding characteristics towards various PTM-peptides and proteins. We analysed their reactivity to four linear peptides (fibrinogen α 27–43, fibrinogen β 36–52, vimentin 59–74 and enolase 5–20) and three cyclic peptides (CCP1, CCP2 and CCP4) carrying three different modifications: citrulline (cit), homocitrulline (hcit) and acetyllysine (ac). Likewise, reactivity to their arginine (arg), respectively, lysine (lys)-containing controls was determined (online supplementary table S2, figure 2 and online supplementary figure S5). Noteworthily, none of the mAbs was exclusively reactive towards the PTM that was originally used for the isolation of the autoreactive B cell. In fact, all mAbs showed reactivity towards at least two different PTMs, whereas several mAbs recognised all three PTMs (1F2, D9, 2C4 and 2F5) within the same antigenic backbone (figure 2A,B). No binding was observed for the non-modified control peptides indicating PTM-specific reactivity.

To further validate these findings, we next analysed the cross-reactivity of the mAbs towards modified proteins, using three PTM-proteins (fibrinogen, OVA and vinculin) as well as carbamylated-FCS (figure 2C,D). The results obtained largely confirmed the results of the peptide-ELISA studies. We observed no reactivity of the ACPA and AAPA mAbs to the unmodified control proteins, but extensive cross-reactivity to the PTM-proteins (figure 2C,D). The cross-reactive nature of the antibodies was further confirmed in another experimental setting examining three mAbs in western blot analyses. These antibodies (2G9, 7E4 and 2C4) were selected on the basis of their differential binding patterns in the peptide and protein ELISAs. The results obtained by western blot indicated that monoclonal AMPA-IgG recognise different epitopes within the PTM-fibrinogen α, β and λ chains (figure 2E). More importantly, 7E4 recognised citrullinated-fibrinogen and acetylated-fibrinogen, as also observed in ELISA. Likewise, in agreement with the ELISA data, 2C4 reacted to all three PTM-variants of fibrinogen, whereas 2G9 mainly reacted to citrullinated-fibrinogen (figure 2E).

To substantiate and further characterise the cross-reactive nature of the ACPA-IgG and AAPA-IgG in a third experimental setting, we performed cross-inhibition studies using 2G9, 7E4 and 2C4 in combination with both modified peptides, C(C/Hcit/Ac)P2

Table 1 Monoclonal AMPA variable region sequences.

Tetramer*	AMPA	Patient	IGH-CDR3aa†	(nt) mut HC V-gene†	IGHV†	Identity (%)†	IGHD†	IGHJ†	IGL-CDR3aa†	(nt) mut LC V-gene†	LC	IGKV/ IGLV†	Identity(%)†	IGLJ†
CCP2	3F3	1	CARGTYLPVDESAAFDVW	56	IGHV1-2*02	80.56	IGHD2-8*01	IGHJ3*01	CQQYYEAPYTF	39	κ	IGKV4-1*01	87.54	IGKJ2*01
CCP2	2E4	1	CARGSFLERPESVPFHPW	71	IGHV1-2*02	75.35	IGHD3-3*01	IGHJ3*01	CLQYHAEPYTF	61	κ	IGKV4-1*01	79.46	IGKJ2*01
CCP2	2D11	2	CARRGGKDNVWGDW	21	IGHV5-51*01	92.71	IGHD3-3*01	IGHJ4*02	CQQYNDWPVTF	11	κ	IGKV3-15*01	96.06	IGKJ2*01
CCP2	2G9	2	CVRWGEDRTEGLW	63	IGHV4-34*02	78.60	IGHD2-21*02	IGHJ5*02	CMQRLRFPLTF	31	κ	IGKV2-40*01	89.56	IGKJ4*01
CCP2	633–1F2	2	CVRGGSLGIFGGSVGYW	45	IGHV7-4-1*01	84.72	IGHD3-10*02	IGHJ4*03	CQSYYRGDWVL	47	λ	IGLV6-57*02	84.19	IGLJ3*02
CCP2	D9	3	CARDLSKIFPLYYGMDVW	54	IGHV3-30-3*01	81.25	IGHD3-3*01	IGHJ6*02	CHHYGFSPCSF	26	κ	IGKV3-20*01	90.78	IGKJ2*04
Cit-fibrinogen	7E4 (22)	4	CVRIRGGSSNWLDPW	63	IGHV4-39*01	77.08	IGHD2-15*01	IGHJ5*02	CAAWNGRLSAFVF	48	λ	IGLV1-51*01	83.86	IGLJ1*01
CCP2	1E7	5	CARGIGLGDVIICEGFDVW	48	IGHV4-30-4*01	83.45	IGHD3-10*01	IGHJ3*01	CQSFDSSGLIF	30	λ	IGLV6-57*01	90.38	IGLJ2*01
CCP2	1F2	5	CARGFGSAEELVCYGMDVW	50	IGHV4-30-4*04	82.76	IGHD2-15*01	IGHJ6*02	CQSYDVSGLVF	15	λ	IGLV6-57*01	95.53	IGLJ2/J3*01
CCP2	2E2	5	CARLQCSNGLCYLGGDTFDIW	29	IGHV4-34*01	89.82	IGHD2-8*01	IGHJ3*02	CQQYVSYSTF	18	κ	IGKV1-5*01	93.55	IGKJ1*01
CCP2	1D10	5	CARGLGKTSLWGVDAFDVW	55	IGHV4-30-4*08	81.10	IGHD3-16*02	IGHJ3*01	CQQSNSSSSITF	43	κ	IGKV1-39*01	85.66	IGKJ4*01
CCP2	2F10	5	CARALGKPLVWGVDSFDVW	38	IGHV4-30-4*01	86.94	IGHD2-15*01	IGHJ3*01	CQQSNSTLSLTF	36	κ	IGKV1-39*01	87.10	IGKJ4*01
Ac-vimentin	2C4	6	CATRHHDDIWGHSSVIFFDTW	57	IGHV4-39*01	80.76	IGHD3-16*01	IGHJ5*02	CQSADSEGLDILF	54	λ	IGLV3-25*03	80.65	IGLJ2/J3*01
Ac-vimentin	2F5	6	CATRHYDDIRGRSSVIFFETW	53	IGHV4-39*01	81.79	IGHD3-16*01	IGHJ5*02	CQSSDSTGEDILF	44	λ	IGLV3-25*03	84.23	IGLJ2/J3*01

*Tetramer used for single B-cell isolation via flow cytometry.
†Determined by IMGT/V-QUEST.
AMPA, anti-modified protein antibody.

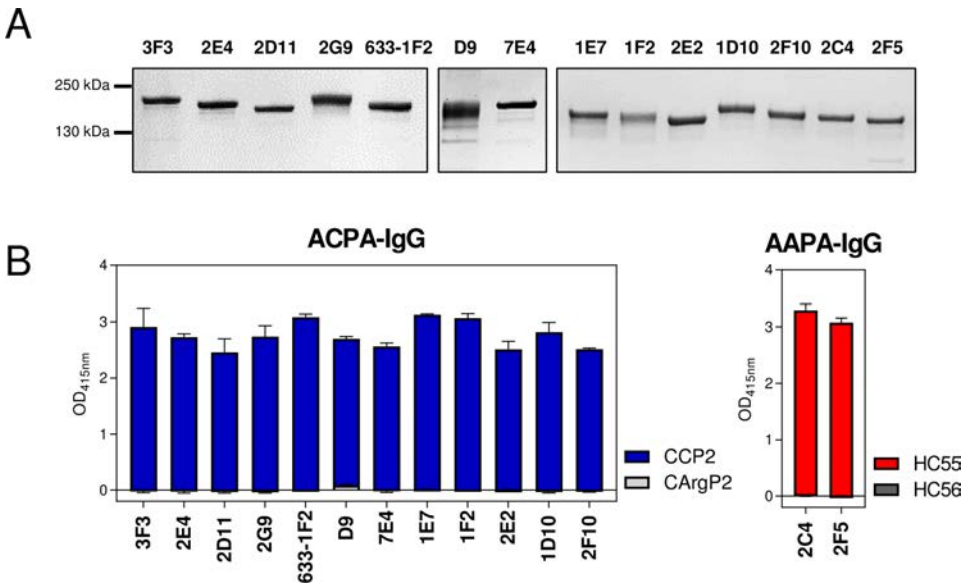

Figure 1 Production of 14 monoclonal AMPA-IgG. (A) SDS-PAGE of purified monoclonal AMPA-IgG using 4%–15% gradient protein gels (BioRad). The size was determined using the PageRuler Plus Prestained Protein Ladder (Thermo Fisher). Molecular weights are higher than 150 kDa and vary between monoclonals due to the expression of different amounts of *N*-linked glycans within their V-domains. (B) Stacked bar graph of the CCP2/CArgP2 (patent protected sequences) and acetylated-vimentin (HC55)/lysine-vimentin (HC56) peptide ELISA of 12 purified monoclonal ACPA-IgG and two AAPA-IgG, respectively. Reactivities were determined by the OD at 415 nm represented on the y-axis. The data represent the mean and SE of three technical replicates. AAPA, anti-acetylated protein antibody; ACPA, anti-citrullinated protein antibody; AMPA, anti-modified protein antibody.

and C(C/Hcit/Ac)P4 as well as proteins, citrullinated-fibrinogen, carbamylated-fibrinogen and acetylated-fibrinogen. The cross-inhibition studies showed that the reactivity of 7E4 to CCP2 and CCP4 could be inhibited by the citrullinated-peptide itself and by its acetylated counterpart, while almost no inhibition could be observed after incubation with CHcitP2/CHcitP4 (figure 3A and online supplementary figure S5). Similarly, reactivity towards citrullinated-fibrinogen and acetylated-fibrinogen could be inhibited by both the citrullinated version as well as the acetylated version of fibrinogen (figure 3B). In agreement with titration ELISAs showing some reactivity of 7E4 towards carbamylated-fibrinogen at high concentrations (online supplementary figure S2a), binding of 7E4 to citrullinated-fibrinogen and acetylated-fibrinogen could be inhibited after preincubation with high amounts of carbamylated-fibrinogen (figure 3B). Thus, together, these cross-inhibition results show that the mAb reactivity towards one particular PTM can be inhibited by another PTM and thereby confirm the reactivity data obtained by ELISA. Likewise, as depicted in figure 3A,B, similar findings were made for 2G9 and 2C4 reaffirming the outcome of the reactivity patterns observed by the peptide-/protein-ELISAs (figures 2 and 3A,B).

Altogether, these data indicate that all ACPA and AAPA mAbs analysed cross-react to a varying extent to at least one other PTM and hence should be regarded as anti-modified protein antibodies (AMPA) rather than as antibodies with a single specificity.

Human B cells expressing CP-reactive BCRs are activated upon stimulation with different PTM-antigens

The data described above show a high degree of cross-reactivity of AMPA towards several modifications and hence suggest that also CP-reactive B cells could react to multiple PTMs. To determine whether such B cells can indeed be activated by several PTMs, we next expressed three different IgGs (7E4, 3F3 and 2G9), isolated from CP-reactive B cells of patients with RA, in a membrane-bound (mIgG) state on a human reporter B-cell line. To this end, we used the human lymphoma Ramos B-cell line in which the genes encoding the endogenous IgD and IgM heavy-chain and light-chain sequences and the gene encoding for AID have been deleted (MDL-AID). This 'triple KO' cell line is unable to show BCR-signalling as it lacks an endogenous BCR. Moreover, it cannot modify a transduced BCR as it lacks AID. On transduction, Ramos B-cell lines showed GFP and BCR-expression, indicating a successful transduction and expression of CP-reactive BCRs. Indeed, binding of the CCP2 antigen, but not of the arginine containing control peptide CArgP2, was observed after incubating the transduced B cells with these antigens (online supplementary figure S3). Next, we used the cells to study BCR-activation via phosphorylation of intracellular Syk (pSyk) 5 min after stimulation with different PTM-antigens. The non-transduced MDL-AID KO cell line (BCR-GFP-) was taken along as a negative/gating control. As depicted in figure 4 and online supplementary figure S4, Syk was phosphorylated after stimulating the 7E4, 3F3 and 2G9 Ramos B-cell transfectants with the respective PTM-antigen. To quantify B-cell activation, the percentage of pSyk+GFP+ cells was determined. 7E4 mIgG carrying B cells readily reacted to stimulation with citrullinated peptides (25.25%±7.142%) and to stimulation with acetyllysine-containing peptides (22.35%±7.990%). In contrast, the cells did not respond to stimulation with a homocitrulline-containing peptide (0.9450%±0.8560%) (online supplementary table S6, figure 4B). These data indicate that the results obtained in the 'non-functional assays' described above translate to the functional activation of 7E4 CP-reactive B cells. More importantly, these results also show that such B cells respond to several PTMs. Similar results were obtained in the activation assays using 3F3-derived and 2G9-derived B cells, showing activation on stimulation with citrullinated peptides (3F3: 28.85%±2.475%; 2G9: 15.00%±4.950%) and with homocitrullinated peptides (3F3: 21.30%±2.828%; 2G9: 14.49%±6.944%). In line with our results obtained by ELISA, these cell lines did not respond to

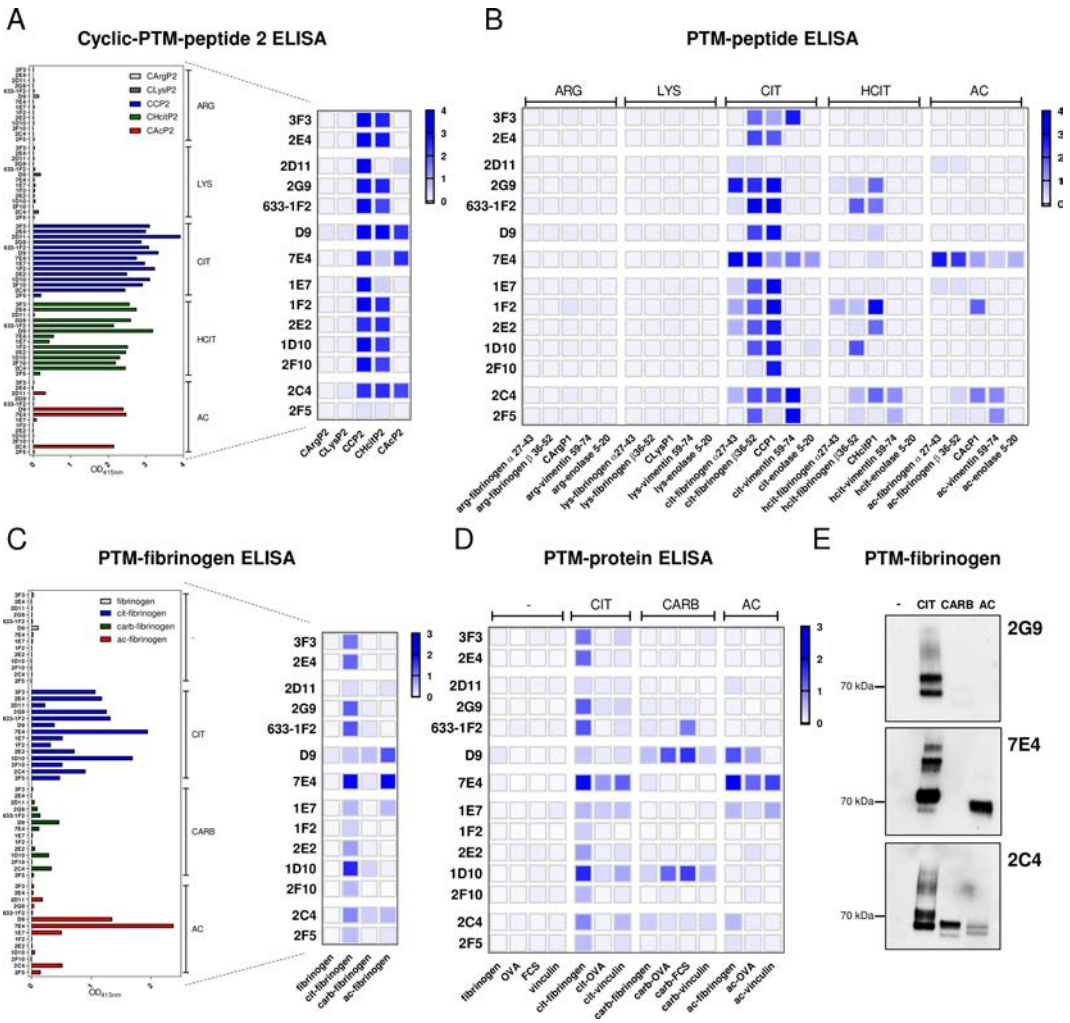

Figure 2 Cross-reactivity of monoclonal AMPA-IgG determined by ELISA and western blot analysis. (A) Bar graph and heatmap of a cyclic-PTM-peptide 2 (C(C/Hcit/Ac)P2) ELISA of 14 monoclonal AMPA-IgG. Monoclonal AMPA-IgG reactivity towards the CCP2 (patent protected sequence) peptide in five modifications (citrulline, homocitrulline, acetyllysine, arginine, lysine) was tested. (B) Heatmap of PTM-peptide ELISAs of 14 monoclonal AMPA-IgG. Monoclonal reactivity to four linear PTM-peptides (fibrinogen α 27–43, fibrinogen β 36–52, vimentin 59–74 and enolase 5–20) and the CCP1 peptide in five modifications (arg, lys, cit, hcit, ac) was analysed. (C) Bar graph and heatmap of PTM-fibrinogen ELISA of 14 monoclonal AMPA-IgG. Monoclonal AMPA-IgG reactivity to the fibrinogen protein in four different versions (unmodified, cit, carb and ac) was tested. (D) Heatmap of PTM-protein ELISAs of 14 monoclonal AMPA-IgG. Monoclonal reactivity to fibrinogen, OVA and vinculin proteins in four different modifications (unmodified, cit, carb and ac) as well as to carb-FCS and unmodified FCS was analysed. Reactivities were determined by the OD at 415 nm represented on the x-axis (bar graphs) or by colour (blue, high OD values, light grey, low OD values) within the heatmaps. Monoclonal AMPA-IgG were tested in a concentration of 10 µg/mL. 2D11 was analysed in a concentration of 20 µg/mL within the cyclic-PTM-peptide 2 ELISA. All ELISA experiments were repeated independently 2–3 times. (E) Western blot analysis of monoclonal AMPA-IgG 2G9, 7E4 and 2C4. Binding towards citrullinated-fibrinogen, carbamylated-fibrinogen and acetylated-fibrinogen and to the unmodified version (-) was analysed under reducing conditions (separately to the α, β and λ chain). Western blot analysis was repeated three times within independent experiments. AMPA, anti-modified protein antibody; FCS, fetal calf serum; OVA, ovalbumin; PTM, post-translational modification.

acetyllysine-containing peptides (3F3: 0.8250%±0.2470%; 2G9: 0.0000%±0.0000%) (online supplementary table S6, figure 4C). To expand the findings described above to the recognition of protein antigens, we next analysed the ability of the different modified forms of fibrinogen to stimulate the CP-reactive B cells. As shown in figures 2B, 3F3 and 2G9 bind solely to citrullinated-fibrinogen in ELISA. In agreement, Ramos cells transduced with these IgG sequences displayed only reactivity to this modification (online supplementary figure S4). More importantly, and in agreement with the data presented in figure 2B, Ramos B cells transduced with 7E4 responded to citrullinated-fibrinogen and also displayed reactivity towards the acetylated counterpart (online supplementary table S7, figure 4B), indicating that CP-reactive B-cells can respond to several PTM-proteins.

Together, these data show that autoreactive B cells expressing a BCR directed against one type of modification can also be activated by other PTMs.

DISCUSSION

Insights into the dynamics of autoimmune responses are vital to understand the breach of tolerance to self-antigens and the 'evolution' of the autoimmune response conceivably underlying the disease. Even though the ACPA-response is considered as the dominating AMPA-response linked to the most

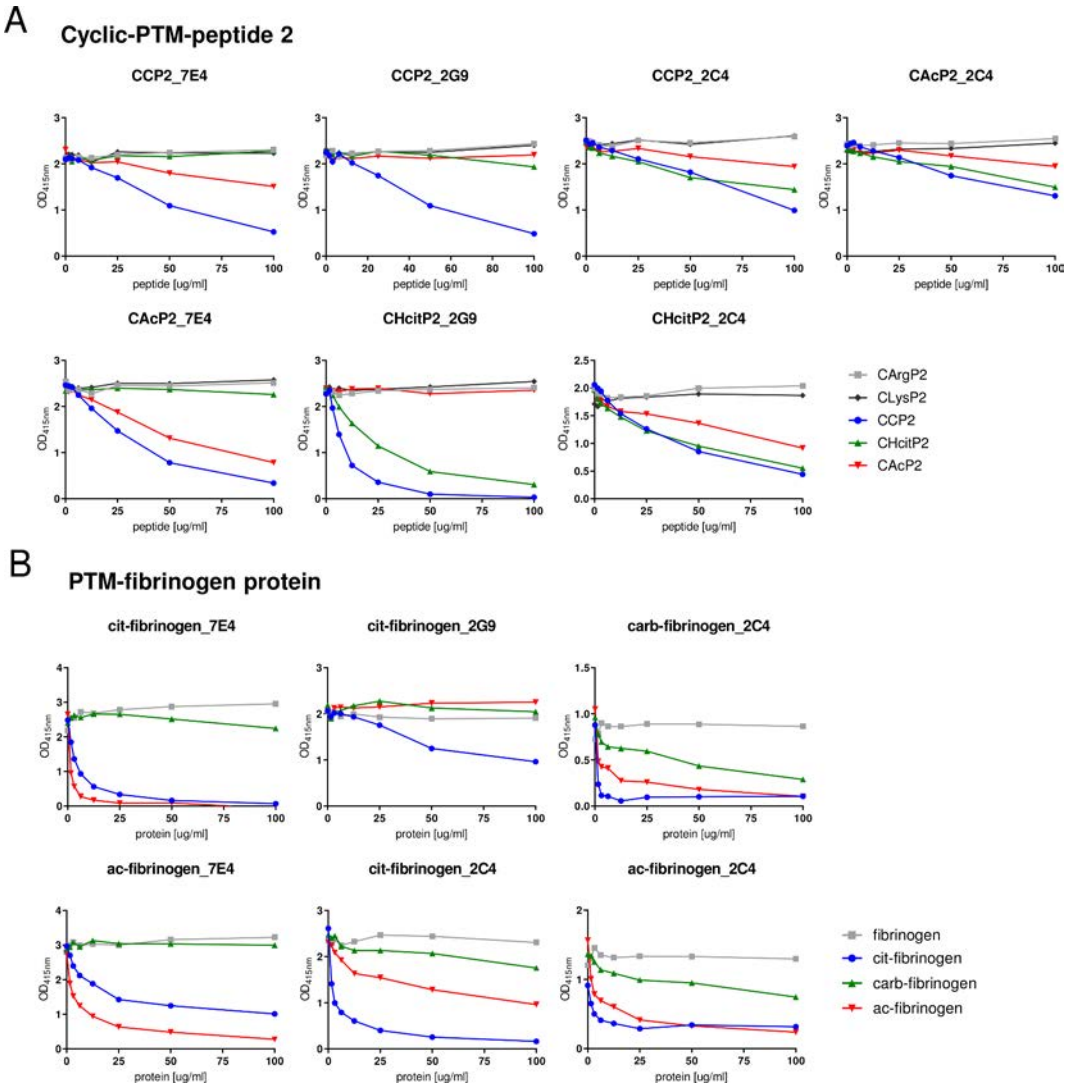

Figure 3 Cross-inhibition studies of monoclonal AMPA-IgG determined by ELISA. (A) Cross-inhibition ELISA with cyclic-PTM-peptide 2 as an inhibitor depicted for 7E4 using CCP2 as well as CAcP2-coated plates, for 2G9 using CCP2 and CHcitP2-coated plates and for 2C4 using CCP2, CHcitP2 and CAcP2-coated ELISA plates. Cross-inhibition was performed with increasing concentrations of the C-PTM-P2 peptide in all three modifications (cit, hcit and ac) and with the negative control peptides CArgP2 and CLysP2. The C-PTM-P2 peptide sequences are patent protected. (B) PTM-fibrinogen cross-inhibition ELISA curves of 7E4 for citrullinated-fibrinogen coated and acetylated-fibrinogen coated plates, of 2G9 for a citrullinated-fibrinogen coated plate and of 2C4 for citrullinated-fibrinogen coated, carbamylated-fibrinogen coated and acetylated-fibrinogen coated plates. Cross-inhibition was performed with increasing concentrations of all four different versions of fibrinogen (unmodified, cit, carb and ac). Monoclonals were tested in concentrations that bound within the linear range of the respective peptide or protein titration ELISA (online supplementary figure S2). Binding is represented by the OD at 415 nm on the y-axis. Cross-inhibition studies were performed two times within independent experiments. Light grey octagon: arginine; dark grey diamond: lysine; blue circle: citrulline; red triangle: acetyl; green square: homocitrulline/carbamyl. AMPA, anti-modified protein antibody; CCP2, Cyclic-citrullinated-peptide 2; PTM, post-translational modification.

prominent genetic risk factors for RA (the HLA-SE-alleles), it is clear that autoantibody responses present in patients with RA extend towards several modifications, such as acetylation and/or carbamylation. AMPA-responses are currently considered to consist of different autoantibody classes that are largely distinct in origin and development. Nonetheless, AMPA also display a certain degree of cross-reactivity and often occur concurrently in individual patients. Recently, we made the crucial observation that vaccinating mice with an acetylated protein leads to the formation of autoantibodies against carbamylated proteins, indicating that different AMPA-responses can evolve from the exposure to only one type of modification. These data provide a conceptual framework for the simultaneous presence of different AMPA-responses in RA by showing that the inciting antigen responsible for the induction of, for example, ACarPAs does not have to be carbamylated, but could be represented by an acetylated protein. We now show that human monoclonal ACPA and AAPA isolated from AMPA positive patients with RA (online supplementary figure S6) are highly cross-reactive towards various PTM-antigens (figure 2). Noteworthily, all ACPA-IgG and AAPA-IgG analysed were able to recognise at least two diverse modifications. This finding has general importance, as it indicates that ACPA, ACarPA and AAPA should be considered as AMPA that are not specific for one type of PTM. Furthermore, our results indicate that besides the affinity of the mAb towards a particular modification also the antigenic backbone and consequently the flanking regions around a modification can contribute

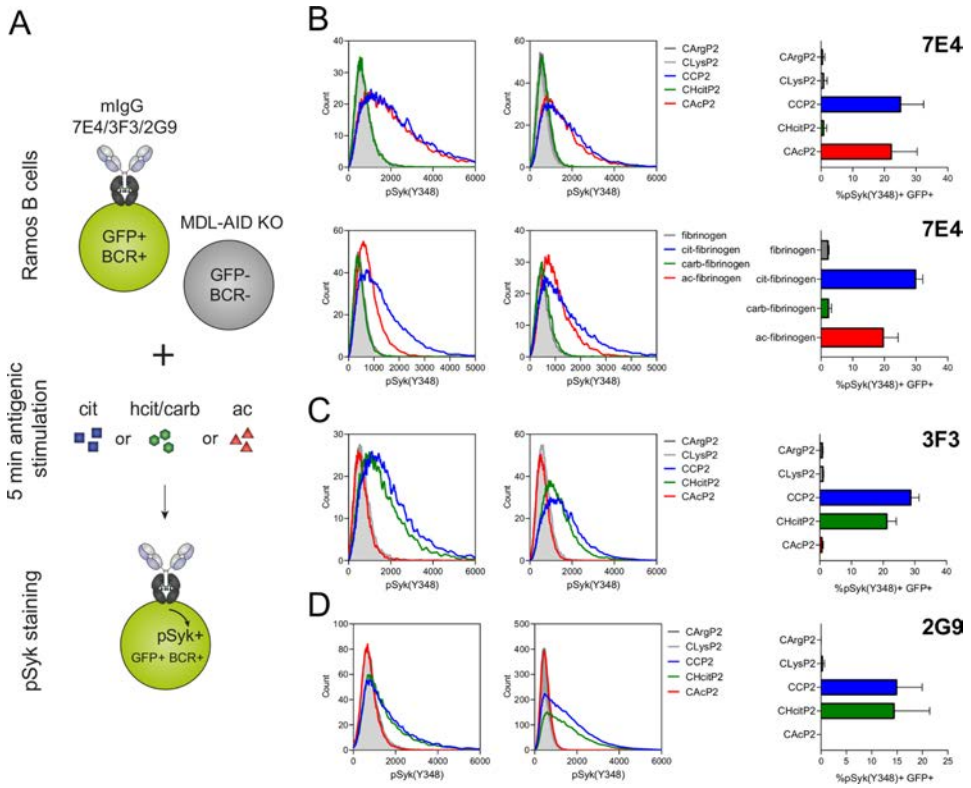

Figure 4 B-cell receptor signalling (pSyk expression) of CP-reactive BCR+GFP+ Ramos B-cell transfectants after stimulation with PTM-antigens. (A) Schematic depiction of the experimental activation assay design. GFP+mIgG-BCR+ Ramos B-cell transfectants and the untransfected GFP-BCR- control MDL-AID KO cell line were stimulated for 5 min with PTM-antigens. BCR activation was determined as the proportion/percentage of GFP+pSyk(Y348)+ B cells. Stimulation with cit-antigens leads to an 'ACPA' response (blue), hcit/carb-activation results in an 'ACarPA' response (green) and ac-antigen activation leads to an 'AAPA' response (red). (B) Histograms of two biological replicates and a bar graph (n=2) showing the percentage of pSyk(Y348)+GFP+7E4 mIgG Ramos B-cells after stimulation with cyclic-PTM-peptide 2 and PTM-fibrinogen. (C) Histograms of two biological replicates and a bar graph (n=2) showing the percentage of pSyk(Y348)+GFP+3F3 mIgG Ramos B cells after stimulation with cyclic-PTM-peptide 2. (D) Histograms of two biological replicates and a bar graph (n=2) showing the percentage of pSyk(Y348)+GFP+2G9 mIgG Ramos B cells after stimulation with cyclic-PTM-peptide 2. The C-PTM-P2 sequences are patent protected. All activation assays were repeated 2–3 times within independent experiments. CArgP2: dark grey; CLysP2: light grey; CCP2: blue; CHcitP2: green; CAcP2: red. Unmodified fibrinogen: light grey; cit-fibrinogen: blue; carb-fibrinogen: green; ac-fibrinogen: red. AAPA, anti-acetylated protein antibody; ACarPA, anti-carbamylated protein antibody; ACPA, anti-citrullinated protein antibody; AID, activation-induced cytidin deaminase; BCR, B-cell receptor; CP, citrullinated proteins; GFP, green fluorescent protein; KO, knockout; PTM, post-translational modification.

to the reactivity-pattern of AMPA-IgG. Depending on the antigen tested (CCP2-peptide or fibrinogen protein), and thus the flanking amino acids around a modification, the AMPAs showed a higher reactivity towards one or another PTM as detected in titration and cross-inhibition ELISAs (figure 3 and online supplementary figure S2). We consider it unlikely that these observations can solely be explained by the number of modifications per protein, which likely differ per PTM generated and might explain the higher mAb reactivity to carb-FCS compared with carb-fibrinogen, as this pattern is not consistent across different antibodies analysed. Nonetheless, it is clear that additional analyses are required to elucidate the potential contribution of flanking regions to the reactivity of AMPA towards PTMs.

Most importantly, our data show that B-cell lines transfected with a BCR derived from one type of defined 'ACPA' can not only be activated by citrullinated, but also by other PTM-antigens. For these studies, we implemented a unique and novel tool by expressing different CP-reactive IgG as BCRs in human Ramos B cells, an accepted model cell line to study BCR responses on stimulation.[25] This enabled us to study human autoreactive B-cell responses on the cellular level. Our observations support the notion that B cells expressing a BCR against citrullinated antigens could be activated by other, non-citrulline containing PTM-antigens. Conceptually, these results are highly relevant to further understand and define the antigens that could be recognised in inflamed joints or at other locations in the body (mucosal tissues) which could be involved in the induction of autoimmunity. Likewise, these findings point to the possibility that a first encounter with a particular PTM can initiate an AMPA-response and determine the direction of it, conceivably dictating a progression towards 'ACPA-dominated', 'ACarPA-dominated' or 'AAPA-dominated' B-cell responses. It is tempting to hypothesise that subsequent antigenic contacts, with different PTM-antigens, could (re)direct the B-cell response towards other modifications, or reinforce the original direction of the AMPA-response. In this way, the concurrent presence of multiple AMPA-reactivities, as observed in many patients with RA, can be explained, and the observation that in other patients the response can be dominated by one AMPA-response towards, for example, citrullinated, carbamylated or acetylated proteins. It would be interesting to investigate the extent of cross-reactivity in

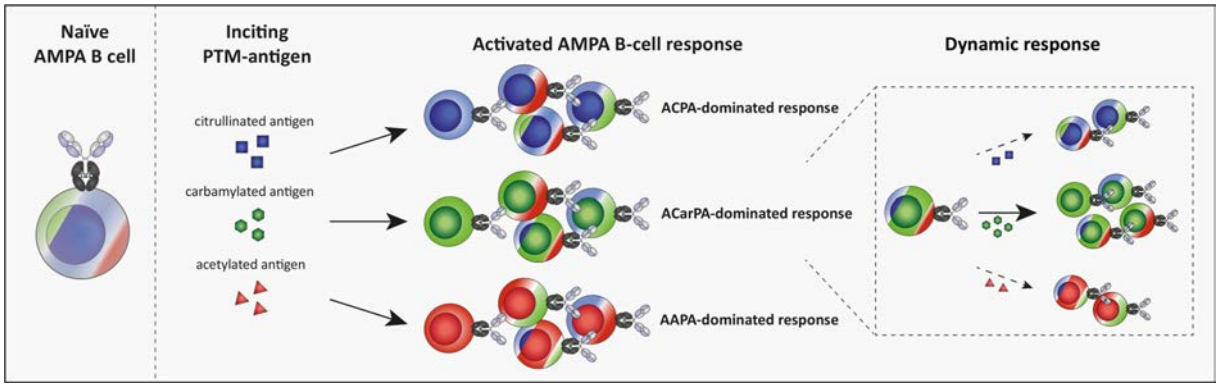

Figure 5 Schematic depiction of an hypothesis proposing the course of autoreactive AMPA B-cell responses. Naïve B cells expressing BCRs directed against PTM-antigens display reactivity towards citrullinated (blue), carbamylated (green) or acetylated (red) antigens. The inciting trigger could represent either a citrullinated, carbamylated or acetylated antigen. Dependent on this initial priming, the B cells are directed towards an 'ACPA-dominated', 'ACarPA-dominated' or 'AAPA-dominated' B-cell response. On subsequent encounter of other PTMs, the AMPA-response can be (re)directed towards another AMPA-class (dynamic response) or the original direction of the AMPA-response can be reinforced (outgrowth of, e.g., 'ACarPA-dominated' B-cell responses). AAPA, anti-acetylated protein antibody; ACarPA, anti-carbamylated protein antibody; ACPA, anti-citrullinated protein antibody; BCR, B-cell receptor; PTM, post-translational modification.

different disease stages, ranging from health to arthralgia, undifferentiated arthritis and RA within future studies. Here, we suggest that AMPA B-cell responses should be considered dynamic responses without a 'fixed' categorisation into different AMPA-classes. We speculate that the inciting and subsequent encounters with particular PTM-antigens define the course of the autoreactive B-cell responses, resulting in the heterogeneous reactivity-pattern observed in RA (figure 5).

Thus, our data disclose a strong relationship and high cross-reactivity between various autoantibodies and their B cells in patients with RA, explaining the concurrent presence of ACPA, ACarPA and AAPA responses. These findings are important to further our understanding of the breach of B-cell tolerance in RA and to unmask the antigens recognised in inflamed tissues.

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Contributors All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

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TRANSLATIONAL SCIENCE

Antibody-mediated inhibition of syndecan-4 dimerisation reduces interleukin (IL)-1 receptor trafficking and signalling

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ABSTRACT

Objective Syndecan-4 (sdc4) is a cell-anchored proteoglycan that consists of a transmembrane core protein and glucosaminoglycan (GAG) side chains. Binding of soluble factors to the GAG chains of sdc4 may result in the dimerisation of sdc4 and the initiation of downstream signalling cascades. However, the question of how sdc4 dimerisation and signalling affects the response of cells to inflammatory stimuli is unknown.

Methods Sdc4 immunostaining was performed on rheumatoid arthritis (RA) tissue sections. Interleukin (IL)-1 induced extracellular signal-regulated kinases (ERK) phosphorylation and matrix metalloproteinase-3 production was investigated. Il-1 binding to sdc4 was investigated using immunoprecipitation. IL-1 receptor (IL1R1) staining on wild-type, sdc4 and IL1R1 knockout fibroblasts was performed in fluorescence-activated cell sorting analyses. A blocking sdc4 antibody was used to investigate sdc4 dimerisation, IL1R1 expression and the histological paw destruction in the human tumour necrosis factor-alpha transgenic mouse.

Results We show that in fibroblasts, the loss of sdc4 or the antibody-mediated inhibition of sdc4 dimerisation reduces the cell surface expression of the IL-1R and regulates the sensitivity of fibroblasts to IL-1. We demonstrate that IL-1 directly binds to sdc4 and in an IL-1R-independent manner leads to its dimerisation. IL-1-induced dimerisation of sdc4 regulates caveolin vesicle-mediated trafficking of the IL1R1, which in turn determines the responsiveness to IL-1. Administration of antibodies (Ab) against the dimerisation domain of sdc4, thus, strongly reduces the expression IL1R1 on arthritic fibroblasts both in vitro and an animal model of human RA.

Conclusion Collectively, our data suggest that Ab that specifically inhibit sdc4 dimerisation may support anti-IL-1 strategies in diseases such as inflammatory arthritis.

Key messages

What is already known about this subject?
- Syndecan-4 (sdc4) is expressed in degenerative and inflammatory joint pathologies.
- Sdc4 contributes to interleukin (IL)-1 signalling in cartilage during osteoarthritis.

What does this study add?
- This study explains how sdc4 impacts on IL-1 signal transduction.
- It shows that blocking sdc4 protects from rheumatoid arthritis (RA)-like joint destruction by inhibiting IL-1 signalling.

How might this impact on clinical practice or future developments?
- The study suggests that blocking of sdc4 by specific antibodies (Ab) might be a novel therapeutic strategy in the treatment of RA.
- The data show that inhibitory Ab should be targeting the dimerisation domain of sdc4 that is involved in IL-1 signalling.

INTRODUCTION

Human rheumatoid arthritis (RA) is a systemic autoimmune disease that primarily affects the joints and that is characterised by chronic inflammation, progressive cartilage destruction and bone erosions.[1] Resident fibroblast-like synoviocytes (FLSs) in the joints have been implicated prominently in the progression of disease, in which early cartilage damage appears to be of pivotal importance.[2] These FLS exhibits an autonomously aggressive phenotype that is maintained in the absence of continuous inflammatory stimulation.[3] The transformation of FLS in RA is associated with altered secretion of soluble factors such as cytokines and chemokines, as well as an enhanced response to inflammatory mediators such as interleukin (IL)-1. Although clinical studies targeting IL-1 have shown only limited efficacy in established disease,[4] a number of studies using both pharmacological inhibition of IL-1 and IL-1-deficient mice have identified IL-1 as an important trigger of early arthritic cartilage damage, with FLS being both an important source of and target cell for IL-1.[5] We have shown previously that the loss of syndecan-4 (sdc4) protects mice from cartilage damage in animal models of osteoarthritis (OA)[6] and RA.[7] In this context, we also showed that chondrocytes lacking sdc4 exhibited a reduced responsiveness to IL-1 with reduced IL-1-mediated ERK phosphorylation.[6] In these studies, antibodies (Ab) raised against the dimerisation domain of sdc4 exhibited a blocking effect on IL-1-induced ERK signalling, but the mechanism by which the loss or inhibition of sdc4 interfered with IL1 signalling remains

unclear.[6] Interestingly, recent evidence suggests that trafficking of the IL-1 receptor (IL1R1) involves the formation of caveolin vesicles[8] and that sdc4 may regulate the caveolin-mediated endocytosis of signalling molecules such as Rac1,[9] as well as of cell surface receptors such as integrins.[10]

Based on these data, we studied the mechanisms by which sdc4 regulates IL-1 signalling and explored the possibility of specifically interfering with sdc4-regulated IL-1 signalling through the delivery of specific Ab. We report that IL-1 binds to sdc4 and leads to its dimerisation, which regulates caveolin vesicle-mediated trafficking of IL1R1. Administration of Ab against the dimerisation domain of sdc4, thus, strongly desensitises arthritic fibroblasts against IL-1, providing a novel tool for therapeutic intervention in IL-1-mediated diseases.

RESULTS

Scd4 reduces IL-1β signalling by direct binding to the heparan sulfate side chains and induction of sdc4 dimerisation

We first investigated the expression of sdc4 in human RA as well as in the human TNF-alpha transgenic (hTNFtg) mouse, an established animal model of the disease. As shown in figure 1A, scd4 was highly expressed in RA synovium, with very prominent staining in the most superficial lining layer that mediates the attachment to and degradation of articular cartilage. In contrast, only very few scd4 expressing cells were found in synovial tissues from patients with OA. As determined by quantitative real-time PCR, the upregulation was also evident at the messenger RNA (mRNA) level, where rheumatoid arthritis synovial fibroblast (RASF) expressed 3.5-fold higher levels of scd4 than osteoarthritis synovial fibroblast (OASF). These data suggested that the inflammatory environment in the RA synovium leads to a strong and sustained upregulation of scd4 in synovial fibroblasts. To further investigate if chronic exposure to tumour necrosis factor-alpha (TNFα) results in a sustained upregulation of scd4 in synovial fibroblasts, hTNFtg mice were analysed for the expression of scd4. As seen in immunohistochemistry, there was a strong expression of scd4 in the synovial membranes of hTNFtg (figure 1B) mice, whereas only negligible staining for scd4 was found in synovial tissues of wild-type (wt) animals. In vitro, synovial fibroblasts isolated from hTNFtg mice showed more 21.6-fold higher expression of scd4 than wt controls (figure 1B).

TNFα and IL-1β both have been shown to be of importance for FLS activation in RA. In western blot analyses, we found that loss of sdc4 in FLS did not affect of ERK activation in response to TNFα (figure 1C). However, sdc4 deficiency in FLS significantly reduced IL-1β-dependent phosphorylation of ERK1/2 (figure 1D). Next, we asked the question if reduction in IL-1β induced ERK signalling in sdc4$^{-/-}$ FLS would translate into reduced expression of disease-relevant matrix metalloproteinases. When investigating the expression of matrix metalloproteinase-3 (MMP-3) in cell culture supernatants of TNFα and IL-1β stimulated FLS from wt and sdc4$^{-/-}$ mice, we found that, again, TNFα-induced expression of MMP-3 was not different between wt and sdc4$^{-/-}$ FLS (figure 1E). However, stimulation of sdc4$^{-/-}$ FLS with IL-1β led to significantly lower levels of MMP-3 compared with those from IL-1-stimulated wt controls (wt: 151.41±5.02, sdc4$^{-/-}$: 77.13±8.36; p=0.001)(figure 1F).

These findings made us wonder whether there is a direct interaction between sdc4 and the cytokines IL-1β and TNFα. To this end, we transfected human embryonal kidney cells 293 (HEK) cells either with FLAG-tagged full-length sdc4 (flag-sdc4wt) or with a FLAG-tagged mutant of sdc4 lacking the attachment sites for glucosaminoglycan (GAG) side chains (flag-sdc4S3A). We found that unlike TNFα, which showed no interaction with either flag-sdc4wt or with flag-sdc4S3A, IL-1β bound to flag-sdc4wt but not to flag-sdc4S3A (figure 1G), indicating that the GAG chains of sdc4 mediated the binding of IL-1. Next, we were interested to see if IL-1β could induce dimerisation of sdc4, which has been described to be a key step in sdc4-mediated signal transduction.[11] Interestingly, IL-1β-induced dimerisation of sdc4 was also observed in FLS lacking the IL1R1 (figure 1H), suggesting that the dimerisation of sdc4 is a direct effect of IL-1 binding and not a secondary effect due to IL-1 receptor signalling. As shown in figure 1I, stimulation of NIH3T3 fibroblasts with IL-1 resulted in dimerisation of sdc4, which was stabilised using the chemical cross-linker BS^3. Obviously, no dimers were detectable in the sdc4$^{-/-}$ fibroblasts and in unstimulated NIH3T3 fibroblasts, even after crosslinking. HEK cells transfected with wt-sdc4-plasmid served as positive control.

Sdc4 regulates IL1R1 trafficking upon IL-1 stimulation

These data made us wonder about the mechanisms by which sdc4 regulates the sensitivity of FLS to IL-1. Using flow cytometry analyses, we found that the loss of sdc4 was associated with a significant reduction of IL1R1 surface expression on FLS (wt: 9.77±0.77, sdc4: 1.94±0.19, IL1R1: 0.52±0.04; p=0.0006) (figure 2A). Importantly, mRNA levels of IL1R1 were unaltered in sdc4$^{-/-}$ cells compared with wt (figure 2B), indicating that sdc4 does not regulate IL1R1 expression but rather affects translational processes and/or trafficking of the IL1R1. The lack of sdc4 had no influence on the expression of the TNF receptor 1 (TNFR1) either on the cell surface, as determined by fluorescence-activated cell sorting (FACS) analysis (wt: 7.38±0.5, sdc4: 7.47±0.43, IL1R1: 8.47±0.20; p=0.9056) (figure 2C), or at the mRNA level (figure 1D). To investigate the time kinetics of IL1R1 surface presentation, we performed FACS analyses of the IL1R1 at 10 min as well as at 3 and 5 hours following IL-1 stimulation. We observed a significant reduction in Il1R1 at the cell surface in wt fibroblasts after 10 min (0.92±0.02, p=0.0286), which was recovered after 3 hours (1.04±0.07). This effect was not observed in sdc4$^{-/-}$ fibroblasts (sdc4 after 10 minutes (t10): 0.89±0.04, sdc4 after 3 hours (t3h): 0.85±0.04) (figure 2E). Because the trafficking of the IL1R1 involves the formation of caveolin vesicles, we next investigated whether inhibition of caveolin vesicle formation either by treatment with nystatin or by small interfering RNA (siRNA) has similar effects on IL-1 induced ERK phosphorylation as the knockout or inhibition of sdc4. Indeed, when we treated wt FLS with nystatin, FLSs lost their ability to respond to IL-1 with a phosphorylation of ERK (figure 2F). As caveolin-1 (Cav-1) is a key component of caveolin vesicles (16), and phosphorylation of Cav-1 constitutes a key step in caveolin vesicle translocation (17), we also knocked down Cav-1 with specific siRNA and found that Cav-1-deficient FLS, in a similar way as nystatin-treated cells, lost their ability to phosphorylate ERK in response to IL-1 (figure 2G). In line with these observations, treatment of wt FLS with nystatin significantly reduced the presentation of the IL1R1 on the cell surface (wt: 6.15±0.40, wt nystatin: 3.31±0.11; p=0.009) but had only minor additional effects on sdc4 deficient cells (sdc4: 3.90±0.27, sdc4 nystatin: 2.58±0.19) (figure 2H). At the mRNA level, we found no differences in Cav-1 expression between wt and sdc4$^{-/-}$ FLS (wt: 0.99±0.17, sdc4: 0.99±0.18; p=0.995) (figure 2I). However, when studying the presence of phosphorylated Cav-1 by sucrose gradient centrifugation, we found that sdc4$^{-/-}$ FLS contained far less phospho-Cav-1 than wt cells after stimulation with IL-1β (figure 2J).

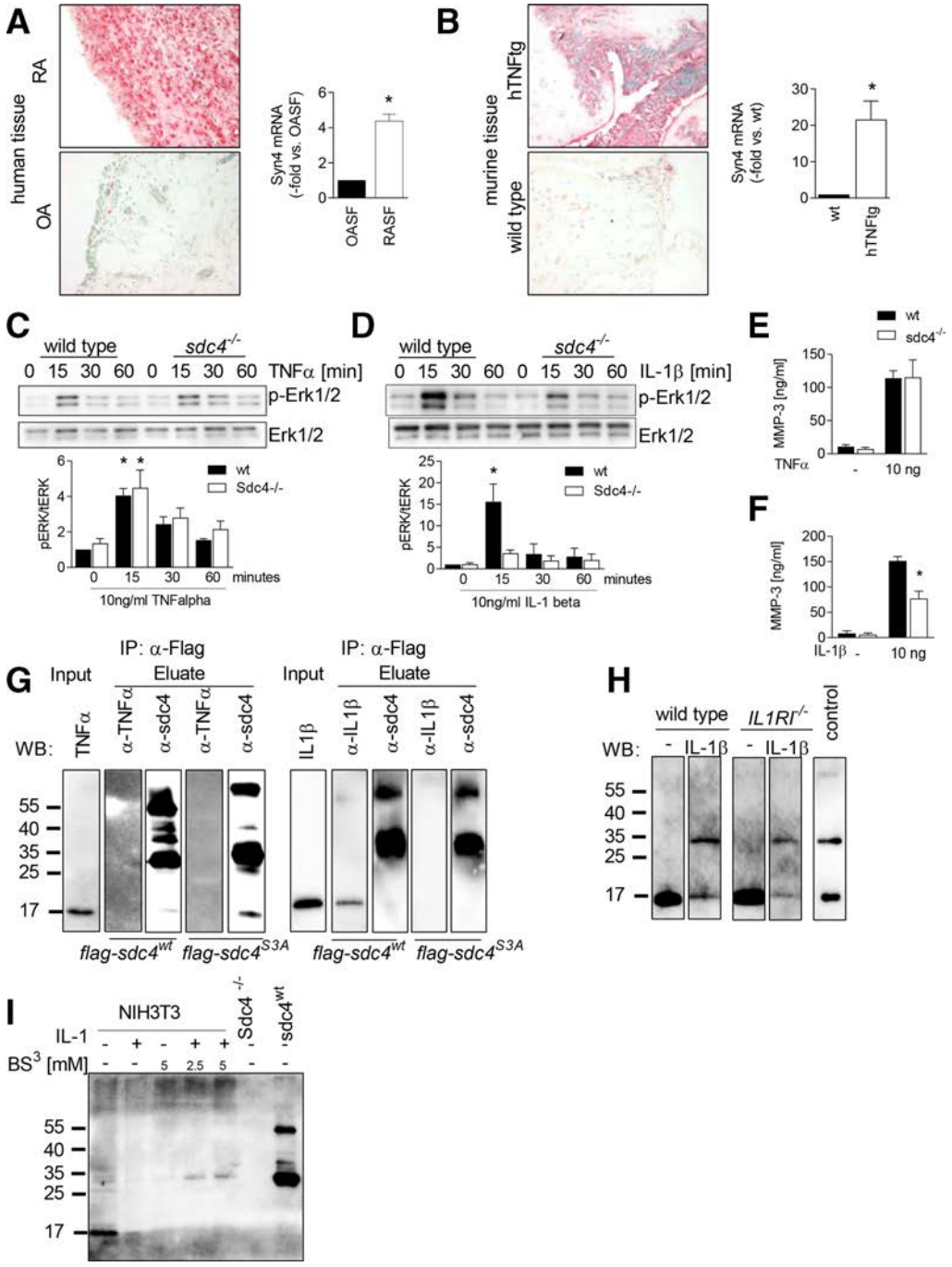

Figure 1 Sdc4 reduces IL-1β signalling by direct binding to the HS side chains and induction of sdc4 dimerisation. (A) Representative images of enhanced sdc4 staining in tissue sections from rheumatoid patients compared with osteoarthritic patients (red, ×200 magnification) (n=10). Quantitative real-time PCR of sdc4 mRNA levels in RASF and OASF revealed upregulation of sdc4 in patients with RA (n=10, p<0.05). (B) Representative immunohistochemical staining of synovial tissue sections of hTNFtg and wt mice with monoclonal sdc4 antibody showed a high sdc4 expression in the Pannus tissue (red, ×200 magnification) (n=8). Quantitative real-time PCR showed a 21.6-fold upregulation in synovial fibroblasts of hTNFtg mice as compared with synovial fibroblasts of wt mice normalised to HPRT (n=4). (C) TNFα stimulation of wt and sdc4$^{-/-}$ synovial fibroblasts revealed no difference in ERK1/2 activation (n≥3). (D) Sdc4$^{-/-}$ synovial fibroblasts showed a reduced ERK1/2 activation in response to IL-1 stimulation compared with wt (n≥3). (E) wt and sdc4$^{-/-}$ synovial fibroblasts were stimulated with TNFα (n≥3) (F) or IL-1β and supernatants were used for MMP-3 ELISA (n≥3). (G) Representative western blot of HEK cells were transfected with FLAG-tagged full-length sdc4 (flag-sdc4wt) or with a FLAG-tagged mutant of sdc4 holding no GAG side chains (flag-sdc4S3A). IL-1, however, could be detected in the eluate of the GAG side chain-holding construct (flag-sdc4wt), but not from the side chain-lacking construct (flag-sdc4S3A). (H) Representative western blot of IL-1-induced sdc4 dimerisation is independent of IL1R1. (I) Representative western blot of sdc4 dimerisation induced by IL-1β. A,B were analysed for statistical significance using Welch's t-test. C–E were analysed using analysis of variance with post hoc t-test. *p<0.05. GAG, glucosaminoglycan; HEK, human embryonal Kidney cells 293; HPRT, hypoxanthine-guanine phosphoribosyl transferase; HS, heparan sulfate; hTNFtg, human TNF-alpha transgenic; IL, interleukin; IP, immunoprecipitation; MMP-3, matrix metalloproteinase-3; mRNA, messenger RNA; OA, osteoarthritis; OASF, osteoarthritis synovial fibroblast; pERK, phospho-ERk; RA, rheumatoid arthritis; RASF, rheumatoid arthritis synovial fibroblast; tERK, total-ERK; sdc4, syndecan-4; TNFα, tumour necrosis factor alpha; WB, western blot; wt, wild type.

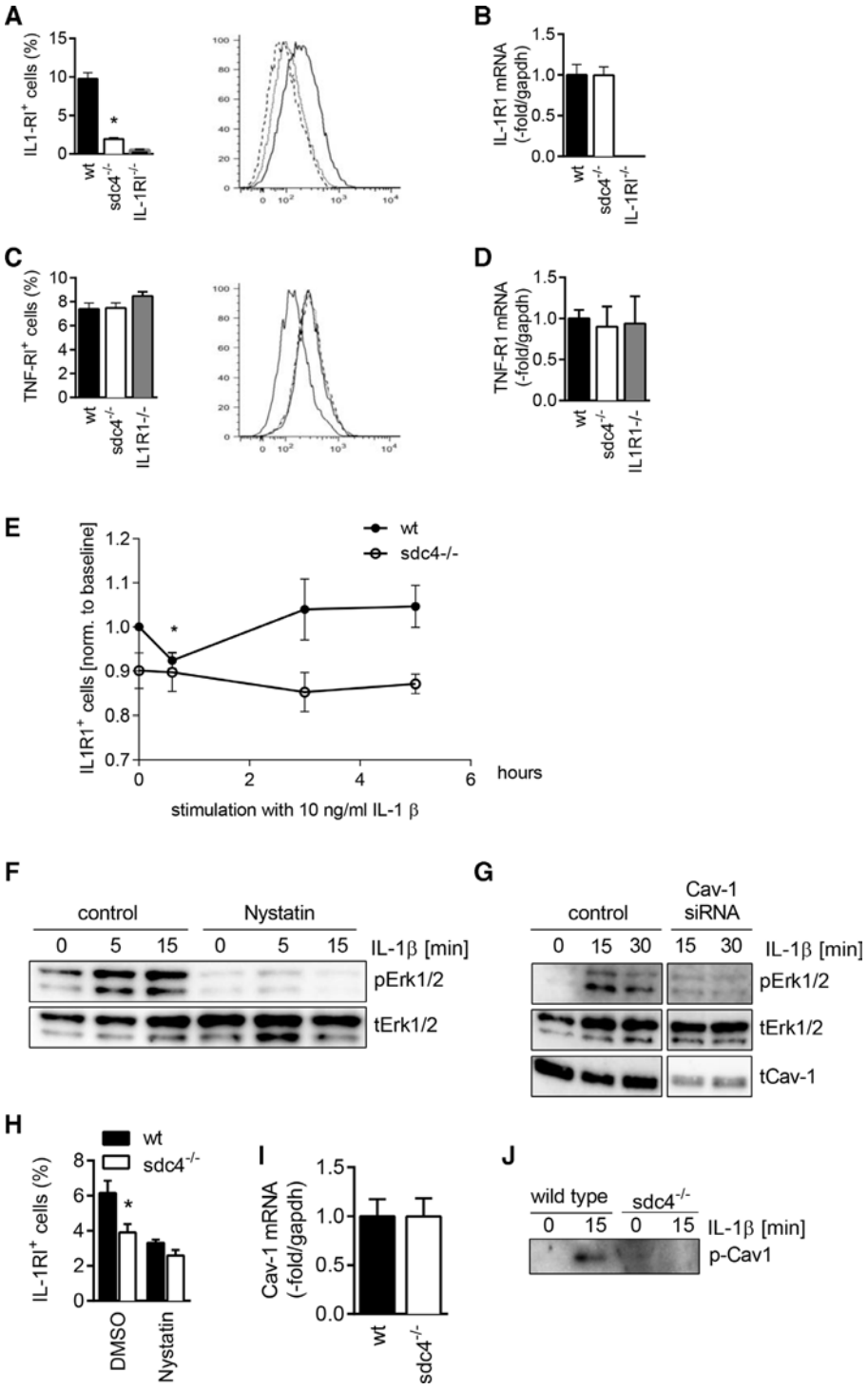

Figure 2 Sdc4 regulates IL1R1 trafficking on IL-1 stimulation. (A) FACS analysis revealed that IL1R1 surface presentation was significantly reduced in sdc4-deficient synovial fibroblasts (n=3). (B) Quantitative real-time PCR showed no difference in IL1R1 mRNA expression levels comparing wt and sdc4$^{-/-}$ synovial fibroblasts normalised to GAPDH (n≥5). (C) TNFR1 surface presentation was comparable between wt and sdc4$^{-/-}$ synovial fibroblasts as shown by FACS analysis (n=3). (D) TNFR1 mRNA expression was equal comparing wt, sdc4$^{-/-}$ and IL1R1$^{-/-}$ synovial fibroblasts by quantitative real-time PCR (n≥5). (E) FACS analysis of IL1R1 surface presentation was significantly reduced in wt synovial fibroblasts after 10 min of IL-1 stimulation, whereas no change was observed in sdc4-deficient fibroblasts (n=4). (F) The caveolin inhibitor nystatin reduced activation of ERK1/2 in wt synovial fibroblasts in response to IL-1 stimulation. (G) Knockdown of Cav-1 using RNAi abolished activation of ERK1/2 in wt synovial fibroblasts. (H) Preincubation with nystatin reduced IL1R1 surface presentation in wt synovial fibroblasts to the levels on sdc4$^{-/-}$ cells (n=3). (I) Quantitative real-time PCR showed no difference in Cav-1 mRNA levels comparing wt and sdc4$^{-/-}$ FLS (n≥5). (J) Sucrose gradient centrifugation was used to isolate caveolin vesicles after stimulation with IL-1. The western blot analysis showed a reduced caveolin phosphorylation in wt compared with sdc4$^{-/-}$ synovial fibroblasts. Data were analysed for statistical significance using analysis of variance with post hoc t-test. *p<0.05. Cav-1, caveolin-1; FACS, fluorescence-activated cell sorting; DMSO, dimethylsulfoxid; FLS, fibroblast-like synoviocyte; IL, interleukin; sdc4, syndecan-4; IL1R1, IL-1 receptor; mRNA, messenger RNA; pERK, phospho-extracellular-signal regulated kinases; siRNA, small interfering RNA; tERK, total-extracellular-signal regulated kinases; TNF, tumour necrosis factor; TNFR1, TNF receptor 1; wt, wild type.

Sdc4 blocking antibody reduces IL1R1 surface presentation, leading to reduced RA symptoms in the hTNFtg RA mouse model

As these data indicated that sdc4 is an important regulator of IL-1 signalling, we tested whether the application of specific Ab raised against the extracellular dimerisation motif of sdc4[6 12] would result in a reduction of ERK phosphorylation as a marker for IL-1 signalling activation. Incubation of FLS with the blocking Ab clearly inhibited the IL-1-induced ERK 1/2 phosphorylation to a comparable extent as the loss of sdc4 (figure 3A). Moreover, FACS analyses of FLS treated with the blocking sdc4 Ab or IgG revealed that anti-sdc4-Ab-treated cells exhibited less IL1R1 on their surface than did IgG treated FLS (wt: 7.30±0.29, sdc4: 4.29±0.21; p=0.001) (figure 3B).

Additionally, we observed a reduction in sdc4 dimers as early as 5 min after IL-1 stimulation (untreated: 1.27±0.30, IL-1: 0.36±0.13; p=0.0091) (figure 3C). These data indicate that the dimerisation of sdc4 is involved in IL1R1 trafficking after IL-1 stimulation. Incubation of FLS with the blocking scd4 Ab or the combination of IL-1 and the blocking scd4 Ab exhibited no additional effect (figure 3C).

These data raised the question of whether the timely application of our blocking anti-sdc4 Ab downregulates IL1R1 and prevents cartilage destruction in hTNFtg mice to a similar extent as we found in Scd4$^{-/-}$ mice previously[7] and as also observed in mice that lack IL1R1. Indeed, injection of the Ab into 8-week-old hTNFtg mice three times per week for 4 weeks protected the treated joints nearly completely from cartilage damage (figure 3D) with decreased pannus area (IgG: 21.92±3.63, α-sdc4-Ab: 6.795±2.56; p=0.0144, n=4), cartilage erosion (IgG: 36.19±4.96, α-sdc4-Ab: 10.19±3.99; p=0.0065, n=4), decreased proteoglycan loss (IgG: 68.82±4.17, α-sdc4-Ab: 18.14±4.47; p=0.0002, n=4) and decreased expression of MMP-3 (IgG: 4.43±0.62, α-sdc4-Ab: 0.68±0.48; p=0.0007, n=4) (figure 3D). The extent to which the antibody inhibited cartilage damage was comparable to that in hTNFtg mice that lacked sdc4 (10) or IL1R1 (figure 3D). This was also seen in histomorphometric analyses, where the inhibition of sdc4 and the loss of the IL1R1 gave very similar results compared with those from the hTNFtg background (figure 3D).

DISCUSSION

In this work, we have investigated how sdc4 modulates the response of fibroblasts to the inflammatory cytokine IL-1 and asked the question if specific, antibody-mediated targeting of sdc4 may alter the response of fibroblasts to IL-1, particularly during chronic destructive arthritis. Since various proteins have been found to bind to the GAG chains of sdc4,[13] and IL-1β binding to GAG chains of both the heparan sulfate type and the chondroitin sulfate type have been described before,[14] the demonstration that IL-1 can bind to the side chains of sdc4 was no surprise. However, we could show that binding of IL-1 to the sdc4 GAG chains also results in the dimerisation of sdc4, which has been suggested to constitute a prerequisite for sdc4-mediated intracellular signalling.[15 16] This is of importance because a clear function of the extracellular domain in dimerisation had not previously been shown, while in our studies, Abs against the dimerisation domain of sdc4 not only inhibited dimer formation but also reduced IL-1-induced ERK phosphorylation in a similar way as did the loss of sdc4, suggesting that sdc4 dimer formation by itself facilitates the IL-1 response of fibroblasts. It may be hypothesised that IL-1-induced dimerisation of sdc4 results from the interaction of IL-1 with its cognate receptor rather than from the direct binding of IL-1 to sdc4. This was not seen when whole cell lysates were used, most likely reflecting the dynamics of sdc4 internalisation in response to IL-1. However, dimerisation of sdc4 in response to IL-1 was also seen in IL-1R-deficient cells, suggesting that the IL-1R is not required for IL-1 to induce scd4 dimerisation and, thus, signalling through this pathway. Recent data suggest that trafficking of the IL1R1 involves the formation of caveolin vesicles.[8] In line with these data, we found that the inhibition of caveolin vesicle formation either by treatment with nystatin[17] or through RNA interference (RNAi) has similar effects on IL-1-induced ERK phosphorylation as had the knockout of sdc4 or the Ab-mediated inhibition of sdc4 dimerisation. An interaction of sdc4 with caveolin vesicles has been previously proposed,[18] and it has been shown that sdc4 is involved prominently in caveolin-mediated integrin recycling. In line with these findings, we detected a reduced presence of IL1R1 on the cell surface in sdc4-deficient fibroblasts, which was dependent on caveolin vesicle formation. The time course of IL1R1 surface presentation in combination with sdc4 dimer detection indicate that the presence of sdc4 critically regulates the surface presentation of IL1R1. Thus, it may be hypothesised that on induction of IL1 signalling, the IL1R1 is reduced from the cell surface at the same time as sdc4 dimerisation is reduced. To show the in vivo relevance of the described mechanism, we applied the blocking anti-sdc4 antibody to hTNFtg mice that are a model for human RA disease. Importantly, treatment with the blocking antibody resulted in a similar phenotype as found in the IL1R1 knockout, as well as the sdc4$^{-/-}$ hTNFtg mouse.[7] However, the effect of the blocking sdc4 antibody was not as strong as the effect of the complete knockout, which might be explained by residual sdc4 on the cell surface that was not blocked by the sdc4 antibody, as we did not titrate for the maximum effect of the blocking antibody.

Collectively, our data demonstrate that dimerisation of sdc4 is critically involved in IL1 signal transduction and IL1R1 surface presentation via caveolin-dependent mechanisms, and those Ab that specifically inhibit sdc4 dimerisation may support anti-IL-1 strategies in diseases such as inflammatory arthritis.

MATERIALS AND METHODS

Blocking sdc4 antibodies

The blocking sdc4 Ab were generated against the peptide NAQPGIRVPSEPKELEENEVIPKRAPSDV of the extracellular part of sdc4. Rabbits were immunised with the synthetic peptide, and the Ab were purified by Pineda (Berlin, Germany). The polyclonal Ab of two different immunised rabbits were tested for sdc4 specificity using western blot (online supplementary figure 1a and c). The specificity of the antibody used for the treatment of hTNFtg mice was also tested in immunohistological stainings of wt and sdc4 ko fibroblasts (online supplementary figure 1b). To test the reproducibility of generating blocking sdc4 Ab when using the extracellular part of sdc4 for immunisation, we tested the polyclonal Ab of another immunised rabbit for the capability to inhibit IL-1-induced ERK1/2 phosphorylation (representative western blot, online supplementary figure 1d).

Animals and treatment hTNFtg mice (strain Tg197) have been described previously[19] and were maintained on the C57BL/6 genetic background. We generated all data from sex-matched and age-matched littermates. The hind paws of 8-week-old hTNFtg mice were treated with either our blocking sdc4 Ab or an IgG control (Chrome Pure Rabbit IgG, Dianova) by periarticular (p.a.) injections of 50 µL into the hind paw, three times

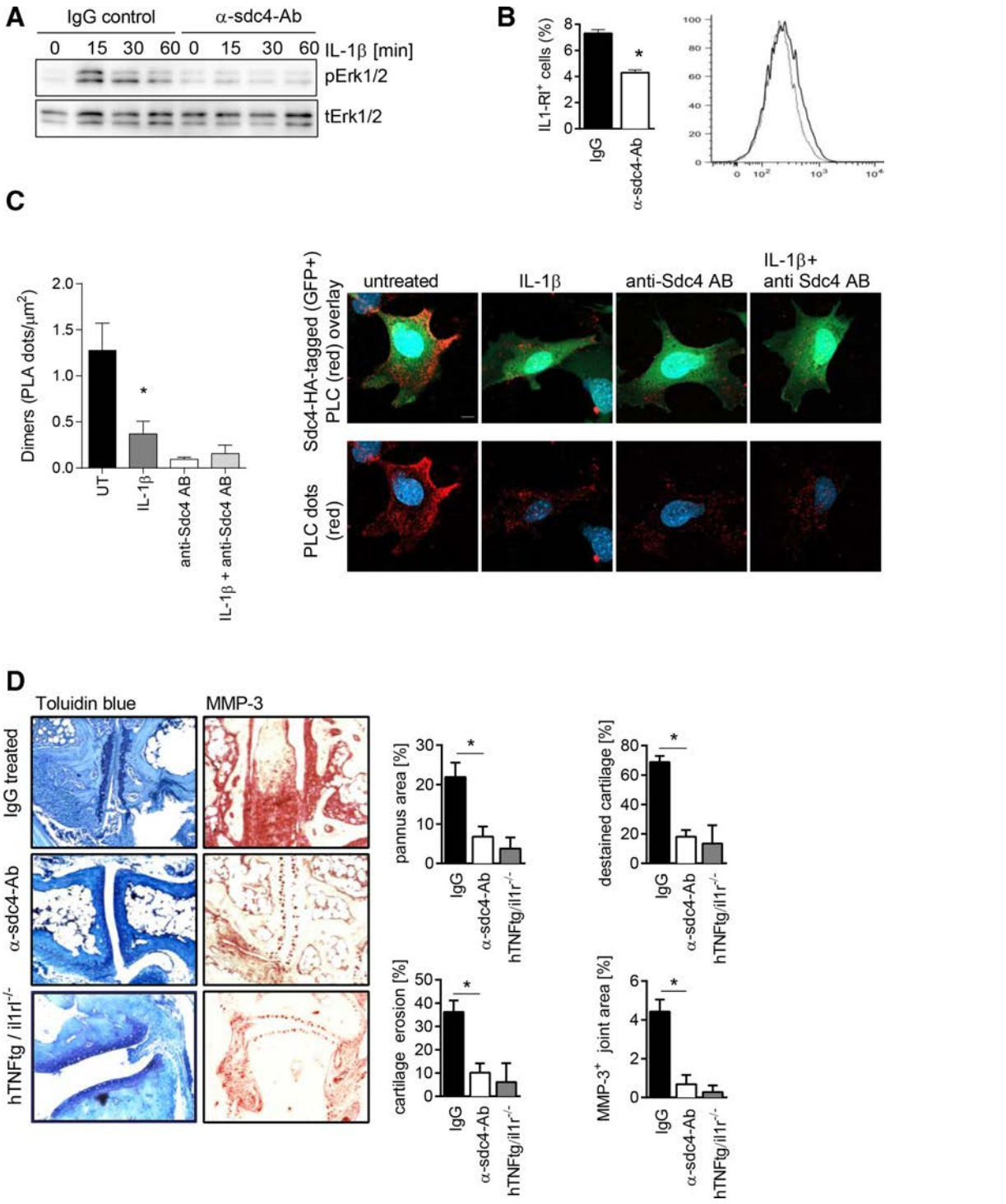

Figure 3 Sdc4 blocking antibody reduces IL1R1 surface presentation, leading to reduced RA symptoms in the hTNFtg RA mouse model. (A) Inhibition of sdc4 function by a polyclonal antibody directed against the membrane proximal part of the extracellular domain of sdc4 (anti-sdc4-Ab) resulted in a diminished ERK1/2 activation in wt synovial fibroblasts. (B) FACS staining revealed that a pre-incubation of wt synovial fibroblasts with anti-sdc4-Ab led to a significant reduction of IL1R presentation compared with IgG treated cells (n=3). (C) Sdc4 dimerisation is inhibited by IL1β stimulation and the blocking α-sdc4 antibody. proximity ligation assays in NIH/3T3 cells transduced with HA tagged sdc4 reveal fewer dots per area (red) in the presence of both IL1β and sdc4 antibody (n=4). (D) hTNFtg mice were treated with our blocking anti-sdc4-Ab or non-specific IgG (IgG control) from week 8 to week 12 of age, while hTNFtg X IL1R1$^{-/-}$ mice served as control. representative toluidine blue and MMP-3 stainings of hind paw sections of 12-week-old hTNFtg mice are shown. Anti-sdc4-Ab treatment of hTNFtg mice caused a strong decrease in MMP-3 levels (red) and inflammation, as well as cartilage damage mean areas of synovial Pannus tissue, destaining of the cartilage (in per cent of total cartilage) and cartilage erosion (in per cent of cartilage), and MMP-3 positive joint area in the tarsal joints was measured. Treatment of hTNFtg mice with anti-sdc4-Ab showed decreased Pannus formation and reduced MMP-3 levels (red), resulting in the preservation of cartilage in comparison to IgG-treated hTNFtg mice (n≥4, at week 12). B–D were analysed for statistical significance using analysis of variance with post hoc t-test. *p<0.05. anti-sdc4-Ab, anti-sdc4-antibody; hTNFtg, human TNF-alpha transgenic; IL, interleukin; MMP-3, matrix metalloproteinase-3; RA, rheumatoid arthritis; sdc4, syndecan-4; wt, wild type.

per week for 4 weeks. Afterwards, mice were euthanised and the hind paws were prepared for histological analysis.

Arthritis assessment

Histological analysis was conducted on 4% phosphate-buffered paraformaldehyde-fixed, decalcified paraffin sections of hind paw joints. Paraffin sections were stained with toluidine blue. Quantification of the pannus area, destained cartilage and cartilage erosion was performed using a Zeiss Observer Z1 microscope (Carl Zeiss) and the Zeiss AxioVision V.4.7.1 software.

Immunohistochemistry

MMP-3 staining was performed using anti-MMP-3 antibody (Abcam), and subsequent detection was performed using the alkaline phosphatase reagent from the Vector Red SK-5100 substrate (Vector Laboratories). For IL-1R staining, the anti-IL-1R antibody (Bioss) was used. The staining was visualised using the DAB reagent from the DAB peroxidase (horseradish peroxidase (HRP)) kit SK-4100 (Vector Laboratories). Quantification was performed using Image-Pro Plus. IL-1R-positive cells (%) were measured by dividing the number of positive stained cells by the total cell number. To calculate the MMP-3-positive joint area (%), a rectangle of fixed size was laid over each joint and the positive stained area was divided by the total area of the rectangle.

Histology

Tissue samples from patients with RA and OA were fixed in 4% paraformaldehyde overnight, embedded into paraffin and sectioned into 5 µm slices. Tissue sections were pretreated with 1× trypsin/EDTA (PAA Laboratories, Pasching, Austria) for 20 min at 37°C, blocked with 10% horse serum and stained with a monoclonal scd4 antibody (Santa Cruz, clone 5G9) for human tissue sections overnight at 4°C. Immunohistochemistry was performed with an alkaline phosphatase technique using Vectastain ABC-A, Vector Red Substance and secondary biotinylated Ab (Vector Laboratories, Burlingame, California, USA). Nuclei were counterstained with methyl green (Vector Laboratories).

Immunocytochemistry

RASF and OASF were seeded on glass cover slips, fixed with 4% paraformaldehyde including 0.1% Tween 20, blocked with 1% bovine serum albumine (BSA) and stained with the monoclonal scd4 antibody 5G9 and an Alexa488 goat antimouse antibody (Molecular Probes) for detection of scd4 expression. Propidium iodide (Sigma) was used to stain the nuclei.

Murine synovial fibroblasts

Murine synovial fibroblasts were isolated from hind paws. Joints were dissociated and digested with Collagenase IV (Worthington) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (PAA). The murine fibroblast cell line NIH/3T3 was used for transduction experiments in the proximity ligation assay (PLA) assay. All cells were cultured in DMEM with 10% FCS at 37°C and 5% CO_2 until passages 4–6. Cells were stimulated for the indicated time period with 10 ng/mL murine IL-1β (R&D Systems) or 10 ng/mL human TNFα (R&D Systems).

Immunoprecipitation

Immunoprecipitation was performed using anti-FLAG M2 Magnetic Beads (Sigma) according to the manufacturer's protocol. Therefore, we transfected HEK cells either with FLAG-tagged full-length sdc4 (flag-sdc4wt) or with a FLAG-tagged mutant of sdc4 in which all GAG-binding serine residues were replaced by alanine (flag-sdc4S3A) and that therefore had no GAG side chains.

The next day, cells were incubated with 100 ng IL-1β or TNFα (both R&D Systems) for 1 hour at 37°C in DMEM medium without FCS. IL-1 was detected using an IL-1β antibody (#12242, Cell Signalling) and TNF using a TNFα antibody (#3707, Cell Signalling). True Blot secondary Ab (Rockland) were used to ensure that IgG fragments from the Ab attached to the beads were not detected.

Isolation of caveolae

Isolation of caveolae was done according to the protocol of Ostrom and Insel[20] by sucrose gradient centrifugation. All the buffers used were identical to the protocol, and proteinase inhibitors as well as phosphatase inhibitor were present in the lysis buffer, sucrose/Modified Barth's solution (MBS) and Triton-X 100 buffer. After cell homogenisation of a complete, confluent cell culture flask, the lysate was loaded on the described sucrose gradient and centrifugation was performed at 260 000*g* for 18 hours at 4°C. Then, the gradient was separated into 250 µL fractions and ethanol precipitation was performed.

As lipid rafts and caveolae were more buoyant than other cellular parts, fraction 2 between the 35% to 5% sucrose borderline was analysed using western blot analysis.

Western blotting

Total cell extracts were obtained using NP-40 buffer (150 mM sodium chloride, 1% NP-40, 50 mM Tris–HCl, pH 8) containing protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche). The extracts were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (GE Healthcare). The proteins were detected with appropriate Ab using the ECL detection system (GE Healthcare) or Super Signal West Femto (Life Technologies). Ab against the following proteins were used: Scd4 (Pineda), Cav-1 (Abcam), p-Cav-1 (Tyr14), ERK1/2, p-ERK1/2, IL-1β, and TNFα (all from Cell Signalling).

FACS analysis

Confluent FLS cells were washed once with PBS and harvested by incubating the monolayer in 10 mL PBS containing 0.02% EDTA and detaching cells by firmly rocking. Unspecific staining was reduced by incubating the resuspended cells in Hank's Balanced Salt Solution (HBSS) containing 1% BSA, 2% bovine FCS and 0.1% NaN_3 in the presence of 0.2 µL/well Fc blocking antibody (BD Biosciences) for 10 min on ice. For TNFR1 staining, 40 µL cell suspension in HBSS plus supplements was incubated with anti-TNFRI antibody (Abcam) or the equal concentration of isotype control rabbit IgG (R&D Systems). A secondary anti-rabbit A488 antibody (Life Technologies) was used. For IL1R1 staining, the anti-IL1R1 PE antibody (BD Biosciences) or the respective isotype control (BD Biosciences) was used. For both stainings, dead cells were excluded by incubating with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies) before recording on a FACS Canto II cytometer.

ELISA

wt and $sdc4^{-/-}$ synovial fibroblasts were cultured in DMEM (Sigma) supplemented with 10% FCS (PAA). Confluent cells were stimulated with 10 ng murine IL-1β or 100 ng murine TNFα (R&D Systems) for 8 hours. Cell culture supernatants

were assessed for total MMP-3 by ELISA (R&D Systems) according to the manufacturer's protocol.

siRNAs and transfection

Cav-1 siRNA was synthesised by Dharmacon (Lafayette, Colorado, USA) under the reference ON-TARGETplus Mouse Cav1 siRNA (J-058415-05-0050). Transfection of siRNAs was performed using N-TER nanoparticle siRNA transfection system (Sigma) according to the manufacturer's instructions. wt synovial fibroblasts were transfected again after 48 hours, and cells were used after 72 hours for western blot analysis.

Inhibition of caveolin vesicle formation via nystatin

Formation of caveolin vesicles was inhibited using Nystatin (Sigma). Nystatin powder was dissolved in DMSO in a concentration of 10 mg/mL and was used in a final concentration of 20 µg/mL dissolved in FCS-supplemented DMEM. Cells were pretreated with nystatin for 45 min prior to stimulation and overnight for flow cytometric analysis.

Semiquantitative PCR from human and murine synovial fibroblasts

Total RNAs from SF was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. cDNAs were synthesised by reverse-transcriptase using the cDNA synthesis kit (Life Technologies) with oligo dT primers. Primer sequences were as follows: Cav-1 forward 5′-AAC ATC TAC AAG CCC AAC AAC AAG G-3′ and reverse 5′-GGT TCT GCA ATC ACA TCT TCA AAG TC-3′, glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) forward 5′-AGC AAG GAC ACT GAG CAA GAG AGG 3′ and reverse 5′-GGG TCT GGG ATG GAA ATT GTG AGG 3′, IL-1R forward 5′-CCC GAG GTC CAG TGG TAT AAG AAC 3′ and reverse 5′-ACT CCG AAG AAG CTC ACG TTG TC 3′, tnfrI forward 5′-ACC TGT CAG TGA GGT AGT CCC AAC 3′ and reverse 5′-ACA GAA TCG CAA GGT CTG CAT TG 3′, human scd4 forward 5′-cgg gca gga atc tga tga ctt tga-3′ and reverse: 5′-gct tca cgc gta gaa ctc att ggt-3′.

Proximity ligation assay

Gene synthesis of the complete murine sdc4 cDNA was performed by GeneArt (Invitrogen). Full-length sdc4 cDNA was mutated such that the HA epitope was inserted between I^{32} and D^{33} of the extracellular domain. The cDNA was then cloned into the lentiviral vector pLNT-SFFV-MCS-eGFP and lentivirus produced in HEK293t cells using conventional procedures. NIH3T3 fibroblasts (HPA Laboratories) were then transfected using the supernatant transfer method. NIH3T3 expressing sdc4-HA-tagged (green fuorescent protein $(GFP)^+$) and untransfected cells were grown on eight-well chamber slides. Cells were treated for 5 min with 50 µL serum-free Optimem containing either 1:10 rabbit anti-sdc4 Ab (#1283, Lot 1405, 575 ng/µL), IL-1β (10 ng/mL) or both. Following treatments, the medium was taken off, cells were washed in PBS at room temperature (RT), fixed in 4% PFA for 15 min at RT. After a 5 min wash in PBS, PFA was quenched by incubating cells with 0.1 M NH_4Cl for 10 min at RT. Proximity ligation assay was performed as per manufacturer's instructions (Duolink, Sigma Aldrich) using mouse IgG1 anti-HA Ab (Covance) and plus and minus anti-mouse probes. Images were taken using confocal microscopy (Carl Zeiss LSM 700) with 63× oil objective and were analysed using ImageJ software (National Institutes of Health). Results were expressed as number of PLA dots per μm^2 of GFP^+ cells.

Crosslinking

In order to stabilise the formed scd4 multimers, crosslinking was applied to cell surface proteins subsequent to multimerisation experiments. Therefore, cells were washed four times with ice-cold PBS directly after IL-1 stimulation and then incubated with 1–5 mM of the crosslinking reagent BS3 (Thermo Fischer, Dreieich, Germany) at room temperature for 30 min. Finally, 15 mM Tris–HCl (pH 7.5) was added for 15 min to quench the reaction, and after washing with PBS, the cells were directly lysed or frozen at −80 C.

Statistical analysis

All data are means±SEM. Biological replicates are stated as n. Mean values of technical replicates are stated as n=1. Statistical analysis was performed using GraphPad Prism Software V.6 (Graph Pad Software, San Diego, California, USA). Differences between groups were examined for statistical significance using an analysis of variance with post hoc t-test and Bonferroni correction for multiple testing, or Welch's t-test ($p<0.05$).

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Contributors LG performed the main experiments. MB performed the interleukin (IL)-1 time-dependent IL1R1 reduction in fluorescence-activated cell sorting (FACS). AK-P performed the histological mouse model evaluation. UK established the IL-1 receptor (IL1R1) FACS protocol. JS performed the phosphocaveolin western blot. DB performed the human TNF-alpha transgenic/IL1R1 breeding. KM performed the sdc4 stainings in murine and human rheumatoid arthritis sections. FE helped in writing the manuscript and interpreting the data. JW and GDR performed the dimerisation experiments. TP and JB drafted the manuscript, evaluated the data and steered the project.

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Competing interests None declared.

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

Patient consent for publication Not required.

Ethics approval The ethics committees of the University Hospital Muenster approved all studies. Samples of synovial tissues from patients with rheumatoid arthritis or osteoarthritis were obtained from joint replacement surgery (Ethics Committee Muenster, approval number 2009-049-f-s). All animal experiments were authorised by the Animal Use Committee in Muenster under the file reference 84-02.04.2014.A465.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information. All relevant data are included in the article and will be made available upon request.

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CLINICAL SCIENCE

GO-DACT: a phase 3b randomised, double-blind, placebo-controlled trial of GOlimumab plus methotrexate (MTX) versus placebo plus MTX in improving DACTylitis in MTX-naive patients with psoriatic arthritis

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ABSTRACT

Objectives To assess the efficacy of golimumab in combination with methotrexate (MTX) versus MTX monotherapy in psoriatic arthritis (PsA) dactylitis.

Methods Multicentre, investigator-initiated, randomised, double-blind, placebo-controlled, parallel-design phase 3b trial in 11 Portuguese rheumatology centres. Patients with PsA along with active dactylitis and naive to MTX and biologic disease-modifying antirheumatic drugs (bDMARDs) were randomly assigned to golimumab or placebo, both in combination with MTX. The primary endpoint was Dactylitis Severity Score (DSS) change from baseline to week 24. Key secondary endpoints included DSS and Leeds Dactylitis Index (LDI) response, and changes from baseline in the LDI and MRI dactylitis score. Analysis was by intention-to-treat for the primary endpoint.

Results Twenty-one patients received golimumab plus MTX and 23 MTX monotherapy for 24 weeks. One patient from each arm discontinued. Patient inclusion was halted at 50% planned recruitment due to a favourable interim analysis. Median baseline DSS was 6 in both arms. By week 24, patients treated with golimumab plus MTX exhibited significantly greater improvements in DSS relative to MTX monotherapy (median change of 5 vs 2 points, respectively; p=0.026). In the golimumab plus MTX arm, significantly higher proportions of patients achieved at least 50% or 70% improvement in DSS and 20%, 50% or 70% improvement in LDI in comparison to MTX monotherapy.

Conclusions The combination of golimumab and MTX as first-line bDMARD therapy is superior to MTX monotherapy for the treatment of PsA dactylitis.

Trial registration number NCT02065713

Key messages

What is already known about this subject?

- Psoriatic dactylitis is associated with higher psoriatic arthritis disease activity and articular erosions.
- Treatment algorithms are controversial due to the absence of randomised controlled trials assessing dactylitis as a primary endpoint, especially in the context of methotrexate (MTX) versus tumour necrosis factor inhibitors /MTX combination.

What does this study add?

- The GO-DACT trial showed that the combination of golimumab plus MTX is associated with significantly greater clinical improvements in dactylitis in comparison with MTX monotherapy.

How might this impact on clinical practice or future developments?

- GO-DACT provides evidence that combining golimumab plus MTX is more efficacious than MTX monotherapy in improving psoriatic arthritis (PsA) dactylitis.
- GO-DACT showed that application of the innovative Dactylitis Severity Score (DSS) and Leeds Dactylitis Index (LDI) response indices (DSS20, 50 and 70 and LDI20, 50 and 70) allowed discrimination between treatment arms, which could be useful for future PsA trials.
- The GO-DACT trial provides data in an area of previously limited evidence to inform the creation of clinically useful treatment algorithms, aiming at the optimal care of patients with PsA.

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic inflammatory disease of substantial phenotypic heterogeneity. Such heterogeneity poses challenges in

management, particularly in deriving a sufficient evidence base to address clinical subtypes. Dactylitis is a hallmark of PsA[1] for which therapeutic strategies remain empirical.[2] Commonly, non-steroidal anti-inflammatory drugs (NSAIDs) and local corticosteroid injections are employed.[3] Patients with PsA with dactylitis have higher disease activity and increased erosion risk.[4–6] Guidelines by the Group for Research and Assessment of Psoriasis and Psoriatic Arthritis recommend conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), such as methotrexate (MTX), as a first-line on NSAIDs failure, but allow for expedited biologic disease-modifying antirheumatic drugs (bDMARDs) based on individual decisions.[7] European League Against Rheumatism recommends the use of tumour necrosis factor inhibitors (TNFi) or biologics targeting interleukin (IL)-12/IL-23 or IL-17 pathways in patients with dactylitis that impacts function and quality of life.[8]

Across randomised controlled trials (RCTs) of bDMARDs efficacy in peripheral PsA, dactylitis has never been studied as a primary endpoint; current practice arises from the analysis of dactylitis as a secondary outcome.[3 9 10] Golimumab, a human monoclonal antibody TNFi, has been approved for the treatment of active PsA.[11] In GO-DACT, a phase 3b trial, we assessed the efficacy of golimumab in combination with MTX versus MTX monotherapy for improving psoriatic dactylitis as a primary endpoint.

METHODS

Study design

GO-DACT was a multicentre, investigator-initiated, randomised, double-blind, placebo-controlled, phase 3b trial of golimumab plus MTX versus placebo plus MTX, in MTX-naive and bDMARDs-naive patients with PsA and active dactylitis. The study was conducted between August 2014 and June 2017 in 11 rheumatology centres in Portugal. The protocol was previously published.[12]

Patients were centrally randomised in blocks of 4 (2:2) by computer-generated random sequence to receive subcutaneous injections of 50 mg golimumab or placebo, administrated every 4 weeks for 24 weeks, both in combination with MTX. Patients and investigators were blind to treatment by providing identical prefilled syringes (MSD Pharmaceutics). MTX was started orally, 15 mg/week and increased 5 mg every 4 weeks until a maximum dose of 25 mg/week, as tolerated. For gastrointestinal intolerance, patients could be switched to a subcutaneous formulation. After the last golimumab injection, each subject was monitored for safety for 60 days (online supplementary figure 1). A planned interim efficacy analysis was performed when 50% of the estimated recruitment had completed 24 weeks follow-up.

Patient population

Patients over 18 years of age with a diagnosis of PsA according to Classification for Psoriatic Arthritis criteria[1] ≥1 digit with tender dactylitis and ≥1 other site of active inflammation (joints, enthesis, spine, skin or nails), naive to MTX and bDMARDs therapy and refractory to at least two NSAIDs at optimal dosage for 3 months were eligible for inclusion. Informed consent was obtained from all subjects before trial activity. The trial was conducted in accordance with the ethical principles of the Declaration of Helsinki, good clinical practice and approved by Portuguese Ethics Committee for Clinical Research, National Authority of Medicines and Health Products and National Data Protection Committee.

Key exclusion criteria were contraindications for the use of any TNFi or MTX, and factors that could interfere with trial evaluations or patient safety. A maximum of two previous local corticosteroids injections were allowed, administrated at least 4 weeks prior to screening. NSAIDs dose had to be stable throughout the trial. Cessation of other csDMARDs and corticosteroids, according to their recommended washout periods, was required.[12]

Trial procedures and endpoints

The primary endpoint was the change from baseline in Dactylitis Severity Score (DSS) at 24 weeks. Each digit with dactylitis was evaluated in a scale of 0–3 (0=no dactylitis, 1=mild dactylitis, 2=moderate dactylitis, 3=severe dactylitis), where scores greater than 0 indicate the presence of dactylitis and the total score was calculated as the sum of scores for all 20 digits (0–60).[13]

Key secondary endpoints included the change from baseline in Leeds Dactylitis Index (LDI), based on the ratio of the circumference of the affected digit and of the contralateral correspondent digit, multiplied by a tenderness score (graded 0–3 on a Ritchie Index) for each digit with dactylitis[14]; and the number of patients with tender and non-tender dactylitis and with dactylitis remission (DSS=0). New dactylitis response indices, defined as the percentage of patients achieving at least 20%, 50% or 70% of improvement in the DSS (DSS20, 50 or 70); and as at least 20%, 50% or 70% of improvement in the LDI (LDI20, 50 or 70) from baseline, were assessed in this trial. Enthesitis was evaluated resorting to the Leeds Enthesitis Index (LEI)[15] and the Spondyloarthritis Research Consortium of Canada (SPARCC) enthesitis score.[16] Enthesitis remission was defined as the absence of tender enthesis, according to LEI.

Additional key secondary endpoints comprised: 68 tender and 66 swollen joint counts,[17] patient-reported outcomes for global assessments of disease activity and pain and psoriasis evaluation using the Psoriasis Area Severity Index (PASI), Body Surface Area (BSA) score and Nail Psoriasis Severity Index (NAPSI) for the target nail. Other efficacy endpoints included physical function and health-related quality of life (psoriasis and global health), composite disease activity and response indices of PsA, as previously described.[12] All clinical efficacy outcomes were collected at every trial visit by a trained rheumatologist blind to treatment.

High-resolution magnetic resonance imaging (MRI), providing better spatial resolution and anatomical definition, was performed for the most affected digit and conventional MRI of the corresponding hand or foot, at baseline and week 24.[12] Images were read by an experienced musculoskeletal radiologist, blind to treatment and chronologic sequence of images. High-resolution dactylitis images were scored according to the presence (0) or absence (1) of eight imaging features (synovitis, bone oedema, subcutaneous oedema, flexor tenosynovitis, extensor tenosynovitis, plantar/volar plate enhancement, collateral ligament enhancement and erosions), at the metacarpal/metatarsophalangeal (MCP/MTP), proximal interphalangeal (PIP) and distal interphalangeal (DIP) joints. The dactylitis total MRI score was calculated as the sum of the partial scores at each location, as previously described.[18] The psoriatic arthritis MRI score (PSAMRIS), was used to assess the overall MRI changes in the hand (PSAMRIS-H)[19] and foot (PSAMRIS-F).[20] A total of 37 patients performed paired MRIs of the hands (16 patients) or feet (21 patients), and 36 patients paired high-resolution dactylitis images, according to the most active dactylitis location. Seven patients did not undergo hand/foot MRI and one other additionally high-resolution dactylitis protocol, due to MRI equipment failure/unavailability, inability to tolerate or trial discontinuation.

Safety and tolerability were evaluated and recorded throughout.

Statistical analysis

We estimated that a sample size of 90 patients was required to detect a difference in DSS of 2.52 between groups (absolute change from GO-REVEAL trial), assuming a SD of 4.01, with a 0.05 significance level, 80% power and accounting for a dropout rate of 10%.[11 21] An interim analysis was planned when 50% of this sample size was included; when conducted, this detected favourable results for the primary endpoint. Based on these findings, patient inclusion was halted at this milestone. Efficacy endpoints were assessed as changes from baseline or as the proportion of patients achieving responses at 12 and 24 weeks. An intention-to-treat analysis was performed for the primary endpoint, applying the last observation carried forward method and including all randomly assigned patients who received at least one dose of study medication. For the remaining clinical endpoints, a per-protocol analysis was conducted, taking into consideration that only two patients (one in each treatment arm) were lost to follow-up. For safety analysis, all patients receiving at least one dose of study medication were included. All statistical analyses were done by a statistician blind to treatment. Continuous variables were summarised by median and interquartile range (IQR), and comparisons were performed using the non-parametric Wilcoxon rank-sum test. Categorical variables were summarised by frequency and percentage, and significance of difference between the two arms analysed with Fisher's exact test (including a generalised version for variables with more than two categories). All analyses were conducted using R V.3.5.0 software (https://www.R-project.org).

RESULTS

Patients disposition and baseline characteristics

A total of 44 patients with PsA enroled at 11 trial centres were randomised. Forty-two completed the study, with one patient on golimumab/MTX discontinuing due to an adverse event (asthma exacerbation), and another patient on placebo/MTX discontinuing due to an insufficient therapeutic effect (figure 1). The mean MTX dose of golimumab/MTX group was 16.3 mg/week (range: 10–25 mg) and 19.2 mg/week (range: 15–25 mg) in MTX monotherapy group. Baseline demographics and disease activity were well matched; all patients had active dactylitis at baseline, with a median baseline DSS of 6 in both arms (table 1, online supplementary table 1).

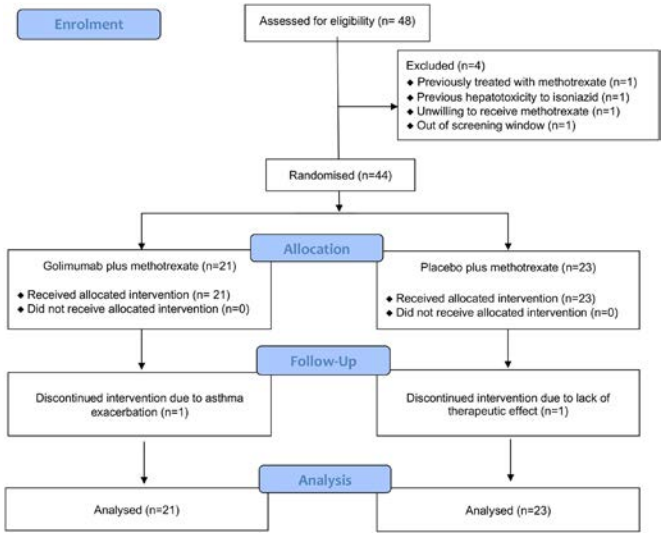

Figure 1 GO-DACT trial consort flow diagram.

Musculoskeletal efficacy

The primary efficacy endpoint was met, whereby patients treated with golimumab/MTX exhibited significantly greater improvements by DSS at week 24 (median change of 5) relative to the placebo/MTX group (median change of 2) (p=0.026), and as early as 12 weeks (p=0.004; figure 2A). Key secondary analyses followed a similar pattern. The proportion of DSS50 and DSS70 responders at week 24 were significantly higher for patients treated with golimumab/MTX (DSS50: p=0.005, DSS70: p=0.010; figure 2B). Greater improvements from baseline and in the proportion of LDI responders were observed in the golimumab/MTX group at 24 weeks (figure 2C). The number of patients achieving dactylitis remission (DSS=0) was low in both treatment groups (6/20, 30% vs 4/22, 18.2%; table 2) and was not significantly different. A total of 66.7% (14/21) patients treated with golimumab/MTX and 21.7% (5/23) treated with MTX monotherapy had absence of tenderness (LDI tenderness=0) at 24 weeks, in the digits previously affected with dactylitis.

The median baseline dactylitis MRI score was balanced between arms: 8.5 (IQR 7) in the golimumab/MTX and 8.0 (IQR 10) in placebo/MTX. At week 24, we observed significantly lower scores in patients treated with golimumab/MTX than in those treated with placebo/MTX (p=0.017). The median change of dactylitis MRI score from baseline was numerically larger for golimumab/MTX (5.5) in comparison with MTX monotherapy (3.5; p=0.273; table 3). Both golimumab/MTX and MTX monotherapy arms reduced bone oedema, subcutaneous oedema, volar and palmar/plantar and collateral enhancement scores at the MCP/MTPs and PIPs, between baseline and week 24. These changes were numerically more prevalent in golimumab/MTX group, but only significantly different between treatment arms for synovitis and bone oedema at PIPs. No change in mean erosion score at the dactylitis digits was observed during the 24 weeks of treatment (table 3). At 24 weeks, the absence of dactylitis associated-inflammatory lesions was observed in 31.0% (9/29) of all patients, 53.8% (7/13) of those receiving golimumab/MTX and 12.5% (2/16) of those receiving placebo/MTX.

GO-DACT patients had a median of 6.5/66 swollen and 7.5/68 tender joints, and moderate Disease Activity Score four variables (DAS28 4v) or high Disease Activity in Psoriatic Arthritis (DAPSA) peripheral disease activity at baseline, despite the absence of inclusion criteria regarding the number of active peripheral joints. DAS28 4v, DAPSA and Psoriatic Arthritis Disease Activity Score (PASDAS) demonstrated improvements of disease activity in the golimumab/MTX in both week 12 (p=0.004; p=0.012; p=0.0007) and week 24 (p=0.013, p=0.039; p=0.008; table 2, figure 2E) that were significantly greater than with placebo/MTX.

Overall, 36.4% (16/44) and 52.3% (23/44) of the patients had baseline enthesitis according to LEI and SPARCC, respectively. Median changes from baseline for both LEI and SPARCC and the percentage of patients with enthesitis remission at week 24 were not significantly different between groups (table 2).

Cutaneous efficacy

PASI and BSA and skin-related quality of life (Dermatology Life Quality Index) improved in both groups at week 24. Patients in the golimumab/MTX arm demonstrated numerically but not significantly greater responses than placebo/MTX. Golimumab/MTX was also associated with improvements in the target

Table 1 Baseline demographic and disease characteristics of the study population

Characteristics	Study population n=44	GLM+MTX n=21	PLB+MTX n=23
Male gender, n (%)	37 (84.0%)	17 (81.0%)	20 (87.0%)
Age at randomisation, years, median (IQR)	45.7 (19.6)	46.2 (15.5)	44.1 (24.6)
Disease duration, median (IQR)	3.9 (6.9)	3.8 (6.7)	4.2 (6.1)
Body mass index (kg/m^2), median (IQR)	26.6 (6.1)	29.0 (4.5)	25.9 (5.4)
Clinical subtype, n (%)			
Symmetric polyarthritis	9 (20.5)	5 (23.8)	4 (17.4)
Predominant arthritis of the distal interphalangeal joints	3 (6.8)	2 (9.5)	1 (4.3)
Asymmetric oligoarthritis	31 (70.5)	13 (61.9)	18 (78.3)
Arthritis mutilans	0 (0)	0 (0)	0 (0)
Predominant axial	1 (2.3)	1 (4.8)	0 (0)
Dactylitis			
DSS, median (IQR)	6 (4)	6 (5)	6 (3.5)
LDI, median (IQR)	64.7 (81.7)	69.4 (73.8)	64.0 (100)
Enthesitis			
Enthesitis, median (IQR)	1 (2)	1 (2)	1 (2)
Enthesitis ≥1 n (%)	23/44 (52.3%)	11/21 (52.4%)	12/23 (52.2%)
LEI, median (IQR)	0 (1)	0 (1)	0 (1)
LEI≥1, n (%)	16/44 (36.4%)	7/21 (33,3%)	9/23 (39,1%)
SPARCC, median (IQR)	1 (2)	1 (2)	1 (2)
SPARCC≥1, n (%)	23/44 (52.3%)	11/23 (47.8%)	12/23 (52.2%)
Peripheral joints			
Tender joints (68), median (IQR)	7.5 (9.25)	8 (9)	6 (8)
Swollen joints (66), median (IQR)	6.5 (6.5)	7 (10)	6 (5)
Psoriasis			
PASI, median (IQR)	3.05 (4.3)	4 (4)	2.4 (2.65)
BSA, median (IQR)	9.75 (21.6)	13 (29.5)	8.2 (15.3)
Target NAPSI, median (IQR)	4 (8)	4 (10)	4 (5)
Physical function			
HAQ-DI, median (IQR)	0.875 (IQR)	0.875 (0.625)	0.875 (1.25)
Health-related quality of life			
DLQI, median (IQR)	3 (4.25)	4 (4)	1 (4)
Composite indices of disease activity			
DAS28 4v, median (IQR)	4.01 (1.68)	3.71 (0.96)	4.14 (1.99)
DAPSA, median (IQR)	24.41 (21.31)	24.3 (20.84)	24.5 (20.20)
PASDAS, median (IQR)	6.13 (2.35)	6.1 (1.83)	6.2 (2.58)
CPDAI, median (IQR)	11 (I3.5)	11.0 (3.5)	11.5 (2.5)

BSA, body surface area; CPDAI, Composite Psoriatic Disease Activity Index; DAPSA, disease activity in psoriatic arthritis; DAS28 4v, Disease Activity Score 4 variablesDLQI, Dermatology Life Quality Index; DSS, Dactylitis Severity Score; GLM, golimumab; HAQ-DI, Health Assessment Questionnaire Disability Index; LDI, Leeds Dactylitis Index; LEI, Leeds Enthesitis Index; MTX, methotrexate; NAPSI, Nail Psoriasis Severity Index; PASDAS, Psoriatic Arthritis Disease Activity Score; PASI, Psoriasis Area and Severity Index; PLB, placebo; SPARCC, Spondyloarthritis Research Consortium of Canada Enthesitis Index;BSA, body surface area; BSA, body surface area; CPDAI, Composite Psoriatic Disease Activity Index; CPDAI, Composite Psoriatic Disease Activity Index; DAPSA, disease activity in psoriatic arthritis; DAPSA, disease activity in psoriatic arthritis; DAS28 4v, Disease Activity Score 4 variablesDAS28 4v, Disease Activity Score 4 variablesDLQI, Dermatology Life Quality Index; DLQI, Dermatology Life Quality Index; DSS, Dactylitis Severity Score; DSS, Dactylitis Severity Score; GLM, golimumab; GLM, golimumab; HAQ-DI, Health Assessment Questionnaire Disability Index; HAQ-DI, Health Assessment Questionnaire Disability Index; LDI, Leeds Dactylitis Index; LDI, Leeds Dactylitis Index; LEI, Leeds Enthesitis Index; LEI, Leeds Enthesitis Index; MTX, methotrexate; MTX, methotrexate; NAPSI, Nail Psoriasis Severity Index; NAPSI, Nail Psoriasis Severity Index; PASDAS, Psoriatic Arthritis Disease Activity Score; PASDAS, Psoriatic Arthritis Disease Activity Score; PASI, Psoriasis Area and Severity Index; PASI, Psoriasis Area and Severity Index; PLB, placebo; PLB, placebo; SPARCC, Spondyloarthritis Research Consortium of Canada Enthesitis Index; SPARCC, Spondyloarthritis Research Consortium of Canada Enthesitis Index.

NAPSI, whereas no changes from baseline to week 12 or 24, were detected in placebo/MTX recipients (figure 2D).

Response indices of disease activity

At week 24, patients' improvement was numerically greater in the golimumab/MTX than placebo/MTX group for Minimal Disease Activity (MDA), Psoriatic Arthritis Response Criteria (PsARC) and Psoriatic Arthritis Joint Activity Index (PsAJAI). Statistically significant improvement was noted for American College of Rheumatology (ACR) 20 and ACR50 responses (figure 2F).

Imaging outcomes

MRI changes were described according to PSAMRIS-H and PSAMRIS-F. DIP readings were applicable only to the hands, and MCP/MTP and PIP readings were grouped together for hands and feet. Osteoproliferation at MTPs/PIPs and periarticular inflammation at PIPs of the feet were excluded due to low image resolution. Golimumab/MTX delivered greater reduction in PSAMRIS inflammatory lesion scores between baseline and week 24, but these differences were only significant in comparison with placebo/MTX for changes in PIP synovitis. Bone erosion and proliferation did not differ significantly between

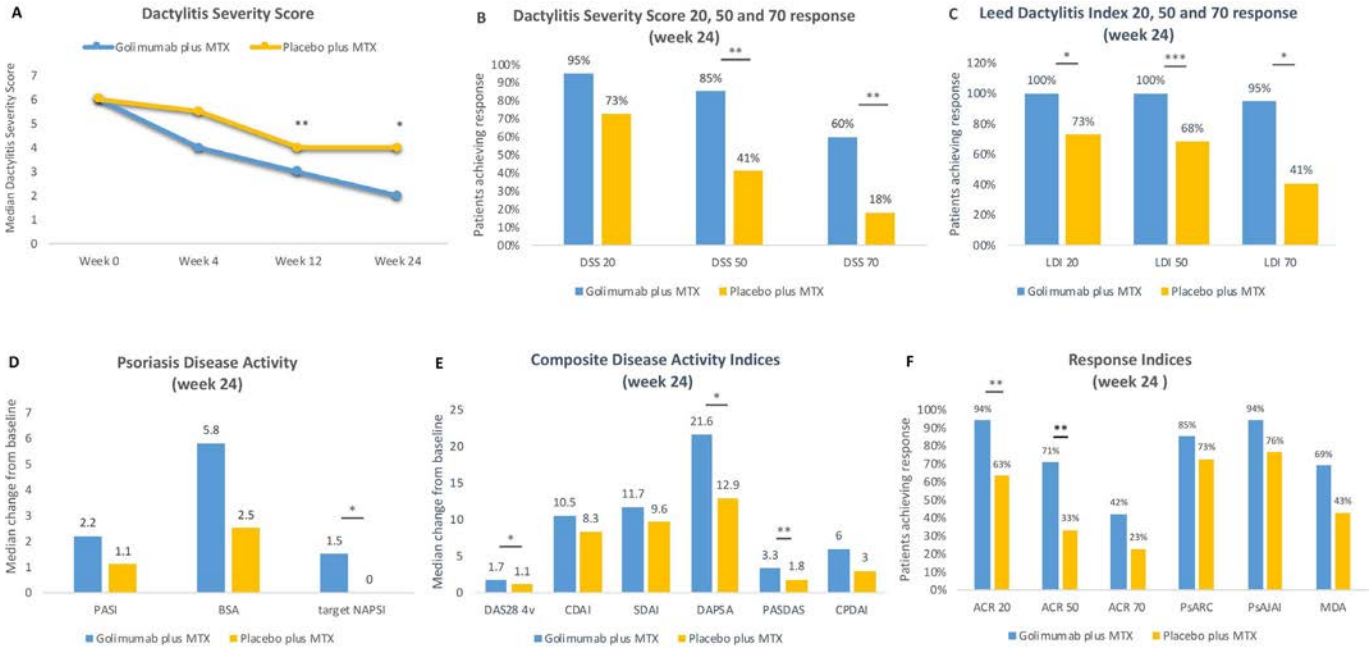

Figure 2 Changes from baseline to week 24 in DSS (A), psoriasis disease activity (D) and composite disease activity indices (E). Proportion of responders at week 24 of DSS 20, 50 and 70 (B), LDI 20, 50 and 70 (C) and response indices (F). ACR, American College of Rheumatology response index; BSA, body surface area; CDAI, clinical disease activity index; CPDAI, composite psoriatic disease activity index; DSS, Dactylitis Severity Score; DAPSA, disease activity in psoriatic arthritis; DAS28 4v, Disease Activity Score four variables; LDI, Leeds Dactylitis Index; MDA, minimal disease activity; MTX, methotrexate; NAPSI, Nail Psoriasis Severity Index; PASDAS: Psoriatic Arthritis Disease Activity Score; PASI: Psoriasis Area and Severity Index; PsAJAI, Psoriatic Arthritis Joint Activity Index; PsARC, Psoriatic Arthritis Response Criteria; SDAI, simplified disease activity index. *p<0.05; **p<0.005; ***p<0.001.

timepoints, regardless of location or treatment (table 4, online supplementary table 3). At 24 weeks, resolution of inflammation, defined as a PSAMRIS of 0 (excluding erosions and bone proliferation), was achieved by 12 patients; 50% (7/14) of patients in golimumab/MTX and 29.4% (5/17) in MTX monotherapy.

Safety

One hundred and two adverse events were reported during the GO-DACT study period, mostly of mild to moderate severity, overall with similar incidence between treatment arms. There were no new safety issues during this trial.

DISCUSSION

Herein we show that the combination of golimumab plus MTX is associated with significantly greater clinical improvements in dactylitis activity than MTX monotherapy. GO-DACT also demonstrated that the application of innovative DSS and LDI response indices (DSS20/50/70 and LDI20/50/70) discriminated between treatment arms, as early as 12 weeks, despite the small trial size. DSS and LDI response indices might be useful instruments for future trials assessing dactylitis. We also observed a trajectory for DSS and LDI response from week 12 to 24 commensurate with a slower achievement of maximal dactylitis response. This has been observed in RCTs with longer follow-up periods[11] ; evaluation of complete resolution of dactylitis in future trials may require follow-up longer than 24 weeks. The follow-up of these patients, according to clinical practice, might bring additional information on the long-term dactylitis remission rates.

Improvements favouring the golimumab plus MTX group occurred across other than dactylitis PsA domains including peripheral arthritis, nail psoriasis and composite measures of disease activity (DAS28 4v, DAPSA and PASDAS). PASDAS showed the ability to discriminate between treatment arms and performed better than the Clinical Disease Activity Index (CDAI), reinforcing previously published golimumab trials and 'real-world' data, suggestive of larger effect sizes for PASDAS.[22 23] Nail psoriasis has not been frequently studied in PsA RCTs.[24–26] Here, we showed significant benefit from golimumab plus MTX and an absence of improvement of target NAPSI in MTX monotherapy-treated patients, supporting previously reported lack of efficacy of MTX.[27] Others though have described improvement of the nail matrix component with MTX.[28]

We included an exploratory imaging evaluation. Evidence that either TNFi or MTX can ameliorate hand/feet PsA MRI features is limited. Both golimumab plus MTX and MTX monotherapy reduced articular and periarticular inflammatory scores either on dactylitis and overall PSAMRIS, while erosions and osteoproliferation scores remained globally unchanged throughout the 24 weeks of the trial. Due to slow progression, erosions or new bone formation changes in PsA are difficult to depict in short-term studies, and MRI complete resolution of inflammation remains a challenging target.[29]

We studied an MTX-naive population to avoid bias from MTX prior non-responders. Although less efficacious alone than in combination with golimumab, we observed small improvements in dactylitis (−2 DSS units from baseline to week 24) and other PsA domains (peripheral arthritis and plaque psoriasis) in MTX monotherapy-treated patients. Furthermore, MTX monotherapy patients consistently attained higher rates of response from week 12 to week 24 for peripheral arthritis and composite indices (ACR, MDA, PsARC, PsAJAI, PASI) of PsA activity, suggesting incremental therapeutic benefits that continued through follow-up. These results are in line with a recently published RCT showing moderate but consistent benefits from MTX.[30]

Table 2 Efficacy outcomes change from baseline to week 12 and 24, for both treatment arms

	12 Weeks			24 Weeks		
Efficacy outcomes	**Median change GLM+MTX**	**Median change PLB+MTX**	**P value**	**Median change GLM+MTX**	**Median change PLB+MTX**	**P value**
Dactylitis						
DSS	-3.5	-1	0.004	-5	-2	0.026
DSS response						
DSS 20, n (%)	19/20 (95)	12/23 (52.2)	0.002	19/20 (95.0)	16/22 (72.7)	0.096
DSS 50, n (%)	17/20 (85)	7/23 (30.4)	0.001	17/20 (85.0)	09/22 (40.9)	0.005
DSS 70, n (%)	7/20 (35)	5/23 (21.7)	0.497	12/20 (60.0)	4/22 (18.2)	0.010
Dactylitis remission (DSS=0), n (%)	2/20 (10)	4/23 (17.4)	0.67	6/20 (30.0)	4/22 (18.1)	0.477
LDI	-58.6	-34.6	0.169	-69.4	-31.1	0.042
LDI response						
LDI 20, n (%)	19/19 (100.0)	18/23 (78.3)	0.053	19/19 (100.0)	16/22 (72.7)	0.023
LDI 50, n (%)	17/19 (89.5)	13/23 (56.5)	0.037	19/19 (100.0)	15/22 (68.2)	0.001
LDI 70, n (%)	16/19 (84.1)	9/23 (39.1)	0.004	18/19 (94.7)	9/22 (40.9)	0.011
Enthesitis						
Enthesitis	-0.5	0	0.512	-1	0	0.224
LEI	0	0	0.752	0	0	0.953
SPARCC	-0.5	0	0.589	-1	0	0.216
Enthesitis remission (LEI=0), n (%)	9/11 (81.8)	10/12 (83.3)		11/11 (100.0)	9/11 (90.0)	0.476
Peripheral joints						
Tender joints (68)	-5.5	-2	0.026	-7.5	-5	0.077
Swollen joints (66)	-6.5	-2	0.006	-7	-4	0.060
Psoriasis						
PASI	-2.4	-0.6	0.027	-2.2	-1.1	0.130
BSA	-7	-0.5	0.097	-5.8	-2.5	0.337
Target NAPSI	-2	0	0.044	-1.5	0	0.027
Patient-reported and physician-reported outcomes						
PGA for arthritis activity (0–100 mm)	-20	-12.5	0.874	-34	-16.5	0.190
PGA for psoriasis activity (0–100 mm)	-30	-10	0.846	-10	-9	0.860
Physical function						
HAQ-DI	-0.5	-0.125	0.163	-0.375	-0.188	0.414
Health-related quality of life						
DLQI	-2	-0.5	0.101	-2.5	-1	0.161
Composite indices of disease activity						
DAS28 4v	-1.67	-0.83	0.004	-1.72	-1.15	0.013
DAPSA	-17.05	-9.32	0.012	-21.62	-12.88	0.039
PASDAS	-2.7	-1.39	0.001	-3.27	-1.76	0.008
CPDAI	-2	-2	0.312	-6	-3	0.292
Response indices						
ACR						
ACR 20, n (%)	17/17 (100.0)	9/19 (47.4)	0.001	15/16 (93.8)	12/19 (63.2)	0.047
ACR 50, n (%)	11/19 (57.9)	5/21 (23.8)	0.052	12/17 (70.6)	7/21 (33.3)	0.049
ACR 70, n (%)	6/20 (30.0)	1/22 (4.5)	0.041	8/19 (42.1)	5/22 (22.7)	0.313
MDA, n (%)	10/18 (55.6)	3/23 (13.0)	0.006	11/16 (68.8)	9/21 (42.9)	0.185
PsARC, n (%)	16/20 (80.0)	13/23 (56.5)	0.119	17/20 (85.0)	16/22 (72.7)	0.460
PsAJAI, n (%)	16/18 (88.9)	14/22 (63.6)	0.082	16/17 (94.1)	16/21 (76.2)	0.197
PASI						
PASI 50, n (%)	16/20 (80.0)	10/22 (45.5)	0.029	17/20 (85.0)	12/20 (60.0)	0.155
PASI 70, n (%)	10/20 (50.0)	8/22 (36.4)	0.534	12/20 (60.0)	9/20 (45.0)	0.527
PASI 90, n (%)	5/20 (25.0)	4/22 (18.2)	0.714	5/20 (25.0)	8/20 (40.0)	0.501

ACR, American College of Rheumatology; BSA, body surface area; CPDAI, Composite Psoriatic Disease Activity Index; DAPSA, Disease Activity in Psoriatic Arthritis; DAS28 v4, Disease Activity Score four variables; DLQI, Dermatology Life Quality Index; DSS, Dactylitis Severity Score; GLM, golimumab; HAQ-DI, Health Assessment Questionnaire Disability Index; LDI, Leeds Dactylitis Index; LEI, Leeds Enthesitis Index; MDA, minimal disease activity; MTX, methotrexate; NAPSI, Nail Psoriasis Severity Index; PASDAS, Psoriatic Arthritis Disease Activity Score; PASI, Psoriasis Area and Severity Index; PGA, patient global assessment; PhGA, physician global assessment; PLB, placebo; PsAJAI, Psoriatic Arthritis Joint Activity Index; PsARC, Psoriatic Arthritis Response Criteria; SPARCC, Spondyloarthritis Research Consortium of Canada Enthesitis Index.p<0.05.

Global assessment of safety on golimumab plus MTX was as expected.[31]

Considering the high disease burden of dactylitis in patients with PsA, including a lower chance of achieving MDA[6] and the risk of structural damage,[4 5] and advantages of early TNFi intervention,[32] it seems reasonable to argue that patients with PsA active dactylitis could benefit from first-line TNFi plus MTX therapy. We expect that these results will be reproducible with other TNFi combination therapies.[33]

Limitations in our study include the small number of patients enroled, which can increase the risk of type II errors. However, because the primary endpoint showed significant differences between treatment groups, recruitment was halted at half of the planned enrolment. MRI assessment was included

Table 3 Dactylitis MRI total and partial scores

	Baseline				Week 24						
Dactylitis total score	**Population (n=36)**	**GLM+MTX (n=18)**	**PLB+MTX (n=18)**	**P value***	**Population (n=36)**	**GLM+MTX (n=18)**	**PLB+MTX (n=18)**	**P value†**	**Median change GLM+MTX**	**Median change PLB+MTX**	**P value‡**
Median (IQR)	8 (7)	8.5 (7)	8.0 (10)	0.696	3.5 (4)	2 (3)	5.5 (3)	0.017	−5.5 (4.8)	−3.5 (6.5)	0.273
(observed range)	2 to 20	2 to 17	2 to 20		0 to 17	0 to 17	0 to 7		−14 to 0	−13 to 2	

	MCP/MTP				PIP				DIP			
	GLM+MTX		PLB+MTX		GLM+MTX		PLB+MTX		GLM+MTX		PLB+MTX	
Dactylitis partial scores	**Baseline**	**Change**	**Baseline**	**Change**	**Baseline**	**Change**	**Baseline**	**Change**	**Baseline**	**Change**	**Baseline**	**Change**
Synovitis, mean	0.56	−0.28	0.78	−0.17	0.78	−0.61*	0.5	−0.22	0.38	−0.31	0.31	0.12
(observed range)	(0 to 1)	(−1 to 1)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(0 to 1)
Bone oedema, mean	0.44	−0.28	0.5	−0.39	0.67	−0.56§	0.44	−0.22	0.31	−0.23	0.38	−0.06
(observed range)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 1)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)
Subcutaneous oedema, mean	0.5	−0.44	0.67	−0.39	0.78	−0.61	0.5	−0.33	0.38	−0.31	0.5	−0.12
(observed range)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(-1 to 1)
Flexor tenosynovitis, mean	0.33	−0.22	0.56	−0.17	0.44	−0.22	0.44	−0.17	0.23	−0.08	0.25	−0.19
(observed range)	(0 to 1)	(−1 to 1)	(0 to 1)	(−1 to 1)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 1)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)
Extensor paratenonitis, mean	0	0	0.11	−0.11	0	0.06	0.11	−0.11	0.15	−0.08	0.06	−0.06
(observed range)	(0 to 0)	(0 to 0)	(0 to 1)	(−1 to 0)	(0 to 0)	(0 to 1)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)
Volar and palmar/plantar plate enhancement, mean	0.5	−0.33	0.59	−0.47	0.5	−0.44	0.44	−0.28	0.23	−0.15	0.31	−0.12
(observed range)	(0 to 1)	(−1 to 1)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 1)
Collateral ligament enhancement, mean	0.39	−0.28	0.67	−0.33	0.39	−0.28	0.44	−0.28	0.23	−0.15	0.38	−0.12
(observed range)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(−1 to 0)
Erosions, mean	0.39	0	0.44	0	0.61	−0.06	0.28	0	0.31	0	0.5	0
(observed range)	(0 to 1)	(−1 to 1)	(0 to 1)	(0 to 0)	(0 to 1)	(−1 to 0)	(0 to 1)	(0 to 0)	(0 to 1)	(0 to 0)	(0 to 1)	(0 to 0)

*Differences in the dactylitis total score at baseline, between treatment arms.
†Differences in the dactylitis total score at week 24, between treatment arms.
‡Differences in the median change of the dactylitis total score from baseline to week 24, between treatment arms.
§Differences in the mean change of the dactylitis partial score from baseline to week 24, between treatment arms. $p<0.05$.
DIP, distal interphalangeal joints; GLM, golimumab; MCP/MTP, metacarpal/metatarsophalangeal joints; MTX, methotrexate; PIP, proximal interphalangeal joints; PLB, placebo.

Table 4 PSAMRIS for individual MRI features as assessed by PSAMRIS-H and PSAMRIS-F, for both treatment arms, at baseline and change from baseline to week 24

	MCP/MTP				PIP				DIP			
	GLM+MTX		PLB+MTX		GLM+MTX		PLB+MTX		GLM+MTX		PLB+MTX	
PSAMRIS features	Baseline	Change	Baseline	Change	Baseline	Change	Baseline	Change	Baseline	Change	Baseline	Change
Synovitis, mean	3.8	−2.87	3.44	−1.94	1.93	−1.54*	1.06	0	1	−0.67	1	−0.71
(observed range)	(0 to 12)	(−9 to 0)	(0 to 7)	(−7 to 2)	(0 to 4)	(−4 to 1)	(0 to 3)	(−3 to 3)	(0 to 3)	(−2 to 0)	(0 to 4)	(-2 to 0)
Flexor tenosynovitis, mean	0.56	−0.38	0.47	−0.05	0.56	−0.38	0.47	−0.05	1.4	−1	1	−0.33
(observed range)	(0 to 3)	(−2 to 0)	(0 to 3)	(−2 to 2)	(0 to 3)	(−2 to 0)	(0 to 3)	(−2 to 2)	(0 to 3)	(−2 to 0)	(0 to 3)	(-2 to 2)
Periarticular inflammation, mean	3.14	−1.86	3	−2.41	0.33	0	0.33	0.14	0.6	−0.4	0.5	−0.29
(observed range)	(0 to 24)	(−14 to 0)	(0 to 7)	(−7 to 0)	(0 to 2)	(0 to 0)	(0 to 2)	(0 to 1)	(0 to 2)	(−1 to 0)	(0 to 2)	(-1 to 0)
Bone marrow oedema, mean	4.56	−2.94	3.11	−2.67	4.82	−3.59	3	−0.72	1.33	−1	2	-1
(observed range)	(0 to 23)	(−14 to 0)	(0 to 16)	(−16 to 0)	(0 to 24)	(−22 to 1)	(0 to 16)	(−8 to 4)	(0 to 6)	(−6 to 0)	(0 to 8)	(-4 to 0)
Bone erosion, mean	2.06	0.5	1.47	−0.06	1.47	0	1.53	0.89	6.33	0	2.89	0
(observed range)	(0 to 12)	(−3 to 12)	(0 to 8)	(−2 to 1)	(0 to 13)	(0 to 0)	(0 to 14)	(−1 to 10)	(0 to 14)	(0 to 0)	(0 to 8)	(0 to 0)
Bone proliferation, mean	2.83	0	1.67	0	2.67	0	2.67	0	2.2	−0.8	2	0
(observed range)	(0 to 5)	(0 to 0)	(0 to 5)	(0 to 0)	(0 to 5)	(0 to 0)	(0 to 5)	(0 to 0)	(0 to 4)	(−4 to 0)	(0 to 4)	(0 to 0)

*Difference between treatment groups in the change from baseline to week 24 (p=0.0054); p<0.05.
DIP, distal interphalangeal joints; GLM, golimumab; MCP/MTP, metacarpal/metatarsophalangeal joints; MTX, methotrexate; PIP, proximal interphalangeal joints; PLB, placebo; PSAMRIS, Psoriatic Arthritis MRI Score; PSAMRIS-F, Psoriatic Arthritis MRI Score for the foot.; PSAMRIS-H, Psoriatic Arthritis MRI Score for the hand.

as a secondary exploratory endpoint in a limited number of patients with single imaging reading, which implies caution in data interpretation. Although a golimumab monotherapy arm could also have been included, results from GO-REVEAL suggested a 10% additional benefit in dactylitis improvements from combination with MTX. GO-DACT included several variables to capture disease activity in its different domains, increasing the risk of type I error through multiple comparisons. Nevertheless, the population was well-balanced between groups, and results were consistent between the variables and with the published literature. This was potentiated by the lack of consensus, especially regarding composite disease activity scores.

CONCLUSIONS

GO-DACT provides strong evidence that combination of golimumab plus MTX is more efficacious than MTX monotherapy in improving PsA dactylitis. GO-DACT also exemplified that application of the innovative DSS and LDI response indices (DSS20, 50 and 70 and LDI20, 50 and 70) allowed discrimination between treatment arms, which could be useful for future PsA trials.

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TRANSLATIONAL SCIENCE

Differences in the serum metabolome and lipidome identify potential biomarkers for seronegative rheumatoid arthritis versus psoriatic arthritis

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ABSTRACT

Objectives The differential diagnosis of seronegative rheumatoid arthritis (negRA) and psoriasis arthritis (PsA) is often difficult due to the similarity of symptoms and the unavailability of reliable clinical markers. Since chronic inflammation induces major changes in the serum metabolome and lipidome, we tested whether differences in serum metabolites and lipids could aid in improving the differential diagnosis of these diseases.

Methods Sera from negRA and PsA patients with established diagnosis were collected to build a biomarker-discovery cohort and a blinded validation cohort. Samples were analysed by proton nuclear magnetic resonance. Metabolite concentrations were calculated from the spectra and used to select the variables to build a multivariate diagnostic model.

Results Univariate analysis demonstrated differences in serological concentrations of amino acids: alanine, threonine, leucine, phenylalanine and valine; organic compounds: acetate, creatine, lactate and choline; and lipid ratios L3/L1, L5/L1 and L6/L1, but yielded area under the curve (AUC) values lower than 70%, indicating poor specificity and sensitivity. A multivariate diagnostic model that included age, gender, the concentrations of alanine, succinate and creatine phosphate and the lipid ratios L2/L1, L5/L1 and L6/L1 improved the sensitivity and specificity of the diagnosis with an AUC of 84.5%. Using this biomarker model, 71% of patients from a blinded validation cohort were correctly classified.

Conclusions PsA and negRA have distinct serum metabolomic and lipidomic signatures that can be used as biomarkers to discriminate between them. After validation in larger multiethnic cohorts this diagnostic model may become a valuable tool for a definite diagnosis of negRA or PsA patients.

Key messages

What is already known about this subject?

- Clinical symptoms of (seronegative) rheumatoid arthritis (RA) and psoriatic arthritis (PsA) can be similar. Biomarkers for a correct diagnosis do not exist. Since immunosuppressive drugs have different therapeutic effects on both diseases, a correct diagnosis is important for the success of treatment.

What does this study add?

- The study presents evidence that chronic inflammatory diseases with similar clinical symptoms have significant differences in their metabolomes and lipidomes at systemic level.
- The study identifies novel biomarkers for the differential diagnosis of seronegative RA and PsA.

How might this impact on clinical practice or future developments?

- Better and earlier attribution of patients with arthritis to the correct diagnosis, which will help a faster correct choice of drugs.
- The expansion of nuclear magnetic resonance-based metabolomic and lipidomic analyses to other cohorts of clinically and demographically well-characterised patients with chronic autoimmune diseases may unveil new biomarkers to improve differential diagnosis, therapy response or disease relapses.

INTRODUCTION

The diagnosis of rheumatoid arthritis (RA) is mostly based on clinical symptoms and the serological positivity of rheumatoid factor (RF) and/or anticitrullinated peptide antibodies (anti-CCPs), whereas for psoriasis arthritis (PsA), only clinical and imaging features help in diagnosing the disease. Although most patients with RA are seropositive for RF and/or anti-CCP, in about 15%–20% of cases, the levels of RF and anti-CCP are not elevated, and since the symptoms between RA and PsA can be very similar, making a differential diagnosis between seronegative RA (negRA) and PsA is often difficult. Since the therapeutic strategies for the two diseases are different, early recognition and correct choice of treatment are essential to attain remission or low disease activity and to prevent, or at least to limit, joint damage as well as systemic manifestations.[1–7] Therefore, innovative tools for a reliable diagnosis of negRA versus PsA are needed.

In patients with chronic inflammatory diseases, an altered action of cytokines and other proinflammatory effector molecules added to a prolonged intake of immunomodulatory drugs leads to major remodelling in cellular and tissue metabolism. Such metabolic modifications also have a systemic impact that can be monitored by analysing the changes in the metabolome of biofluids. Assessing several metabolites simultaneously can potentially locate differences between disease profiles, thereby allowing the identification of potential

biomarkers and the discovery of altered metabolic pathways. ^{1}H nuclear magnetic resonance (NMR)-based metabolomic studies of serum, urine and synovial fluid obtained from patients with chronic arthritis have been used for diagnostic, prognostic and following the response to treatment. The development of RA in patients with early arthritis has been associated with increased serum levels of certain metabolites that correlated with the C reactive protein (CRP) titre.[8] High serum levels of lactate, acetylated glycoprotein and cholesterol differentiated healthy individuals from patients with RA regardless of anti-tumor necrosis factor α (TNF) therapy.[9] The urine metabolome from anti-TNF-treated patients with RA identified high levels of histamine, glutamine, thymine, creatinine and xanthine as predictors of a good response to TNF-α blockade.[10] An effective response to methotrexate (MTX) in patients with RA appears to be linked to elevated serum levels of uric acid, taurine, histidine, hypoxanthine and methionine.[11] However, a comparison of the aromatic, sugar and aliphatic regions in the ^{1}H NMR spectra of synovial fluid samples could not distinguish groups of patients with different types of arthritis.[12]

In all the studies comparing the metabolome of patients with RA to the metabolome of other patients with chronic inflammatory arthritis, there is no separate analysis of the negRA group, eventually due to the small size of the studied RA cohorts. Therefore, the potential of using metabolomic and lipidomic profiling to improve the differential diagnosis of PsA and negRA remains largely unexplored. Hence, we carried out ^{1}H NMR-based metabolomic and lipidomic analysis of serum samples from a large cohort of PsA and negRA patients, followed by a validation cohort analysis in order to identify and confirm serum metabolome-based biomarkers as a diagnostic multivariate model for these two pathologies.

PATIENTS AND METHODS

A detailed description of the patient selection, the experimental and statistical methods can be found in the online supplementary materials file 1.

Serum samples were collected from 49 patients with negRA and 73 with PsA at the Division of Rheumatology outpatient clinic of the University Hospital Heidelberg. Clinical and demographic characteristics of the cohort are summarised in table 1.

Study approval and patient and public involvement

Besides their voluntary participation in donating samples patients had no further involvement in the planning or execution of this study.

NMR spectroscopic analysis and metabolite identification

Metabolic analysis of the serum samples was carried out on a Bruker 600MHz NMR spectrometer following previously described procedures.[13] Metabolite identification was performed using the resonance assignments, chemical shifts and coupling patterns published for human serum samples.[14 15] Further details on the NMR analysis can be found in the online supplementary materials file 1.

RESULTS

Metabolomic and lipidomic profile of blood samples from negRA and PsA patients

In the ^{1}H single-pulse NMR spectrum, peaks from both small molecules and macromolecules are observed, resulting in an uneven baseline and the overlap of the signals originating from different compounds. Nonetheless, due to their characteristic spectral profiles, it is possible to use ^{1}H NMR to identify and quantify lipids in the serum (figure 1A). By suppressing the broad signals from lipids and proteins, the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence allows the peaks from low-molecular weight compounds to not be overshadowed by the signals arising from macromolecules. Since they are characterised by sharp signals and a well-defined baseline, CPMG NMR spectra allow better identification and analysis of signals arising from small molecules (figure 1B).

Table 1 Clinical and demographic data of the study participants

	negRA (n=49)	PsA (n=73)	P value
Female:Male	39:10	29:44	<0.0001 (χ^2)
Age (minimum–maximum in years)	64.2 (32–83)	56.2 (30–78)	0.003
Disease duration (minimum–maximum in years)	11.6 (1–41)	9.0 (0–24)	0.042
DAS28-CRP (minimum-maximum)	2.6 (0–6.2)	2.3 (0.96–4.09)	*0.093*
% active (DAS28 >3.2)	30.6%	28.8%	
% remission (DAS28 <2.6)	55.1%	57.5%	
CRP (±SD) (mg/L)	5.7±7.4	6.7±13.8	*0.642*
Rheumatoid factor positive	None	None	
Anti-CCP positive	None	None	
MTX (±SD) (mg)*	13.2±4.8	14.1±3.2	*0.368*
% from total patients	44.9%	50.7%	*0.531* (χ^2)
Glucocorticoid (±SD) (mg)	6.2±5.0	5.6±3.5	*0.651*
% from total patients	38.8%	34.2%	*0.610* (χ^2)
Leflunomide (±SD) (mg)	15.8±4.7	13.6±4.5	*0.266*
% from total	24.5%	15.1%	*0.192* (χ^2)
On immunotherapy	13	35	0.018 (χ^2)
Anti-TNF	15.1%	27.8%	
Anti-IL-6R	3.8%	0%	
Anti-CTLA-4	1.9%	0%	
Anti-IL-12/IL-23	0%	6.3%	
JAK-blockade	5.7%	0%	

p-values above 0.05 are indicated in italic.
*The average dosage of each medication (MTX, glucocorticoid and leflunomide) was calculated only for the patients taking that medication. The p-values bove 0.05 are indicated in italic.
anti-CCP, anticyclic citrullinated peptide antibodies; CRP, C reactive protein; CTLA, cytotoxic T-lymphocyte-associated protein 4 inhibitor; DAS28, disease activity score; IL, interleukin; JAK, janus kinase inhibitor
; MTX, methotrexate; nd, not determined; TNF, anti-tumor necrosis alpha.

Untargeted analysis

Multivariate statistical analysis was performed on the bucketed ^{1}H single-pulse and CPMG NMR spectra in order to determine whether there were characteristic spectral patterns or peaks that distinguished between the two diseases.

Based on the results partial least squares discriminant analysis (PLS-DA) and random forest models, there was no evidence that any of the clinical and demographic covariates could influence the metabolomic patterns of the patients' sera (online supplementary figure SF1). Additionally, there were no significant correlations between the clinical or demographic covariates and the regions of the ^{1}H (figure 1C) and CPMG (data not shown) spectra.

Since principal components analysis-based clustering of metabolomics data is often difficult,[16–18] the ^{1}H and CPMG spectral data-sets were evaluated by PLS-DA. Even though the principal components 1–5 explained 96.9% of the variance of the ^{1}H data or 84.6% of the variance in the CPMG data, a clear clustering that could distinguish between the negRA and

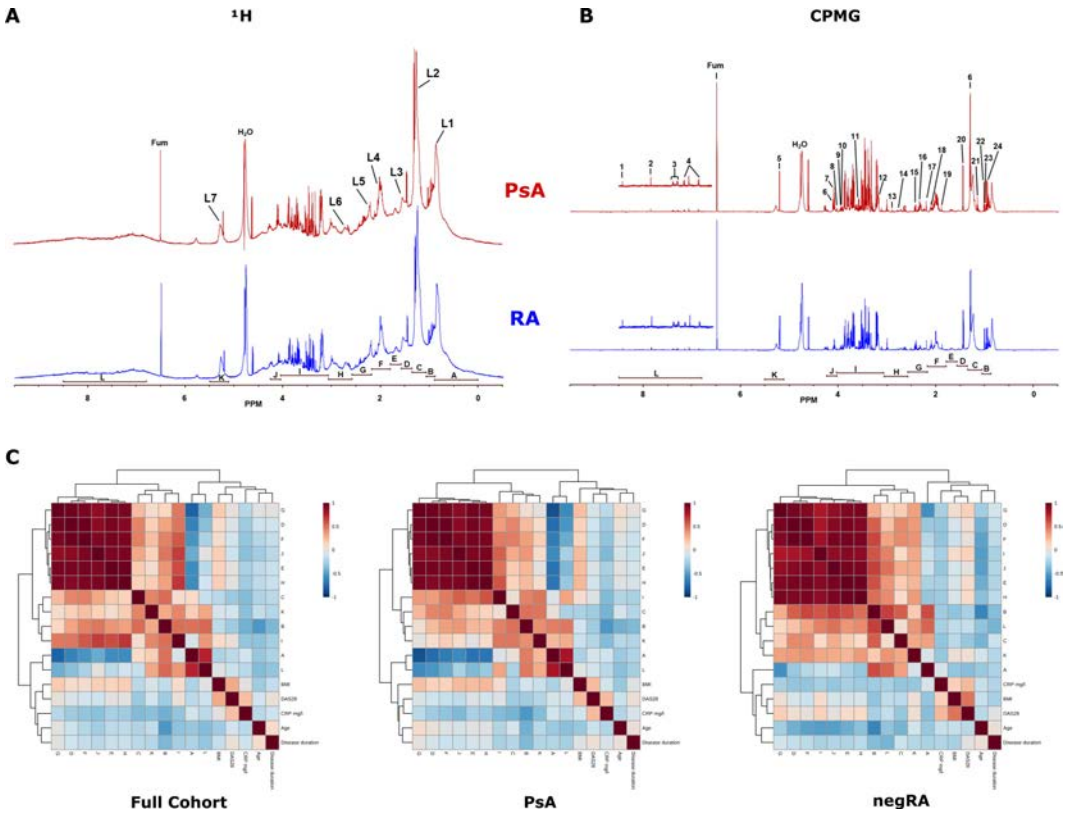

Figure 1 PsA and negRA patients have distinct spectral profiles that do not correlate with clinical and demographic covariates. Representative water-suppressed and baseline-corrected (A) ^{1}H single-pulse and (B) CPMG NMR spectra of blood serum from patients with PsA and negRA assigned with the regions and metabolites and lipid groups included in the untargeted and targeted analysis: (1) formate, (2) histidine, (3) phenylalanine, (4) tyrosine, (5) α-glucose, (6) proline, (7) lactate, (8) creatinine, (9) creatine, (10) creatine phosphate, (11) threonine, (12) choline, (13) sarcosine; (14) citrate, (15) glutamine, (16) succinate, (17) acetoacetate, (18) glutamate, (19) acetate, (20) alanine, (21) β-hydroxybutyrate, (22) valine, (23) isoleucine and (24) leucine. (L1) Lipid methyls, (L2) lipid aliphatic chain, (L3) lipid β-methylenes, (L4) lipid allylic methylenes, (L5) lipid α-methylenes, (L6) lipid polyunsaturated allylic methylenes and (L7) lipid alkenes. Fumarate (10 mM in 99.9% D_2O) was used as an internal standard. (C) Correlograms showing the Pearson correlation coefficients between the clinical or demographic variables and the ^{1}H spectral regions, and hierarchical clustering with Euclidean distance metric for the full discovery cohort, and the split PsA and negRA groups. negRA, seronegative rheumatoid arthritis; NMR, nuclear magnetic resonance; PsA, psoriasis arthritis.

PsA patients was not evident (figure 2A). When assessing the quality of the PLS-DA models, their accuracy was ≤65%, and both the R^2 and Q^2 values were very low (^{1}H-spectra: R^2=0.13, Q^2=0.06; CPMG spectra: R^2=0.16, Q^2=0.08). To improve the diagnostic accuracy based on spectral patterns, we used a random forest classification algorithm, due to its robustness for high dimensional data analysis. In both ^{1}H and CPMG spectra, the algorithm identified similar regions that classified negRA and PsA patients with an out-of-bag error of 0.361 for the classification based on the ^{1}H, and of 0.336 based on the CPMG (figure 2B,C). Nonetheless, the significant spectral regions identified by the random forest algorithm were used to focus the targeted analysis.

Targeted analysis

For the lipidomic analysis, the ^{1}H single-pulse NMR-spectra were used. Due to the broad character of the lipid signals, seven groups of lipid signals (L1–L7) were assessed and designated by the moieties present within[19]: L1: lipid methyls; L2: lipid aliphatic chain; L3: lipid β-methylenes; L4: lipid allylic methylenes; L5: lipid α-methylenes; L6: lipid polyunsaturated allylic methylenes and L7: lipid alkenes (figure 1A). Due to the broadness of the lipid signals, lipid groups were compared in patients as ratios relative to the lipid methyl group L1. In the metabolomic analysis using the CPMG spectra, we chose 24 metabolites that could be clearly identified and quantified and are present in the healthy human sera and have been reported to be altered in chronic arthritis[8 9 11 14 20–24] (figure 1B).

After quantifying the concentration of metabolites and lipid groups, the differences between patient groups were determined by univariate analysis. Among the 24 metabolites, nine had significantly different concentrations between both patient groups, namely the amino acids (AA) alanine, leucine, phenylalanine, threonine and valine and the organic compounds acetate, choline, creatine and lactate. In our analysis, lipid ratios L3/L1, L5/L1 and L6/L1 were found to be statistically different between negRA and PsA patients (figure 3A). There was a clear enrichment of certain metabolic pathways when comparing both groups (figure 3B).

Correlation between serum metabolites and lipids and clinical data of the patients

Age, gender and therapeutic regimen can influence the concentration of metabolites in biological fluids in different diseases,[25 26] thus impacting the definition of the biomarkers to be used in therapy-naïve patients or in patients of different ages. To analyse whether any of the clinical or demographic parameters could

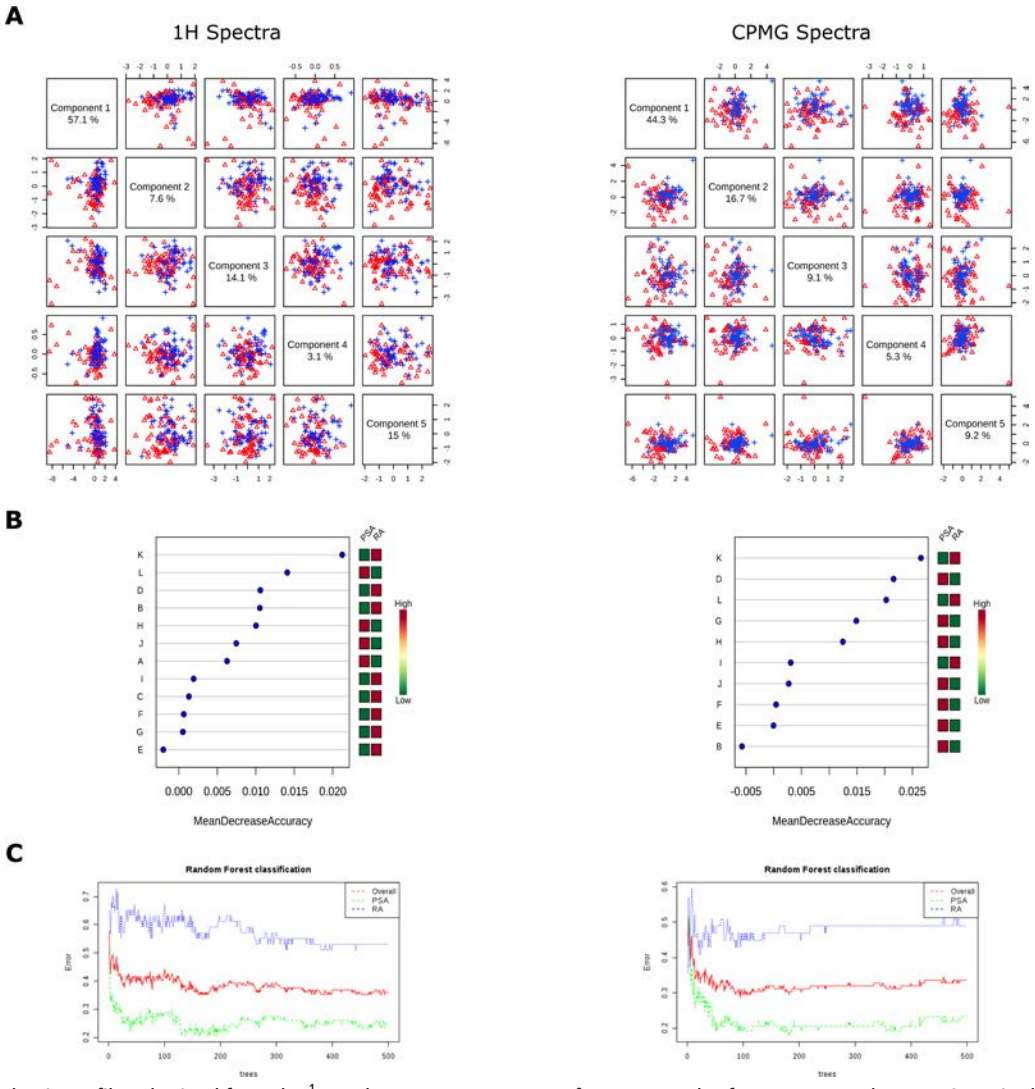

Figure 2 Metabolomic profiles obtained from the ^{1}H and CPMG NMR spectra of serum samples from negRA and PsA patients in the disovery cohort after supervised PLS-DA analysis and random forest analysis. (A) Pairwise scores plots between the five principal components with the corresponding variances shown in the diagonal. (B) Significant features identified by random forest. The features are ranked by the mean decrease in classification accuracy when they are permuted. (C) Cumulative error rates by random forest classification. The overall error rate is shown as the red line; the blue and green lines represent the error rates for each disease. negRA, seronegative rheumatoid arthritis; NMR, nuclear magnetic resonance; PLS-DA, partial least squares discriminant analysis; PsA, psoriasis arthritis.

have influence on the serum concentration of the 24 metabolites or the lipid groups, we carried out a one-way and multiway multivariate analysis of variance (MANOVA) of the associated metabolites and the potential clinical and demographic confounders (see online supplementary tables ST1–ST4). Disease activity was associated with changes in choline concentration and L2/L1 and L7/L1, while disease duration was associated with changes in the concentration of citrate, phosphocreatine, glucose, histidine, tyrosine and valine. Changes in metabolite concentrations and lipid ratios were equally seen when combining age and body mass index classes with the disease groups. Even though RA is a disease mainly affecting women, which contrasts with PsA, the MANOVA analyses combining disease groups and gender did not present any significant differences in the associated metabolites. The same was true when disease and therapy were combined. Univariate analyses did not present any significant correlations between metabolites' concentration or lipid ratios and clinical and demographic variables (figure 3C).

Multivariate diagnostic model for patient classification

Receiver operating characteristic (ROC) analyses of the single metabolites or lipid ratios yielded area under the curve values (AUC) lower than 70% (online supplementary table ST6). Thus, univariate models did not present enough sensitivity and specificity to classify PsA and negRA patients. In order to reach the highest diagnostic accuracy, we built three different machine learning algorithms: random forest, naive Bayes and multivariate logistic regression on the metabolomic and lipidomic profile of 73 PsA and 49 negRA patients. The random forest had an accuracy of 73.3% (Cohen's kappa 40.1%) and the naïve Bayes accuracy was 63.7% (Cohen's kappa 26.5%) to predict the probability of a patient having PsA (ROC curves not shown).

By the stepwise forward–backward selection algorithm, the following diagnostic predictors were included into the diagnostic model: age, gender, L6/L1, L5/L1, L2/L1, alanine, succinate and creatine phosphate.

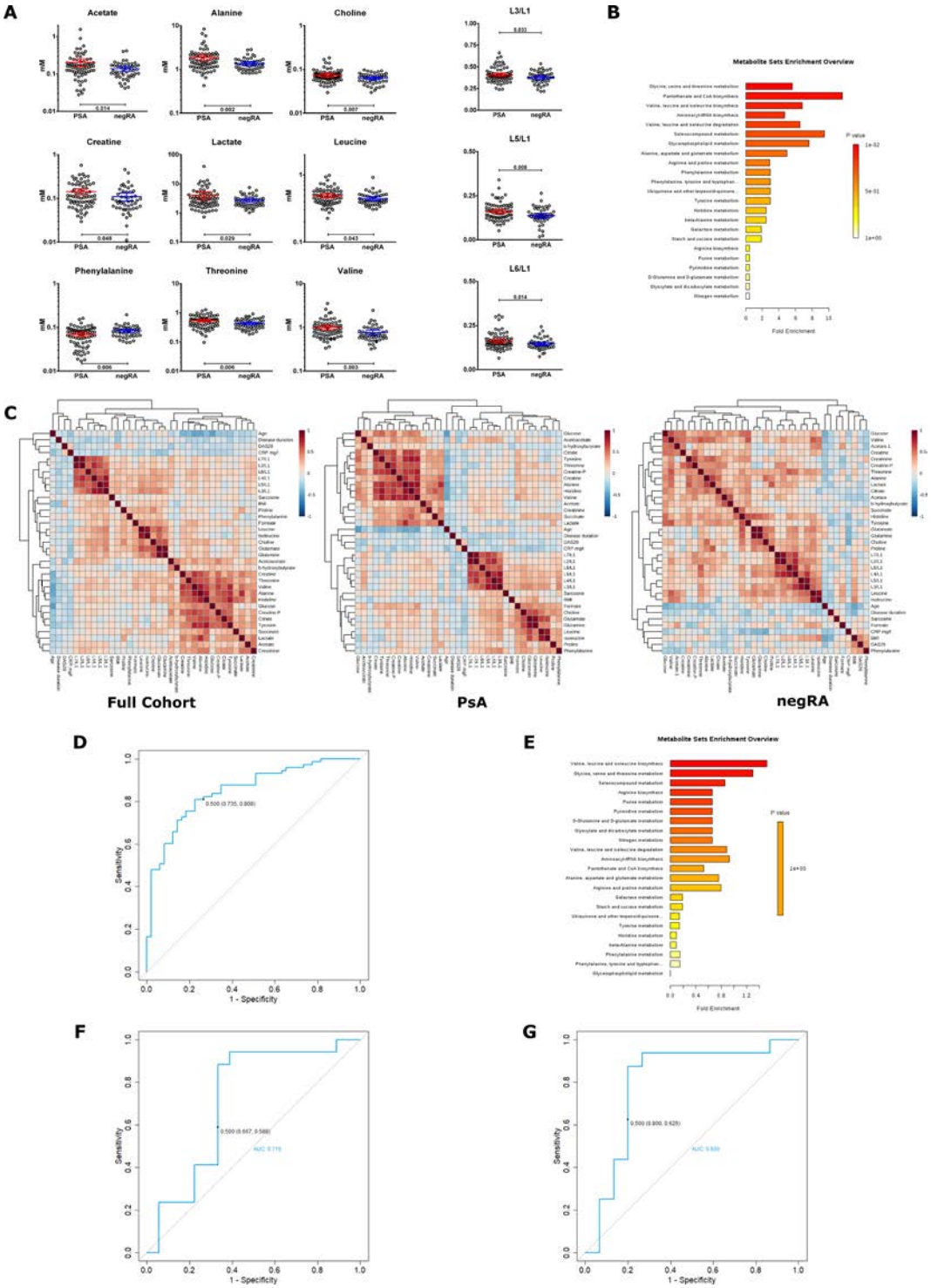

Figure 3 The concentrations of several metabolites and lipid groups allow the distinction between negRA and PsA patients. (A) Dot plots of the metabolites and lipid ratios included in the targeted analysis and that present significant differences between the two patient groups in the discovery cohort. Lines indicate the mean and 95% CI. (B) Summary bar graph for quantitative enrichment analysis showing the changes between negRA and PsA metabolomes in the discovery cohort. (C) Correlograms showing the Pearson correlation coefficients between the clinical or demographic variables and the metabolites, and hierarchical clustering with Euclidean distance metric for the full discovery cohort, and the split PsA and negRA groups. (D) ROC curve for the modelled probability p_{PsA} based on the cross-validation in the discovery cohort. (E) Summary bar graph for quantitative enrichment analysis showing the changes between negRA and PsA metabolomes in the blinded validation cohort. (F) ROC curve for the modelled probability p_{PsA} based on the blinded validation cohort. (G) ROC curve for the modelled probability p_{PsA} based on the reassessed validation cohort. negRA, seronegative rheumatoid arthritis; PsA, psoriasis arthritis; ROC, receiver operating characteristic.

In a first validation procedure, the resulting model was evaluated using a 10-fold cross-validation (CV), which yielded the coefficient estimates in table 2.

Employing these estimates into the regression model yields the following formula:

Table 2 Estimates of the model coefficients

	Estimate	SE	Test statistics*	P value	ORs
(Intercept)	1.046	2.018	0.518	0.604	.
Age	−0.055	0.025	−2.177	0.029	0.947
Gender male	2.412	0.640	3.767	<0.0001	11.155
L6/L1	16.653	8.676	1.919	0.055	17074068.923
L5/L1	16.639	6.820	2.440	0.015	16829326.675
Alanine	2.475	0.756	3.630	<0.0001	15.572
Succinate	−48.819	17.246	−2.831	0.005	0.000
Creatine phosphate	−11.231	4.818	−2.331	0.020	0.000
L2/L1	−1.619	0.681	−2.378	0.017	0.198

*The test statistic and the p value correspond to the Wald test, that is, test if the coefficient is equal to zero.

$$(F1)\log\left(\frac{p_{PsA}}{(1-p_{PsA})}\right) = x = 1.046 - 0.055 \times Age + 2.412 \times Male + 16.653 \times \left[\frac{L6}{L1}\right] + 16.639 \times \left[\frac{L5}{L1}\right] + 2.475 \times [Alanine] - 48.819 \times [Succinate] - 11.231 \times [Creatine\ Phosphate] - 1.619 \times \left[\frac{L2}{L1}\right]$$

The concentrations of each metabolite, age and gender (male=1, female=0) are substituted into the formula. The probability of belonging to the PsA group is then calculated by substituting the result × obtained in F1:

$$(F2)p_{PsA} = \frac{e^x}{(1+e^x)}$$

The probability of a patient belonging to the negRA group is given by:

$$(F3)p_{negRA} = 1 - p_{PsA}$$

To classify patients into the two groups, a cut-off value for the calculated probability (F2) has to be chosen. Usually, the cut-off value 0.5 is applied and a subject is classified to PsA if its estimated probability of having PsA is larger than 0.5, which reflects the idea to classify a subject to the diagnosis that is more likely. The following ROC plot shows the sensitivity and specificity of the model for this cut-off value and an overall area under the ROC curve (AUC) of 84.5%(figure 3D).

To further validate the model retrieved from the cross-validation procedure in a prospective way, a separate blinded sample of 35 new arthritis patients was collected (online supplementary table ST7). This cohort had a similar pathway distribution as the cohort used to build the diagnostic model (figure 3E). Evaluation of the cohort using the cut-off 0.5 yielded a correct prediction of 62.9% of the patients (table 3), and an ROC analysis was performed in which the AUC dropped to 71.6% (figure 3F).

After diagnosis prediction, there was a clinical re-evaluation of the 13 patients for which the predicted diagnosis was different from the one initially done by the rheumatologist. For four of those patients, a definite diagnosis could still not be assigned, as they kept lacking distinctive clinical parameters. Taking this into consideration, we removed those four individuals from the validation cohort and recalculated the prediction match, which increased to 71.0%, and performed a new ROC analysis that resulted in increased sensitivity (62.5%) and specificity (80.0%) (figure 3G).

Table 3 Classification table of the blinded validation cohort (numbers in parenthesis indicate how many individuals lacked distinctive clinical parameters after reassessment)

	Prediction	
Diagnosis	PsA	RA
PsA	10	7 (1)
RA	6 (3)	12

PsA, psoriasis arthritis; RA, rheumatoid arthritis.

DISCUSSION

A definite differential diagnosis between negRA and PsA is often impossible due to lack of clear clinical, serological or radiological parameters. As therapy differs, a reliable diagnosis is important to prescribe the correct treatment. Additionally, the chronic inflammatory processes leading to the characteristic joint destruction in RA and PsA patients may cause major and variant alterations in the metabolism of cells, tissues and organs.[27–29] Such metabolic alterations result in changes in the serum metabolome and lipidome that we were able to quantify with the aim of discovering biomarkers to improve the clinical differential diagnosis between PsA and negRA patients and learn more about the specific metabolomics processes in these chronic arthritides. By means of ^{1}H NMR-based metabolomics and lipidomic analyses, we were able to identify metabolites and lipid groups that differed in concentrations in the sera of negRA and PsA patients. A model was derived from these data to classify the patients into one of the two disease categories and was subsequently validated on separate blinded cohort of patients.

Even though we used different technical and metabolite identification approaches, we reached similar conclusions pertaining to the differences in the levels of the AAs alanine, leucine, threonine and valine between negRA and PsA patients as those reported in a metabolomic analysis of serum samples of healthy individuals, and PsA and total RA patients by mass spectrometry.[22] Serum alanine and valine levels in RA have been associated with synovial B-lymphocyte stimulator expression, and the serum levels of threonine, phenylalanine and leucine associated with synovial expression of IL-1β and IL-8.[24] Free serum AA can be the result of disease-related protein catabolism, but they can also regulate cell functions by controlling intracellular signalling cascades and gene expression.[30] In HeLa cells, alanine, valine and threonine act on the mammalian target of rapamycin complex 1 (mTORC1) in a two-step process in which they prime, and then activate, mTORC1 leading to the phosphorylation of its downstream targets.[31] On activation, mTORC1 is a major inducer of aerobic glycolysis in several cell types.[32 33] It is therefore not surprising that we found strong correlations between lactate levels (the product of aerobic glycolysis) and certain AA, particularly valine and alanine in PsA sera and to a lesser extent

in negRA sera. Furthermore, lactate has been shown to reshape $CD4^+$ T cell phenotype in arthritis towards a proinflammatory profile.[34] The high-energetic demand caused by chronic inflammation in the joint and skin could be related to the higher levels of serum creatine found in PsA patients. Creatine plays a major role in T cell proliferation and cytokine secretion by securing a continuous replenishment of the adenosine triphosphate (ATP) pool.[35]

Short-chain fatty acids (SCFAs), such as acetate, originate from microbiota in the gastrointestinal tract and are involved in a plethora of essential cellular, tissue and organ functions. However, disease-induced dysbiosis leads to altered local and systemic concentrations of SCFA resulting in functional modifications that contribute to disease exacerbation and development of comorbidities. Dysbiosis of the gut microflora has been reported for RA and PsA patients affecting bacteria families that are major SCFA producers.[36–38] However, the role of acetate and other SCFA in inflammatory diseases is still not fully understood as different animal models yield contradictory results. In experimental autoimmune encephalopathy, a model for multiple sclerosis, and in collagen-induced arthritis, a model for autoimmune polyarthritis, dietary supplementation with acetate leads to amelioration of disease scores. However, in another model of polyarthritis, acetate supplementation resulted in increased inflammation and joint destruction[39] and dietary supplementation of healthy mice with acetate resulted in kidney disease with increased serum levels of creatinine and urea, elevated systolic pressure and higher IL-17A and IFN-γ secretion by T-lymphocytes.[40] Nonetheless, SCFAs have a positive effect on increasing bone mass by suppressing osteoclastogenesis.[41]

Choline and acetyl-coenzyme A (CoA) build the neurotransmitter acetylcholine, which is found in the RA synovium.[42] Moreover, on action of choline kinase, choline is used to synthesise the cell membrane phospholipid phosphatidylcholine, which is present in synovial fibroblasts and associates with TNF-α production and migration.[43] Thus, it was not surprising that we detected changes in the serum concentrations of choline, which have been associated to the expression of synovial markers.[24]

The lipid groups L3, L5 and L6 show significant differences between PsA and negRA patients, being all higher in PsA. While L3 and L5 can mostly associate with changes in levels of lipids in the sera, since they reflect the lipid β-methylenes and α-methylenes, common to most medium and long-chain fatty acids, the L6 group reflects polyunsaturated allylic methylenes due to the presence of polyunsaturated fatty acids (PUFAs), which are known to play a central role in the homeostasis of the immune system. PUFAs have been associated with both proinflammatory (ω6-PUFAs) and anti-inflammatory (ω3-PUFAs) features.[44–46]

Even though the univariate analysis pointed to differences in the serum metabolome and lipidome between PsA and negRA patients, none of the identified compounds for itself could clearly and accurately distinguish between the two groups. Therefore, a multivariate approach was pursued that also accounts for possible interactions between the covariates, and a variable selection was performed for noise reduction. Even though the model was able to reach more than 70% prediction match in the blinded validation cohort, there were still four patients with a mixed diagnosis that could not be assigned to any of the groups. Consequently, we must accept that this proposed model will still fail to identify patients presenting clinical features of both diseases. Moreover, for a translation into clinical practice, it still needs to be tested in a larger multinational/multiethnic cohort for its validation in genetically heterologous populations. Nonetheless, our data propose expanding ^{1}H NMR-based metabolomic and lipidomic analyses as a biomarker discovery tool to other autoimmune diseases, for which differential diagnosis, response to therapy or disease prognosis are still hard to determine or predict.[47] Supported by several reports on successful implementation of ^{1}H NMR-based metabolomics as a routine diagnostic tool in clinical settings for non-autoimmune diseases,[23 48 49] our study helps paving the way to extend this technique to the routine diagnostic techniques for autoimmune pathologies. Moreover, our data once again highlight that the metabolomic processes associated with inflammatory rheumatic diseases are different between diseases even when clinical feature are similar. Consequently, metabolomics and lipidomics are starting to feed a completely new field of research in autoimmunity.

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TRANSLATIONAL SCIENCE

Long non-coding RNA HOTAIR drives EZH2-dependent myofibroblast activation in systemic sclerosis through miRNA 34a-dependent activation of NOTCH

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ABSTRACT

Background Systemic sclerosis (SSc) is characterised by autoimmune activation, tissue and vascular fibrosis in the skin and internal organs. Tissue fibrosis is driven by myofibroblasts, that are known to maintain their phenotype in vitro, which is associated with epigenetically driven trimethylation of lysine 27 of histone 3 (H3K27me3).

Methods Full-thickness skin biopsies were surgically obtained from the forearms of 12 adult patients with SSc of recent onset. Fibroblasts were isolated and cultured in monolayers and protein and RNA extracted. HOX transcript antisense RNA (HOTAIR) was expressed in healthy dermal fibroblasts by lentiviral induction employing a vector containing the specific sequence. Gamma secretase inhibitors were employed to block Notch signalling. Enhancer of zeste 2 (EZH2) was blocked with GSK126 inhibitor.

Results SSc myofibroblasts in vitro and SSc skin biopsies in vivo display high levels of HOTAIR, a scaffold long non-coding RNA known to direct the histone methyltransferase EZH2 to induce H3K27me3 in specific target genes. Overexpression of HOTAIR in dermal fibroblasts induced EZH2-dependent increase in collagen and α-SMA expression in vitro, as well as repression of miRNA-34A expression and consequent NOTCH pathway activation. Consistent with these findings, we show that SSc dermal fibroblast display decreased levels of miRNA-34a in vitro. Further, EZH2 inhibition rescued miRNA-34a levels and mitigated the profibrotic phenotype of both SSc and HOTAIR overexpressing fibroblasts in vitro.

Conclusions Our data indicate that the EZH2-dependent epigenetic phenotype of myofibroblasts is driven by HOTAIR and is linked to miRNA-34a repression-dependent activation of NOTCH signalling.

Key messages

What is already known about this subject?

- Enhancer of zeste 2 (EZH2) contributes to the epigenetically stable activation of dermal fibroblasts in systemic sclerosis (SSc).

What does this study add?

- Long non-coding RNA HOX transcript antisense RNA (HOTAIR) drives the specific methylation profile of EZH2 in SSc fibroblasts.
- The HOTAIR/EZH2-dependent profibrotic activation of SSc fibroblasts is mediated by NOTCH through miRNA-34a repression.

How might this impact on clinical practice or future developments?

- This study provides further details into the epigentic stable activation of SSc dermal fibroblasts which will help us understand how to target this pathway therapeutically.

INTRODUCTION

Systemic sclerosis (SSc) is a prototypic fibrotic disease that causes tissue and vascular fibrosis in the skin and internal organs including heart, lungs, kidneys and gastrointestinal tract.[1 2] Tissue fibrosis typically starts in the skin at the level of hands and feet[3] and progresses through the arms and thighs to eventually involve the chest and abdomen in the most severe cases (diffuse cutaneous SSc).[1]

Fibroblasts are the key cellular elements of fibrosis and once explanted from affected tissues they maintain their profibrotic phenotype in vitro, showing increased secretion of collagen and extracellular matrix proteins and higher frequency of alpha-smooth muscle actin (α-SMA) positive cells (myofibroblasts).[4 5] This epigenetic feature has allowed in-vitro studies which have detailed the molecular mechanisms linked to fibrosis including a key role for growth factors like transforming growth factor beta (TGF-β),[6–8] platelet-derived growth factor (PDGF)[9 10] and Notch signalling.[11 12]

The Notch family of cell surface receptors is important for cell-to-cell communication.[13] On ligand binding, the receptor is cleaved by gamma secretase proteases to release the intracellular domain Notch Intracellular Domain (NID),[14] which induces transcription of downstream targets.[15] NOTCH expression is known to be regulated by miRNAs such as miRNA-34a, which function as a suppressor of NOTCH expression.[16 17]

Recently, Tsou *et al*[18] have shown that enhancer of zeste 2 (EZH2) plays an important role in the epigenetic features linked to tissue fibrosis in SSc.

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EZH2 is the enzymatic subunit of the polycomb repressor complex (PRC) that induces methylation of histone 3, therefore silencing target genes. Nevertheless, the signal directing the PRC to specific DNA regions responsible for the phenotype was not elucidated.

Scaffold lncRNAs are RNAs of at least 200 nucleotides with a 'W'-shaped tertiary structure.[19] This allows one domain to recognise specific DNA sequences and the other to bind the PRC, effectively focusing PRC activity in specific promoter regions.[20 21] There is increasing evidence that lncRNA plays an important role in a number of fibrotic conditions including liver,[22] myocardial[23] and renal fibrosis.[24] Particularly, lncRNAs within the HOX loci have been described as master epigenetic regulators within the connective tissue.[25] HOX transcript antisense RNA (HOTAIR) is one of the better characterised lncRNAs within the HOX locus. It has been shown to cooperate with PRC2 in mediating the EZH2-driven repression of homeobox D cluster through the spread of H3K27me3 methylation marker associated with gene silencing.[26–28]

Here, starting from the observation of increased HOTAIR expression in SSc fibroblasts cultured and SSc skin biopsies, we demonstrate that HOTAIR expression is sufficient to induce profibrotic activation of dermal fibroblasts in vitro. Further, we show that this phenotype is driven by NOTCH pathway activation which is mediated by EZH2-dependent repression of miRNA-34a expression.

METHODS

Detailed description of experimental methods is available as online supplementary file 1.

RESULTS

α-SMA positive fibroblasts show increased expression of HOTAIR in vitro and in vivo

Fibroblasts from SSc maintain in vitro their profibrotic phenotype, including increased α-SMA expression, indicating an epigenetically driven activation of these cells.[29 30] Immunofluorescence studies of SSc dermal fibroblasts and healthy control (HC) fibroblasts showed that the increased expression of α-SMA was due to increased number of α-SMA positive cells, rather than homogenous increase of α-SMA expression in all cells (figure 1A). lncRNA from the HOX locus are master regulators of the connective tissue. To investigate the role of this specific group of lncRNAs in the myofibroblast phenotype in SSc, we performed HOX tiling array as described by Rinn *et al*[25] on the RNA extracted from laser capture microdissected α-SMA positive or negative cells from dermal fibroblasts cultured from four SSc skin biopsies (figure 1B). Effectiveness of laser capture microdissection (figure 1B) was validated by quantitative real time (qRT)-PCR showing 3.5-fold increased mRNA levels for α-SMA (figure 1C). HOX tiling array on the extracted RNA showed a number of lncRNA from the HOX locus were upregulated (figure 1D). Interestingly, HOTAIR levels were elevated 2.05-fold in α-SMA-positive fibroblasts. HOTAIR is a known regulator of EZH2[27] and it is highly expressed in the hands and feet of humans.[25] Therefore, it was an interesting target for SSc. We validated the tiling array data by qPCR and observed a 2-fold increased levels of HOTAIR in α-SMA positive cells compared with α-SMA negative cells (figure 1E). SSc dermal fibroblasts (n=6) showed in vitro a 7-fold increase in HOTAIR levels compared with HC dermal fibroblasts (figure 1F). Clinical features and relative HOTAIR levels for each patient fibroblast cell line is found in online supplementary table 1.

To validate this finding in vivo, we analysed SSc skin biopsies for HOTAIR transcript levels both by qRT-PCR and in-situ hybridisation. SSc skin biopsies showed >100-fold increased expression of HOTAIR compared with HC skin (n=4) (figure 1G). Consistent with these findings, in-situ hybridisation of SSc skin biopsies showed increased localisation of HOTAIR in the dermis of SSc skin biopsies compared with HC (n=3) (figure 1H).

HOTAIR drives a profibrotic activation in dermal fibroblasts

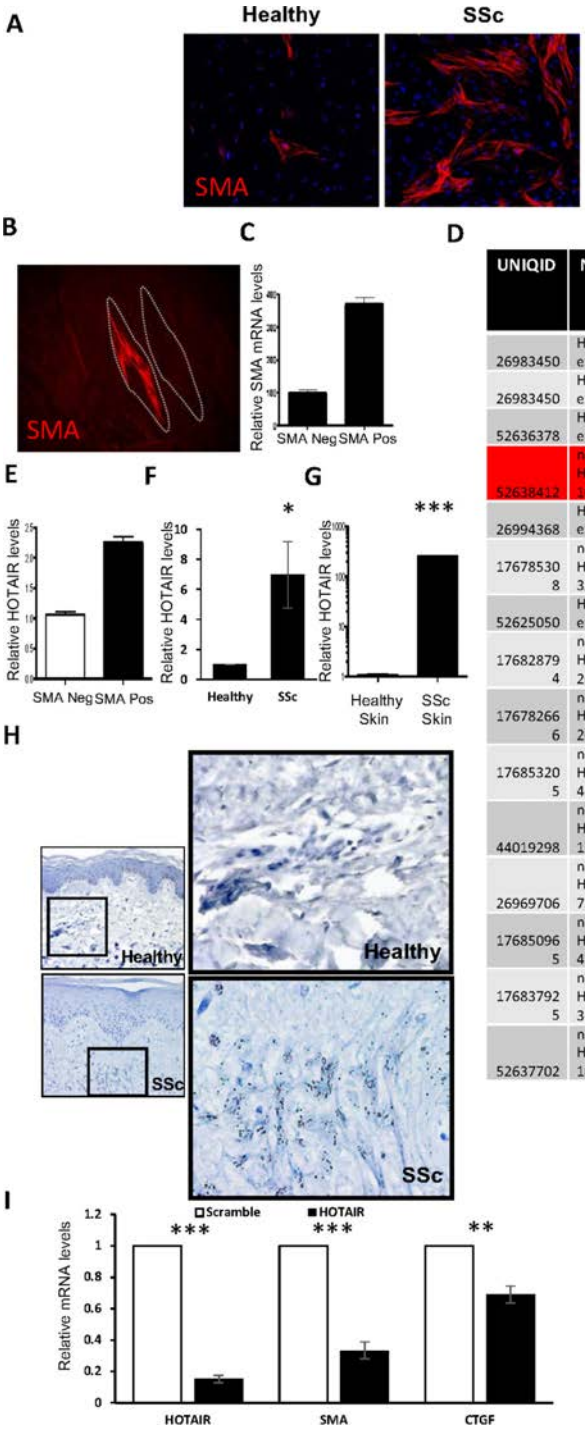
To determine the role of HOTAIR in the profibrotic activation of dermal fibroblasts, we silenced HOTAIR expression in primary fibroblasts using long non-coding antisense (LNA) oligonucleotides for 72 hours. LNA efficiently reduced HOTAIR transcript levels (figure 1I). Silencing HOTAIR also resulted in a significant reduction in α-SMA and connective tissue growth factor (CTGF) transcript levels.

In a complementary approach, we set out to induce stable overexpression of HOTAIR (or scrambled RNA) in human dermal fibroblasts immortalised through retroviral induced expression of HTERT as previously described.[10] Immortalised dermal fibroblasts were infected with lentiviral particles carrying HOTAIR gene in frame with GFP and puromycin-resistance genes. Infected cells were visualised and positively sorted by green fluorescent protein(GFP) (figure 2A,B), to select the cells with highly efficient lentiviral integration, and maintained in media containing puromycin. qRT-PCR analysis for HOTAIR confirmed its increased expression in transduced cells compared with cells infected with lentivirus carrying a scrambled RNA sequence (figure 2C). We screened the scrambled and HOTAIR-expressing fibroblasts for profibrotic markers expression. Overexpression of HOTAIR in fibroblasts resulted in increased levels of collagen type 1A1, 1A2, α-SMA and CTGF transcript levels (figure 2D–G) . This was consistent with increased protein levels of collagen type 1 (3-fold) and α-SMA (2-fold), (figure 2H,I). Accordingly, HOTAIR-expressing fibroblasts showed pronounced expression of α-SMA fibres by immunofluorescence compared with scramble control fibroblasts (figure 2J). These data suggest that HOTAIR expression can induce expression of myofibroblast markers in vitro. Further, we observed that treatment with TGF-β treatment induced a 14-fold increase in α-SMA levels compared with untreated HOTAIR-expressing fibroblasts, which was 2-fold higher than the upregulation observed in scramble fibroblasts treated with TGF-β (figure 2K). This suggests that the overexpression of HOTAIR primes the fibroblasts for TGF-β mediated activation.

HOTAIR induces EZH2-dependent increase of H3K27me3 methylation marker

HOTAIR has been shown to cooperate with PRC2 to induce methylation of target gene promoter.[31 32] One member of the complex is the enzyme EZH2, which is essential for histone 3 trimethylation. Tsou *et al* have shown that EZH2 levels are increased in SSc fibroblasts and inhibition of EZH2 suppresses their profibrotic phenotype.[18] Here, we set out to determine whether HOTAIR overexpression enhances expression of EZH2 and methylation of histone 3 in dermal fibroblasts.

HOTAIR-expressing fibroblasts showed no significant difference in EZH2 transcript when compared with scrambled controls (figure 3A). On the contrary, levels of H3K27me3 were increased by 8-fold in HOTAIR-expressing fibroblasts, consistent with the role of HOTAIR in targeting EZH2 to specific DNA regions rather than increasing its expression (figure 3B,C).



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UNIQID	NAME	ratio SMA +/-	p value
26983450	HOXA10 ex1	2.725567893	0.021528177
26983450	HOXA10 ex1	2.725567893	0.021528177
52636378	HOXC12 ex2	2.199440218	0.011426809
52638412	nc-HOXC11-107A	2.052519859	0.035794884
26994368	HOXA11 ex 1	1.700897114	0.010146431
176785308	nc-HOXD12-3A	1.666198854	0.040167938
52625050	HOXC13 ex2	1.654747861	0.006484339
176828794	nc-HOXD4-26A	1.619463293	0.034180236
176782666	nc-HOXD13-20	0.682450827	0.030584953
176853205	nc-HOXD3-44S	0.678609866	0.036251664
44019298	nc-HOXB4-173S	0.641828172	0.044801201
26969706	nc-HOXA7-730	0.641611795	0.030984426
176850965	nc-HOXD3-41S	0.619602088	0.01058697
176837925	nc-HOXD4-34S	0.562796357	0.049916033
52637702	nc-HOXC11-106S	0.560058921	0.038545651

Figure 1 lncRNA HOX transcript antisense RNA (HOTAIR) is upregulated in activated myofibroblasts. (A) Healthy and systemic sclerosis (SSc) fibroblasts were stained with a mouse alpha-smooth muscle actin (α-SMA) antibody and visualised with an alexa 594-conjugated mouse secondary antibody (red). Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to stain the nuclei (blue). (B) α-SMA positive or negative single cells were dissected by laser capture microscopy on untreated SSc patient fibroblasts. RNA was extracted from α-SMA positive and negative cells. (C) α-SMA transcript levels were assessed by qPCR. Graph represents mRNA levels from three independent repeats. (D) HOX tiling array was performed on the RNA and the table represents the fold differences of each lncRNA within the HOX locus in α-SMA positive cells compared with negative. HOTAIR is labelled in red. (E) HOTAIR transcript levels were assessed from RNA extracted from α-SMA positive and negative cells by qPCR. Graph represents mRNA levels from three independent repeats. (F) RNA was extracted from telomerase reverse transcriptase (Human) (HTERT) immortalised fibroblasts that had been isolated from healthy and patient with SSc skin biopsies. HOTAIR transcript levels were assessed by qPCR. Graph represents HOTAIR mRNA levels from five healthy patients and five patient with diffuse SSc fibroblasts. (G) RNA was extracted from healthy and patient with diffuse SSc skin biopsies. HOTAIR transcript levels were assessed by qPCR. Graph represents HOTAIR mRNA levels from (n=3). (H) HOTAIR in situ hybridisation staining from healthy and patient with SSc skin. (H) Expanded panels represent areas of interest in the dermis regions and black boxes represent the region of the area of interest. (I) RNA was extracted from primary fibroblasts transfected with HOTAIR and scramble control antisense oligonucleotides. HOTAIR, α-SMA and connective tissue growth factor (CTGF) transcript levels were assessed by qPCR. Graph represents mRNA levels from three independent repeats. *p<0.05, **p<0.01, ***p<0.001.

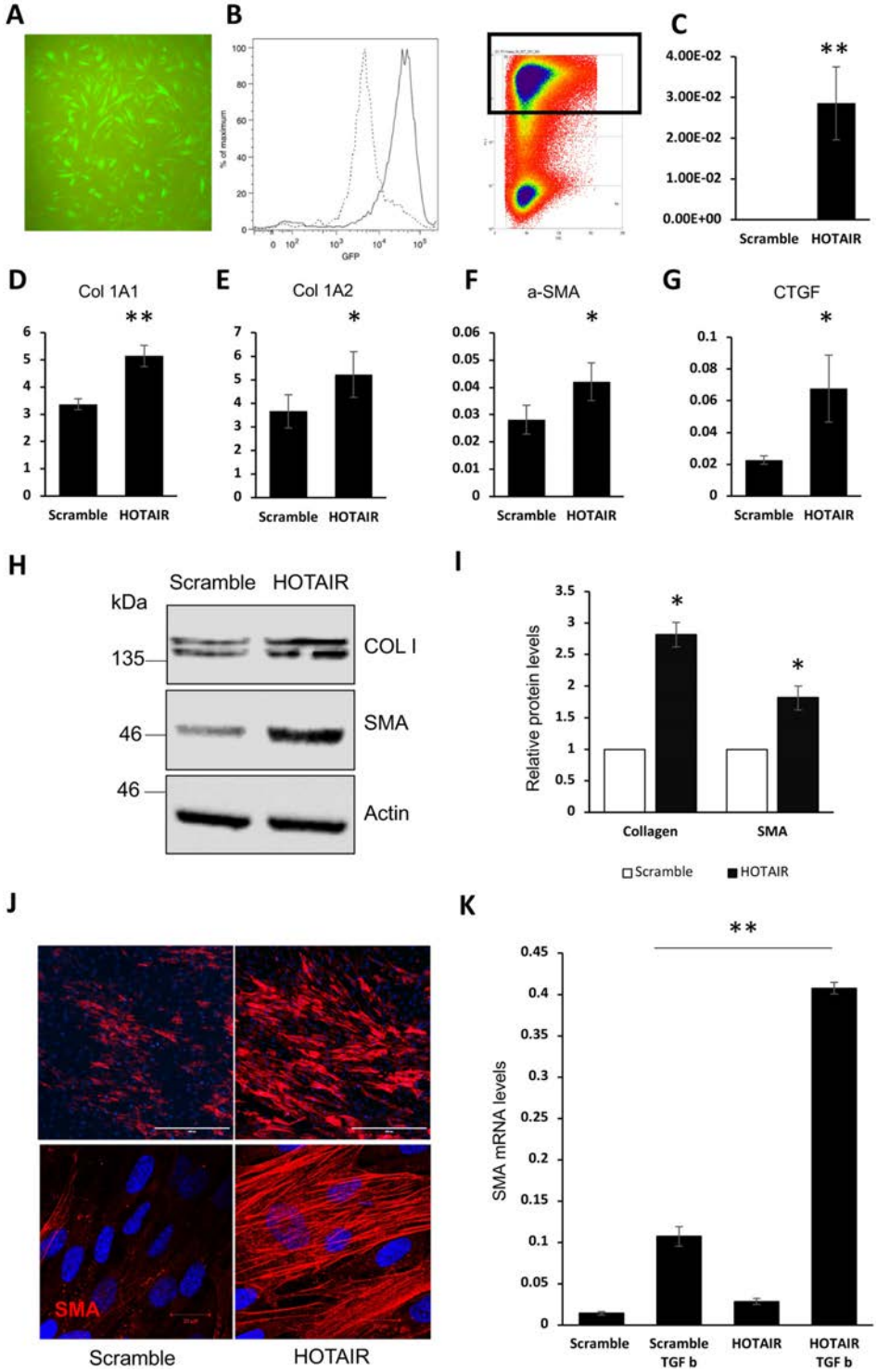

Figure 2 HOX transcript antisense RNA (HOTAIR) drives profibrotic activation of dermal fibroblasts. (A) Representative image of healthy dermal fibroblasts infected with lentiviruses containing the scramble/HOTAIR vectors. Vectors contain a GFP reporter to determine infection efficiencies. (B) Histograms representing GFP cell sorting of fibroblasts-infected lentiviruses containing the scramble/HOTAIR vectors. GFP-positive fibroblasts were collected and cultured. RNA was extracted from fibroblasts stably expressing scramble and HOTAIR vectors. (C) HOTAIR, (D) collagen type 1A1, (E) 1A2, (F) alpha-smooth muscle actin (α-SMA) and (G) connective tissue growth factor (CTGF) transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (H) Protein was extracted from fibroblasts stably expressing scramble and HOTAIR vectors. Lysates were probed with a pan collagen type 1 antibody and an α-SMA antibody by western blot. β-actin was probed for as a loading control. (I) Graph represents densitometry analysis of collagen type 1 and α-SMA western blots from three independent repeats. (J) α-SMA staining of scramble and HOTAIR expressing dermal fibroblasts. Fibroblasts were stained with a mouse α-SMA antibody and visualised with a mouse-specific alexa 594-conjugated secondary (red). Cells were counterstained with DAPI to visualise nuclei (blue). White lines represent 400 µM scale bar. Red lines represent 20 µM scale bar. Scramble and HOTAIR expressing fibroblasts were serum depleted for 16 hours prior to stimulation with TGF-β (10 ng/mL) for 48 hours. (K) RNA was extracted and α-SMA transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. *p<0.05, **p<0.01, ***p<0.001.

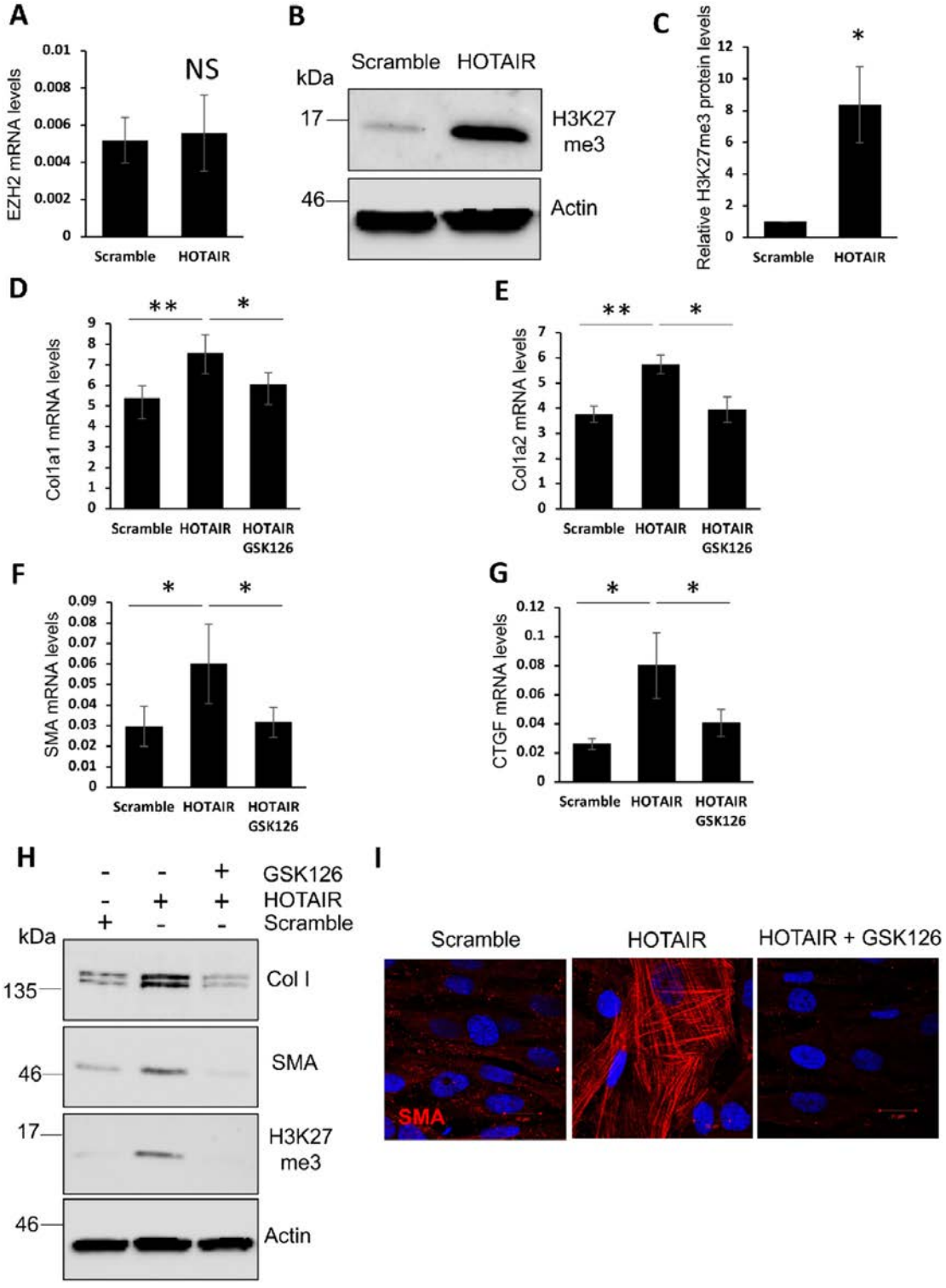

Figure 3 HOX transcript antisense RNA (HOTAIR) drives profibrotic activation through enhancer of zeste 2 (EZH2)-mediated H3K27me3 methylation. RNA was extracted from fibroblasts stably expressing scramble and HOTAIR vectors. (A) EZH2 transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (B) Protein was extracted from fibroblasts stably expressing scramble and HOTAIR vectors. Lysates were probed with H3K27me3-specific antibody by western blot. β-actin was probed for as a loading control. (C) Graph represents densitometry analysis of H3K27me3 western blots from three independent repeats. RNA and protein were extracted from fibroblasts stably expressing the scramble and HOTAIR vectors, in addition to HOTAIR fibroblasts treated with the EZH2 inhibitor GSK126. (D) Collagen type 1A1, (E) collagen type 1A2, (F) alpha-smooth muscle actin (α-SMA) and (G) connective tissue growth factor (CTGF) transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (H) Protein lysates were probed with pan collagen type 1, α-SMA and H3K27me3 antibodies by western blot. β-actin was probed for as a loading control. (I) α-SMA staining of scramble and HOTAIR expressing dermal fibroblasts in addition to HOTAIR fibroblasts treated with the EZH2 inhibitor GSK126. Fibroblasts were stained with a mouse α-SMA antibody and visualised with a mouse-specific alexa 594-conjugated secondary (red). Cells were counterstained with DAPI to visualise nuclei (blue). Red lines represent 20 µM scale bar. *p<0.05, **p<0.01, ***p<0.001. NS, not significant.

To determine whether HOTAIR induced profibrotic activation through EZH2, we employed the EZH2 inhibitor GSK126.[18 33] HOTAIR-expressing fibroblasts treated for 48 hours with GSK126 displayed reduced Col1A1, Col1A2, α-SMA and CTGF gene expression to levels comparable to scramble fibroblasts (figure 3D–G). This correlated with a reduction in collagen type 1 and α-SMA protein levels when HOTAIR-expressing fibroblasts were treated with the inhibitor (figure 3H,I). As expected, expression of H3K27me3 was lost on treatment with GSK126 (figure 3H). Importantly, inhibition of EZH2 with GSK126 also suppressed the increased collagen and α-SMA expression of SSc dermal fibroblasts (online supplementary figure 1) confirming the work of Tsou *et al*.[18]

HOTAIR primes myofibroblast differentiation through Notch activation

Notch signalling plays an important role in the fibrotic phenotype of SSc fibroblasts and in scleroderma animal models.[11 12 34] HOTAIR has previously been shown to enhance Notch expression and signalling in keratinocytes and retinoblastoma tissue.[35–37] In addition, EZH2 is known to play an important role in enhancing Notch1 transcription.[16 38] Therefore, we set out to determine whether HOTAIR expression could drive a EZH2-dependent increase in Notch expression in dermal fibroblasts and could Notch mediate the profibrotic effects of HOTAIR.

RNA levels of Notch1 were twofold higher in the HOTAIR-expressing fibroblasts compared with scramble control (figure 4A). NOTCH activation results in the cleavage of NID, which in turn is responsible for the target gene effects (figure 4B). Accordingly, HOTAIR-expressing fibroblasts showed increased levels of NID compared with the scramble control (figure 4C). In addition, levels of Hes1 (Notch responsive gene) were increased in the HOTAIR-expressing fibroblasts (figure 4D). These data supported the hypothesis that Notch signalling is increased in HOTAIR-expressing dermal fibroblasts.

To determine whether the increased NOTCH signalling observed in HOTAIR-expressing fibroblasts was EZH2 dependent, we looked at Notch1 transcript levels in HOTAIR-expressing fibroblasts treated with the GSK126 for 48 hours (figure 4E). Notch1 transcript levels were significantly reduced on GSK126 treatment. We also observed a reduction in NID levels in HOTAIR-expressing fibroblasts treated with GSK126 (figure 4F). Importantly, SSc fibroblasts showed increased Notch1 transcript levels compared with the healthy fibroblasts, consistent with published data (figure 4G).[11] The addition of the EZH2 inhibitor for 48 hours reduced Notch1 transcript. These data suggest that the ability of HOTAIR to regulate H3 trimethylation is important for Notch1 transcription.

To determine whether Notch signalling plays a role in HOTAIR-induced profibrotic fibroblast activation, we treated HOTAIR-expressing fibroblasts with the gamma secretase inhibitor RO4929097, which is known to block the gamma secretase responsible for cleaving the intracellular domain of NOTCH from the plasma membrane protein.[39] HOTAIR-expressing fibroblasts treated with RO4929097 showed a reduction in Hes1 transcript levels to levels comparable to Scrambled control (figure 4H). Most importantly, RO4929097 treated HOTAIR-expressing fibroblasts showed a reduction in collagen 1A1 and α-SMA expression to levels comparable to scrambled controls both at mRNA and protein levels (figure 4I–L).

We observed similar results with a second distinct gamma secretase inhibitor DAPT, a non-transition state analogue which blocks gamma secretase with a different mechanism of action[40] (online supplementary figure 2A). Following the same experimental approach, we treated HOTAIR-expressing fibroblasts with DAPT and assessed Col1A1 transcript levels. Like R04929097, DAPT reduced Col1A1 transcript levels in the HOTAIR-expressing fibroblasts to levels similar to the scramble control (online supplementary figure 2C). Hes1 transcript levels were also reduced confirming the inhibitor was active (online supplementary figure 2B). mRNA data were confirmed at protein level where we observed a reduction of the HOTAIR-expressing fibroblasts enhanced collagen type 1 and α-SMA protein levels in DAPT-treated cells to levels comparable to scramble controls (online supplementary figure 2D).

HOTAIR derepresses NOTCH1 expression through EZH2-dependent methylation of miRNA-34a

We next wanted to identify the mechanism HOTAIR employs to enhance Notch1 transcription. HOTAIR cannot target EZH2 directly to the Notch1 promoter because this would lead to suppression of Notch1 transcription. Therefore, HOTAIR must target a negative regulator of Notch1 transcription. Kwon *et al* have recently shown that EZH2 increases Notch1 transcription by methylation of miRNA-34a.[16] Hence, we determined miRNA-34a transcript levels in the scramble and HOTAIR-expressing fibroblasts. Expression of HOTAIR supressed miRNA-34a transcript levels in fibroblasts by 50%, which was completely reversed by EZH2 inhibition through GSK126 (figure 5B). We observed similar data in SSc fibroblasts. miRNA-34a transcript levels were reduced in SSc fibroblasts by 60% compared with healthy fibroblasts and this suppression of miRNA-34a was completely reverted on treatment with GSK126 to levels higher than HCs (figure 5C). These data indicate that the observed increased expression of Notch1 driven by HOTAIR in linked to repression of miRNA-34a. To determine the role of miRNA-34a suppression in the increased Notch1 expression of HOTAIR and SSc fibroblasts, we employed miRNA-34a mimics. Overexpression of miRNA-34a in HOTAIR-expressing fibroblasts reduced Notch1 transcript levels by 30% (figure 5D–E). In addition, overexpression of miRNA-34a supressed the levels of NID in HOTAIR expressing fibroblasts (figure 5F). Similar results were observed when miRNA-34a was overexpressed in SSc fibroblasts. Overexpression of miRNA-34a led to a 50% reduction in Notch1 transcript levels in the SSc fibroblasts (figure 5G,H). Accordingly, levels of NID and Hes1 were reduced in SSc fibroblasts were miRNA-34a was overexpressed (figure 5I–J). These data indicated that suppression of Notch1 transcription by overexpression of miRNA-34a in SSc fibroblasts was sufficient to supress Notch signalling. Taken together, these data show that HOTAIR enhances Notch1 expression and signalling through the suppression of miRNA-34a in an EZH2-dependent manner (figure 5A)

HOTAIR-mediated suppression of miRNA-34a is important for SSc fibrosis

We have shown that miRNA-34a suppressed Notch1 expression in both HOTAIR-expressing and SSc fibroblasts. We therefore wanted to investigate whether this led to a reduction in the profibrotic phenotype in both types of fibroblasts. Overexpression of miRNA-34a in HOTAIR-expressing fibroblasts reduced Col1a1, 1A2 and α-SMA transcript levels (figure 6A–C). This correlated with a reduction in collagen type 1 and α-SMA protein levels when HOTAIR-expressing fibroblasts were transfected with the miRNA-34a mimic (figure 6D,E). Similar results were observed in SSc fibroblasts when miRNA-34a was overexpressed.

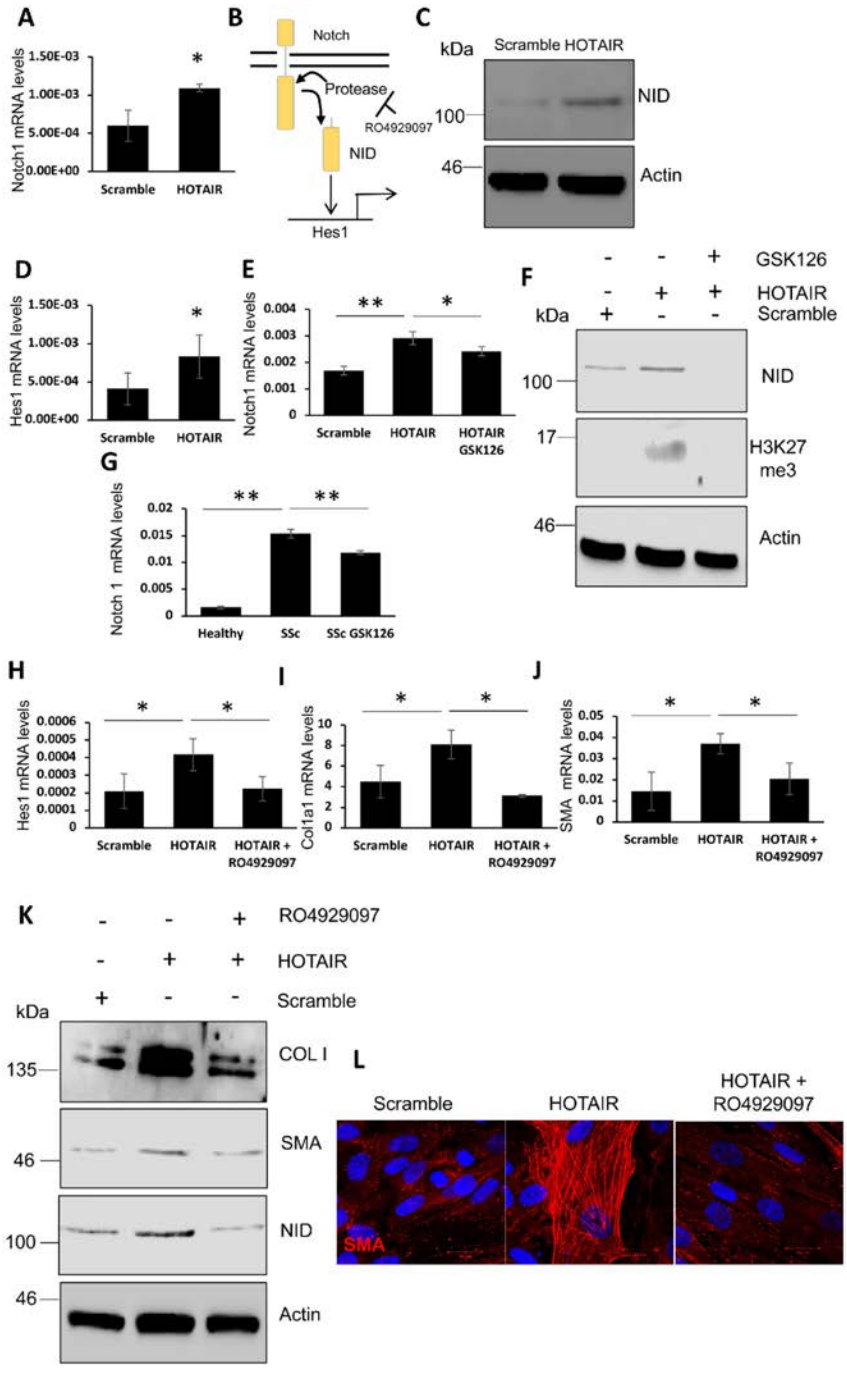

Figure 4 HOX transcript antisense RNA (HOTAIR) drives Notch 1 expression in dermal fibroblasts through enhancer of zeste 2 (EZH2). RNA was extracted from fibroblasts stably expressing scramble and HOTAIR vectors. (A) Notch1 and (D) Notch target gene Hes1 transcript levels were assessed by qPCR. Graph represents mRNA levels from three independent repeats. (B) Schematic of the Notch 1 receptor and the mechanism of activation. (C) Protein was extracted from fibroblasts stably expressing scramble and HOTAIR vectors. Lysates were probed with an antibody specific for the intracellular domain of Notch, Notch Intracellular Domain (NID) by western blot. β-actin was probed for as a loading control. (E) RNA and protein were extracted from fibroblasts stably expressing the scramble and HOTAIR vectors, in addition to HOTAIR fibroblasts treated with the EZH2 inhibitor GSK126. Notch 1 transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (F) Protein lysates were probed with an NID and H3K27me3 antibodies by western blot. β-actin was probed for as a loading control. RNA was extracted from healthy and systemic sclerosis (SSc) fibroblasts, in addition to SSc fibroblasts treated with the EZH2 inhibitor GSK126. (G) Notch 1 transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. RNA and protein were extracted from fibroblasts stably expressing the scramble and HOTAIR vectors, in addition to HOTAIR fibroblasts treated with the gamma secretase inhibitor R04929097. (H) Hes1, (I) Col1A1 and (J) alpha-smooth muscle actin (α-SMA) transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (K) Protein lysates were probed with a pan collagen type 1 antibody, an α-SMA antibody and an antibody specific for the intracellular domain of Notch 1 (NID) by western blot. β-actin was probed for as a loading control. (L) α-SMA staining of scramble and HOTAIR expressing dermal fibroblasts, in addition to HOTAIR fibroblasts treated with the gamma secretase inhibitor R04929097. Fibroblasts were stained with a mouse α-SMA antibody and visualised with a mouse-specific alexa 594-conjugated secondary (red). Cells were counterstained with DAPI to visualise nuclei (blue). Red lines represent 20 µM scale bar. *p<0.05, **p<0.01, ***p<0.001.

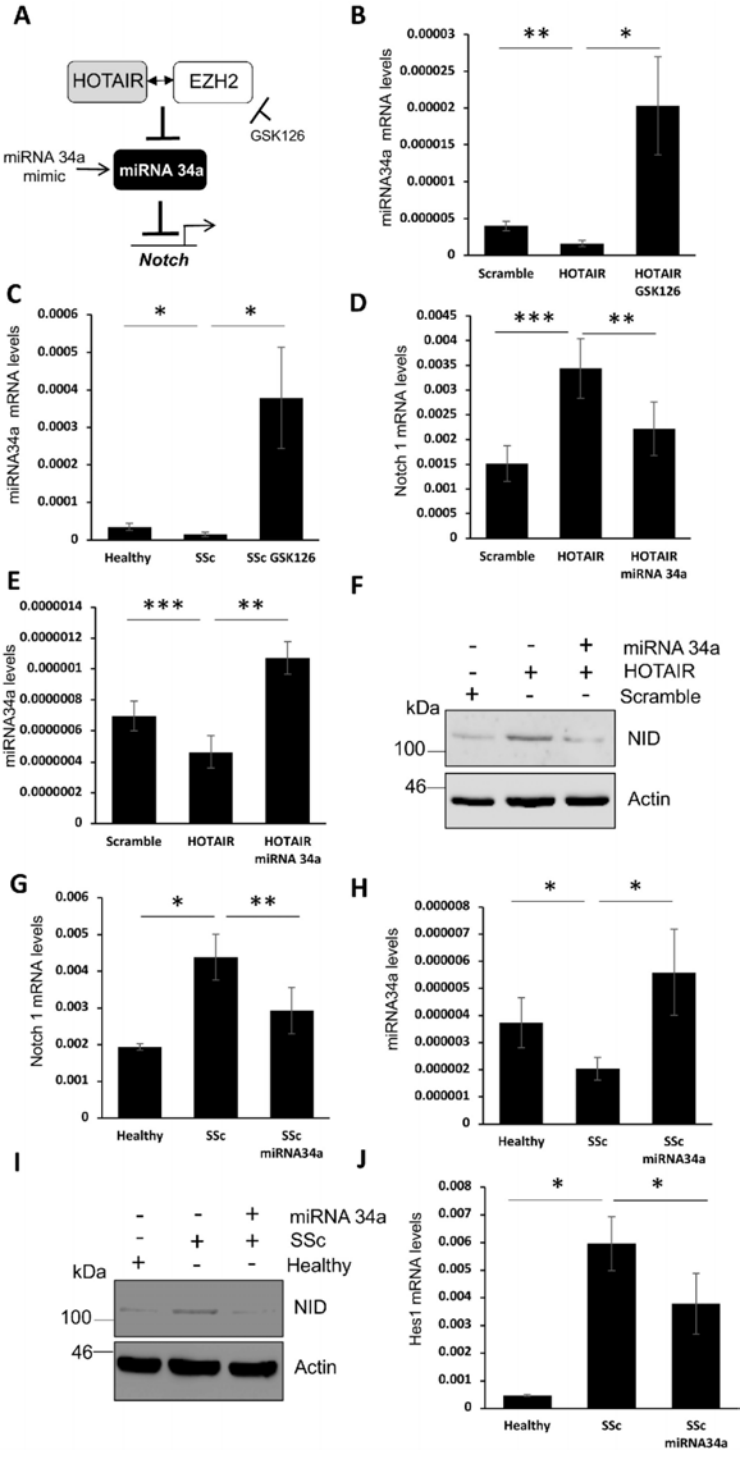

Figure 5 HOX transcript antisense RNA (HOTAIR) drives Notch 1 expression through the methylation of miRNA-34a. (A) Schematic of the mechanism HOTAIR employs to drive Notch transcription. (B) RNA was extracted from fibroblasts stably expressing the scramble and HOTAIR vectors, in addition to HOTAIR fibroblasts treated with the enhancer of zeste 2 (EZH2) inhibitor GSK126. MiRNA-34a transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (C) RNA was extracted from healthy and SSc fibroblasts, in addition systemic sclerosis (SSc) fibroblasts treated with the EZH2 inhibitor GSK126. miRNA-34a transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. RNA and protein were extracted from scramble or HOTAIR expressing fibroblasts transfected with an miRNA-34a mimic or a negative control mimic. (D) Notch 1 and (E) miRNA-34a transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (F) Protein lysates were probed with an antibody specific for the intracellular domain of Notch 1 (Notch Intracellular Domain (NID)) by western blot. β-actin was probed for as a loading control. RNA and protein were extracted from healthy and SSc fibroblasts transfected with an miRNA-34a mimic or a negative control mimic. Notch 1 (G) and miRNA-34a (H) transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (I) Protein lysates were probed with an antibody specific for the intracellular domain of Notch 1 (NID) by western blot. β-actin was probed for as a loading control. (J) RNA was extracted from healthy and SSc fibroblasts transfected with an miRNA-34a mimic or a negative control mimic. Hes1 transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. *p<0.05, **p<0.01, ***p<0.001.

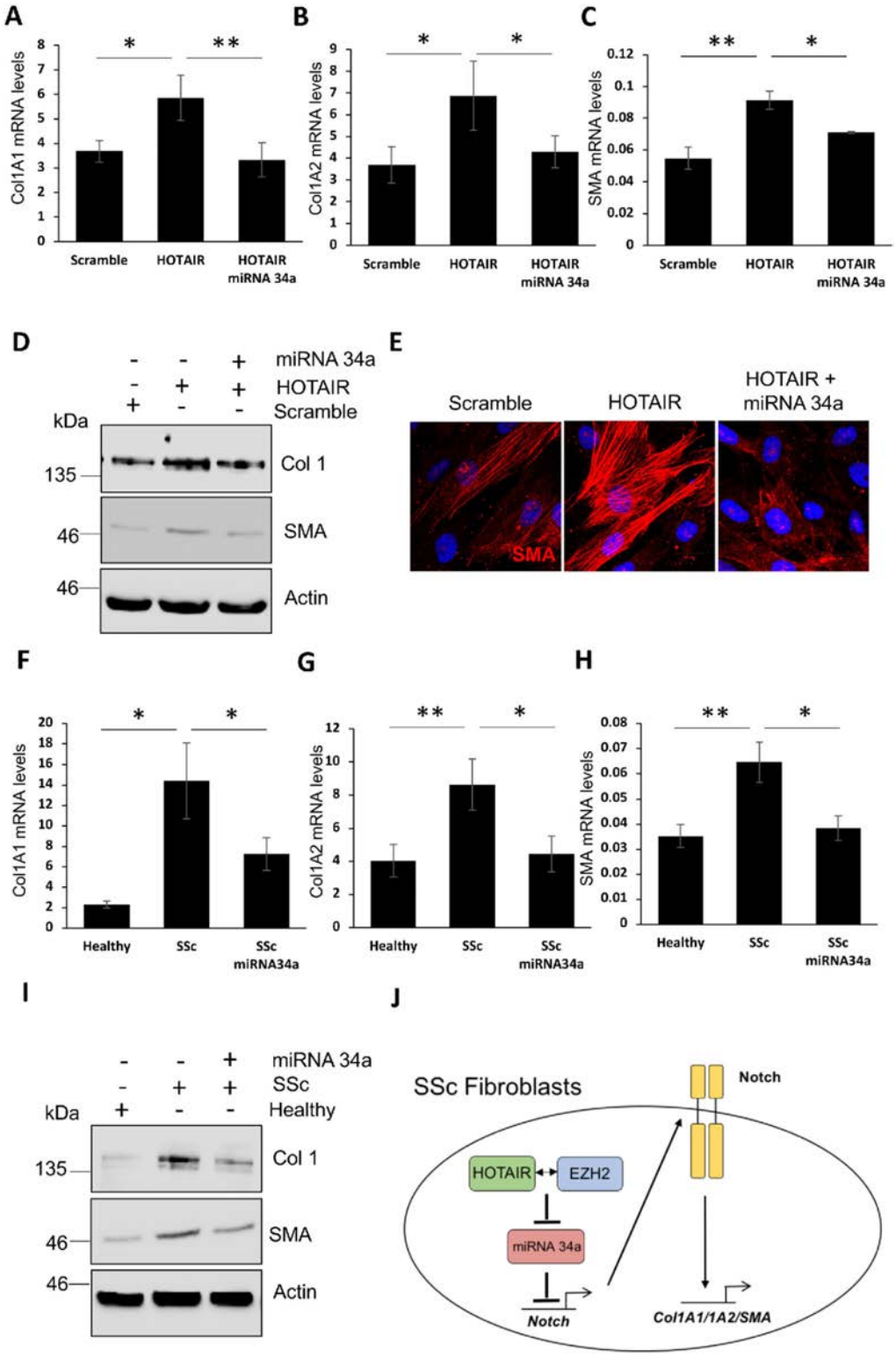

Figure 6 HOX transcript antisense RNA (HOTAIR)-mediated suppression of miRNA-34a is important for systemic sclerosis (SSc) fibrosis. RNA and protein were extracted from scramble or HOTAIR-expressing fibroblasts transfected with an miRNA-34a mimic or a negative control mimic. (A) Col1A1, (B) Col1A2 and (C) alpha-smooth muscle actin (α-SMA) transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (D) Protein lysates were probed with a pan collagen type 1 and α-SMA antibody by western blot. β-actin was probed for as a loading control. (E) α-SMA staining of scramble and HOTAIR expressing dermal fibroblasts, in addition to HOTAIR fibroblasts were transfected with an miRNA-34a mimic or a negative control mimic. Fibroblasts were stained with a mouse α-SMA antibody and visualised with a mouse-specific alexa 594-conjugated secondary (red). Cells were counterstained with DAPI to visualise nuclei (blue). Red lines represent 20 µM scale bar. RNA and protein were extracted from healthy and SSc fibroblasts transfected with an miRNA-34a mimic or a negative control mimic. (F) Col1A1, (G) Col1A2 and (H) α-SMA transcript levels were assessed by qPCR. Graphs represents mRNA levels from three independent repeats. (I) Protein lysates were probed with an collagen type 1 antibody and an α-SMA antibody by western blot. β-actin was probed for as a loading control. (J) Schematic of the role HOTAIR plays in SSc-associated fibrosis. *p<0.05, **p<0.01, ***p<0.001.

Overexpression of miRNA-34a lead to a significant reduction in Col1A1, 1A2 and α-SMA transcript levels (figure 6F–H). This correlated with a reduction in collagen type 1 and α-SMA protein levels when miRNA-34a was overexpressed in SSc fibroblasts (figure 6I).

DISCUSSION

α-SMA expression is a defining marker of myofibroblasts,[41] which are the key cellular elements of tissue fibrosis. The number of myofibroblasts in vivo correlates with severity of disease.[42] It has been well established that fibroblasts cultured from SSc skin biopsies showed increased expression of α-SMA both at RNA and protein levels, which resemble the increased expression of α-SMA induced by TGF-β in dermal fibroblasts derived from HC skin. This observation has supported the studies that have later elucidated the importance of TGF-β in the pathogenesis of tissue fibrosis. When analysed at single-cell level by immunofluorescence, it became apparent that in vitro, the increased expression of α-SMA in SSc fibroblasts, as well as in TGF-β-treated dermal fibroblasts, is due to increased number of α-SMA positive cells (figure 1A) rather than a homogenous increase of α-SMA in all cells. This led us to hypothesise that there is a subpopulation of cells which are epigenetically 'primed' to differentiate into myofibroblasts. Indeed, the most recent single-cell RNA-sequencing analysis of dermal fibroblasts demonstrates the existence of different fibroblast populations in the dermis.[43]

We identified HOTAIR as an epigenetic factor important for the priming of myofibroblasts. Overexpression of HOTAIR in healthy dermal fibroblasts and knockdown of HOTAIR in foreskin fibroblasts modulated expression of α-SMA. A possible limitation of our loss of function studies is that the dermal fibroblasts of these experiments were from a different anatomical location (foreskin). In addition, in vivo studies validating the importance of HOTAIR expression in experimental model of fibrosis will elucidate the importance of this pathway in vivo.

Previous work has shown that SSc fibroblasts extracted from patient skin and cultured for an extended period still have enhanced Notch signalling and this is important for their profibrotic phenotype in vitro and for progression of tissue fibrosis in animal models.[9 10] Evidence presented here shows that HOTAIR is an important epigenetic factor involved in maintaining Notch signalling through enhanced transcription of the receptor. Therefore, HOTAIR may be important for maintaining Notch signalling in SSc fibroblasts in culture. Because of the known HOTAIR function, our data suggested that HOTAIR may play a role in inhibiting gene expression of factors that suppress Notch. The data presented in this study clearly identify miRNA-34a, which is known to suppress Notch1 transcription,[14] as one of the target of HOTAIR-driven gene repression (figure 6J). Nevertheless, it is likely that several other targets are methylated following HOTAIR expression. In addition, it is also possible that miRNA-34a targets a number of other profibrotic targets which requires further investigation.

During this study, Tsou *et al* have shown a critical role of the methyltransferase protein EZH2 in the profibrotic phenotype of SSc fibroblasts. Here, we dissect this phenomenon further by showing that EZH2 methylation profile is driven by HOTAIR and linked to activation of NOTCH. Interestingly, Tsou *et al* found that EZH2 effect on endothelial cells was rescued by NOTCH expression. In this context, our data suggest that EZH2 may play opposite effects in fibroblasts and endothelial cells, which is consistent with fibroblast specific methylation patterns observed in SSc fibroblasts.[44 45]

Beyond NOTCH and TGF-β pathways, other important morphogens have been shown to play a role in the profibrotic phenotype of SSc fibroblasts namely Wnt and sonic hedgehog. Studies elucidating the role of HOTAIR in modulating these pathways would be extremely interesting in future work since we believe they will elucidate the overall role of HOTAIR in tissue homeostasis.

Another recent study identified a novel transcription factor PU.1 as an important regulator of fibrosis. Wohlfahrt *et al* have recently published the importance of PU.1 as transcription factor mediating the TGF-β-induced profibrotic activation in fibroblasts. Nevertheless, in the same study, they have also shown that PU.1 transcription is suppressed by EZH2-dependent methylation.[46] In line with these latter findings, we have observed a reduced expression of PU.1 in our HOTAIR overexpressing fibroblasts (online supplementary figure 3). These data warrant further studies since they suggest that PU.1 and HOTAIR-induced EZH2 methylation may mediate profibrotic activation through distinct or redundant mechanisms. Overall, there is a growing body of evidence to suggest that inhibitors of the PRC2 may represent a viable therapeutic target for SSc.

Our studies explored the effects of HOTAIR upregulation in the fibrosis associated with SSc, but left unanswered the question on how HOTAIR is upregulated in SSc. There is evidence to suggest that Rho GTPase/ROCK signalling is important for HOTAIR expression in breast cancer.[47] Inhibition of ROCK lead to a reduction in HOTAIR transcript levels. Rho GTPases may enhance HOTAIR transcription in SSc fibroblasts. Since it is well established that inhibition of Rho GTPase and ROCK in scleroderma fibroblasts leads to a reduction collagen production and SMA expression,[48] it is possible that Rho GTPase may drive SSc fibrosis through enhanced HOTAIR expression. In our opinion, this is an interesting hypothesis which deserves further investigation.

Overall, our data show that HOTAIR is overexpressed in SSc fibroblasts and this overexpression is important for myofibroblast activation through EZH2/PRC2 H3K27me3 methylation. In addition, our data may offer a potential explanation of why skin fibrosis in patients with SSc starts in the hands and feet which are regions with a physiologically higher expression of HOTAIR in dermal fibroblasts.[25]

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Contributors CWW performed most of the experiments and wrote the manuscript with FdG. HH and FdG performed the LCM experiments. GA, MM and RLR performed some of the gene expression experiments. CWW, FdG and CAF-B designed the experimental plan. FdG, CAF-B and SAJ conceived the study. HC generated LncRNA tiling array data and contributed to manuscript draft.

Competing interests H.Y.C. is a co-founder of Accent Therapeutics, Boundless Bio, and advisor to 10x Genomics, Arsenal Biosciences, and Spring Discovery.

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TRANSLATIONAL SCIENCE

Elevated EPSTI1 promote B cell hyperactivation through NF-κB signalling in patients with primary Sjögren's syndrome

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ABSTRACT

Background Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease characterised by aberrant B cell hyperactivation, whose mechanism is partially understood.

Methods We performed whole transcriptome sequencing of B cells from three pSS patients and three matched healthy controls (HC). Differentially expression genes (DEGs) were confirmed with B cells from 40 pSS patients and 40 HC by quantitative PCR and western blot. We measured the proliferation potential and immunoglobulins production of siRNA-transfected or plasmid-transfected B cells stimulated with cytosine-phosphate-guanine (CpG) or anti-IgM. We also explored Toll-like receptor 9 (TLR9) signalling to reveal the potential mechanism of B cell hyperactivation in pSS.

Results We identified 77 upregulated and 32 downregulated DEGs in pSS B cells. We confirmed that epithelial stromal interaction (EPST1) expression in pSS B cells was significantly higher than that from HCs. EPSTI1-silencing B cells stimulated with CpG were less proliferated and produced lower level of IgG and IgM comparing with control B cells. EPSTI1-silencing B cells expressed lower level of p-p65 and higher level of IκBα, and B cells with overexpressed EPSTI1 showed higher level of p-p65 and lower level of IκBα. Finally, IκBα degradation inhibitor Dehydrocostus Lactone treatment attenuated p65 phosphorylation promoted by EPSTI1.

Conclusion Elevated EPSTI1 expression in pSS B cells promoted TLR9 signalling activation and contributed to the abnormal B cell activation, which was promoted by facilitating p65 phosphorylation and activation of NF-κB signalling via promoting IκBα degradation. EPSTI1 might be implicated in pSS pathogenesis and was a potential therapeutic target of pSS.

Key messages

What is already known about this subject?
► B cell hyperactivation is the hallmark of primary Sjögren's syndrome (pSS).

What does this study add?
► Epithelial mesenchymal interacting protein 1 (EPSTI1) was upregulated in B cells from pSS patients, and promoted B cell proliferation and immunoglobin production.

How might this impact on clinical practice or future developments?
► EPSTI1 might be implicated in pSS pathogenesis and was a potential therapeutic target of pSS.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is a prevalent autoimmune disease characterised by lymphocytic infiltration of salivary and lachrymal glands, leading to xerophthalmia and xerostomia. Moreover, one-third of the patients develop systemic manifestations, such as renal, pulmonary and neurological manifestations.[1]

B cell plays an essential role in the pathogenesis of pSS. B cell population is significantly increased in both peripheral blood and exocrine glands, where ectopic germination centres are frequently observed.[2] The aberrant activation of B cells further induce hypergammaglobulinemia and produce high-level autoantibodies, including antinuclear antibodies, rheumatoid factor, anti-Ro/SSA antibody and anti-La/SSB antibody.[3] Eventually, about 5% of pSS patients develop lymphomas, mostly B cell non-Hodgkin lymphomas.[4–6]

The mechanism of B cell hyperactivation in pSS remains partially understood, which is orchestrated by genetic, epigenetic and environmental factors. Genome-wide association studies identify risk polymorphisms locus in C-X-C chemokine receptor type 5, B Lymphocyte Kinase and PR domain 1, which regulates follicle organisation, B-cell receptor (BCR) activation and plasma cell activation,[7 8] respectively. Moreover, Interferon (IFN)-regulated genes were hypomethylated in B cells from pSS patients.[9] Furthermore, virus infections, including hepatitis C virus (HCV) and Epstein–Barr virus (EBV), are proposed as the trigger of pSS.[10] Genetic and transcriptomic studies reveal the IFN signature in pSS, which also promotes B cell activation[10] and B cell activating factor (BAFF) expression.[2] BAFF is overexpressed in pSS and is a key cytokine for B cell maturation, proliferation and survival. T follicular helper cells assist B cells maturation by secretion of IL-21, and are essential for maintaining germinal centre.[11 12]

In light of the redundancy of the immunoregulation pathway, other potential mechanisms may also play a role in B cell hyperactivation in pSS. To this

end, we performed transcriptome analysis of B cells to identify abnormal gene expression profile of B cells from pSS patients. We also explore the new mechanisms of B cell hyperactivation in pSS patients.

METHODS

Patients

We enrolled 40 pSS patients (38 females, mean age 50.2 years) fulfilled the 2002 American European Consensus Group criteria[13] and 40 healthy controls (HCs) from Peking Union Medical College Hospital (PUMCH) (online supplementary table S1). The study was approved by the institutional review board of PUMCH. All participants provided written informed consent.

RNA processing and real-time quantitative PCR

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using Ficoll-Paque density centrifugation, $CD19^+$ B cells were purified from PBMCs using $CD19^+$ B cells isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's instruction, with a purity of more than 90% by flow cytometry. Total RNA of $CD19^+$ B cells was isolated using TRIZOL (Invitrogen Carlsbad, USA) and were quantified using a NanoDrop2000 spectrophotometer (NanoDrop Technologies, USA). cDNAs were synthesised from 1 µg of total RNA using reverse transcription kit (Takara, Japan). Real-time PCR was performed using 7900HT fast real-time PCR (Applied Biosystems,USA) with triplicate. Expression levels of mRNA were normalised to GAPDH and analysed with 7900HT real-time analysis software V.2.4. The following primers were used for SYBR green-based real-time PCR: epithelial mesenchymal interacting protein 1 (EPSTI1), 5′-ACCCGCAATAGAGTGGTGAAC-3′ (forward) and 5′-GCTATCAAGGTGTATGCACTTGT-3′(reverse); valosin-containing protein (VCP), 5′-CCCTGTGCCTGCTTCTTT-3′ (forward) and 5′-GCTGCTCCCTTTCCACCA-3′ (reverse); GAPDH, 5′-TCAACGACCACTTTGTCAAGCTCA-3′ (forward) and 5′-GCTGGTGGTCCAGGGGTCTTACT-3′ (reverse); ΔCt was calculated by subtracting the Ct values for GAPDH from the Ct value for the gene of EPSTI1. ΔΔCt was calculated by subtracting the control Ct from pSS Ct. The fold change of expression between control and pSS samples was calculated by the equation: $2^{-\Delta\Delta\ Ct}$. Transcriptome analysis of B cells was detailed in the supplementary material (online supplementary material 1).

Transfection

EPSTI1, VCP and control siRNA (100 nM) (RiboBio, China) were mixed with riboFECT CP reagent (RiboBio, China) and then transferred to a 24-well plate containing 1×10^6/mL B cells or Raji B cells according to the manufacturer's protocol. For plasmid transfection, B cells (2.0×10^6/mL) were resuspended in Nucleofector Solution and mixed with ESPTI1-expressing or control PIRES2-EGFP-3Flag plasmid (5 uM) (ObiO Technology, China) at room temperature and were electroporated, then were rinsed with 1 mL pre-equilibrated culture medium and transferred to a 12-well plate.

Antibodies

The following antibodies were used: PE-conjugated Annexin V, 7AAD (BD Biosciences, USA), EPSTI1 mAb (ZZ4), β-Actin Antibody, mouse IgGκ binding protein-horseradish peroxidase (HRP) (Santa Cruz, USA). Phospho-NF-κB p65 (Ser536) Rabbit mAb, NF-κB p65 (D14E12) Rabbit mAb, Phospho-p38 MAPK (Thr180/Tyr182) Rabbit mAb, p38 MAPK (D13E1) Rabbit mAb, Phospho-c-Jun N-terminal kinase (JNK) (Thr183/Tyr185) Rabbit mAb, SAPK/JNK Antibody, Phospho-IκBα (Ser32/36) (5A5) Mouse mAb, IκBα (L35A5) Mouse mAb, BCL6 (D412V) Rabbit mAb (CST, USA). VCP Rabbit mAb (ab109240) (Abcam, UK).

Western blot

The total protein of B cells was extracted with Minute Total Protein Extraction Kit (Invent Biotechnologies, USA), and protein concentrations were determined with BCA Assay kit (Pierce Biotechnology, USA). Cell lysate containing 20 ug of protein was fractionated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with tris-buffered saline-Tween 20 (TBST) containing 5% non-fat milk for 1 hour at room temperature followed by incubation overnight with human primary mAb at 4°C. The membrane was washed three times and incubated with secondary antibody for 1 hour at room temperature. Enhanced chemiluminescence reagent was added after washing the membrane three times. Immunoreactive protein was detected by chemiluminescence with X-AR film (Beijing ComWin Biotech, China).

Immunohistochemistry and immunofluorescence assay

Paraffin-embedded salivary gland tissues were sectioned into 3 um sections. Following deparaffinization, rehydration and heat-induced epitope retrieval, the sections were incubated in appropriate antibody dilution (1:5) overnight at 4°C. The slides were washed five times in TBS and incubated in the antimouse IgG peroxidase antibody for 1 hour, then washed five times in TBS. Avidin-biotin-peroxidase reagents were added and the slides were incubated in a 0.5 mg/mL HRP substrate solution, washed five times in TBS and counterstained for 1 min with hematoxylin. The slides were dehydrated by washing the slides for 1 min each in a series of 75%, 80% and 100% ethanol. Immunofluorescence staining was performed on paraffin sections following a standardised protocol. Quantification of immunofluorescence staining was performed using Image J software (National Institutes of Health, USA).

B cells proliferation assay and immunoglobulin production measurement

$CD19^+$ B cells were activated with anti-IgM (10 µg/mL) and CD40L (500 ng/mL) or CpG (2.5 ug/mL) in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C. The proliferation of $CD19^+$ B cells was measured using Cell Counting Kit-8 (CCK-8) according to the manufacturer's instruction (Dojindo Molecular Technologies, Japan). Briefly, 5×10^4 $CD4^+$ T cells were incubated in a 96-well plate for 72 hours, and were supplemented with 10 µL of CCK-8 reagent for another 2 hours. The plate was read at 450 nm using a microplate reader (Thermo Fisher Scientific, USA) and the absolute cell numbers were calculated with the standard curve. The supernatant was harvested on day 7 and the IgG, IgA and IgM were quantified by ELISA (Bethyl Laboratories, USA).

Apoptosis assay

$CD19^+$ B cells were activated with anti-IgM (10 µg/mL) and CD40L (500 ng/mL) or CpG (2.5 ug/mL) for 72 hours. Cells were washed with Annexin V Binding Buffer (BD Biosciences), incubated with PE-conjugated Annexin V and 7-AAD at room temperature for 15 min, and were immediately analysed with a

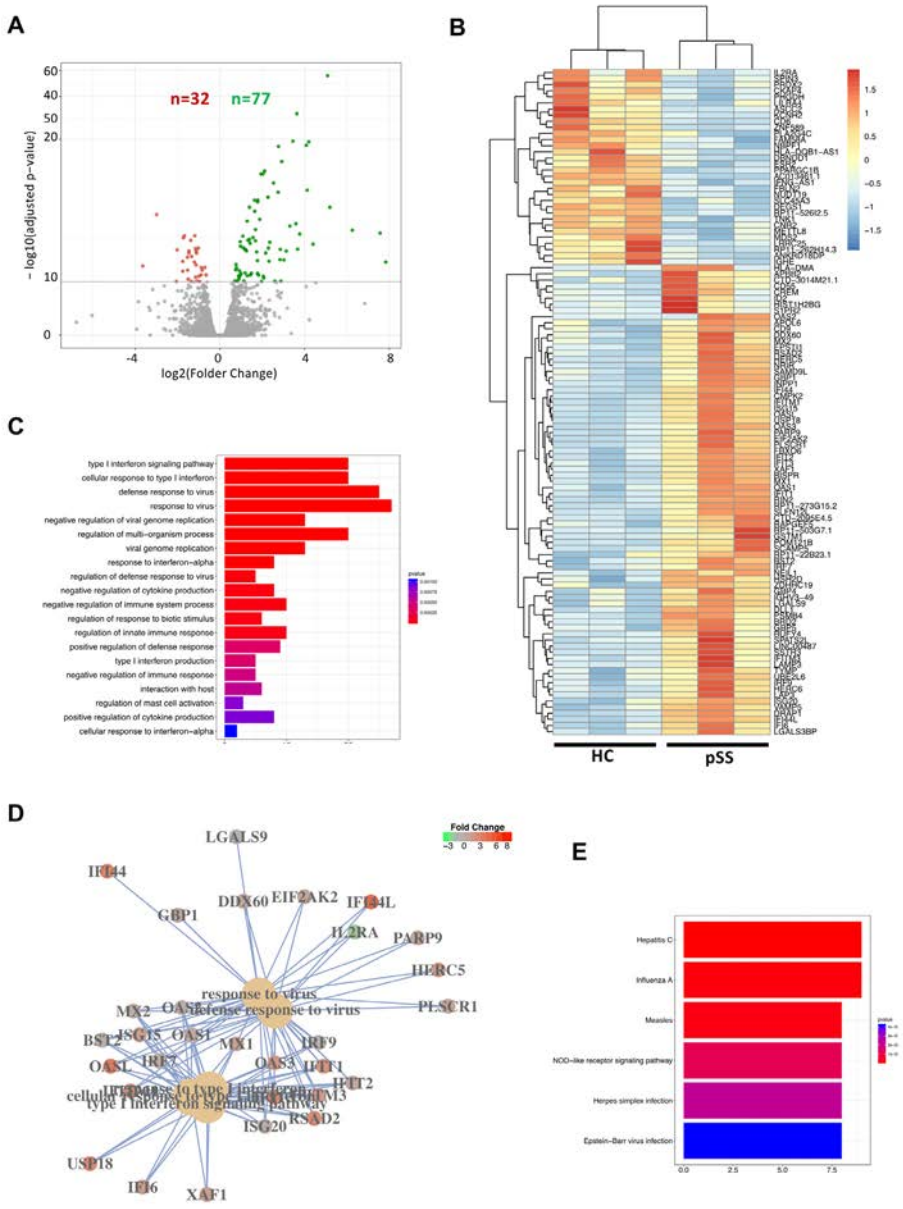

Figure 1 Transcriptome analysis of B cells from primary Sjögren's syndrome (pSS) patients. CD19$^+$ B cells were FACS sorted from PBMCs of pSS patients and healthy controls (HC). RNAseq was performed, data were proceeded by HISAT2, HTSeq, DESeq2 and clusterProfiler R packages. (A) Volcano plot of differential expression genes (DEGs) of B cells from pSS (n=3) and HC (n=3). Green, upregulated (n=77); red, downregulated (n=32). (B) Heatmap of DEGs (n=109) between B cells from pSS and HC. (C) GO biological process enrichment of DEGs of B cells from pSS and HC. (D) Cnet plot of GO biological process enrichment of DEGs of B cells from pSS and HC. (E) KEGG enrichment of DEGs of B cells from pSS and HC.

BD Accuri C6 flow cytometer (Becton Dickinson, USA). Data were processed by FlowJo Software (Tree Star, USA).

Statistical analysis

Student's t-test was used to compare the difference between pSS patients and HC. A two-tailed p-value<0.05 was considered as statistically significant. All statistical analysis was performed using SPSS V.17.0 software (IBM, USA).

RESULTS

Whole transcriptome sequencing of B cells from pSS patients

We normalised sequencing data with DESeq2 (online supplementary fig S1A) and identified 109 DEGs (upregulated n=77, downregulated n=32) between pSS and HC (figure 1A, online supplementary table S2). The cluster analysis of DEGs showed a distinct pattern between pSS group and HC group (figure 1B). Gene Ontology (GO) biological process enrich analysis revealed that 49 GO entries were enriched in pSS, with IFN-α signalling and virus replication signalling as the top GO entries (figure 1C). Consistently, Cnet analysis showed that type I IFN signalling and response to virus were the main clusters (figure 1D). Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrich analysis revealed five virus-related pathways, including HCV and EBV (figure 1E). Additionally, double-stranded RNA binding was the top enriched molecular function of DEGs (online supplementary fig S1B-C).

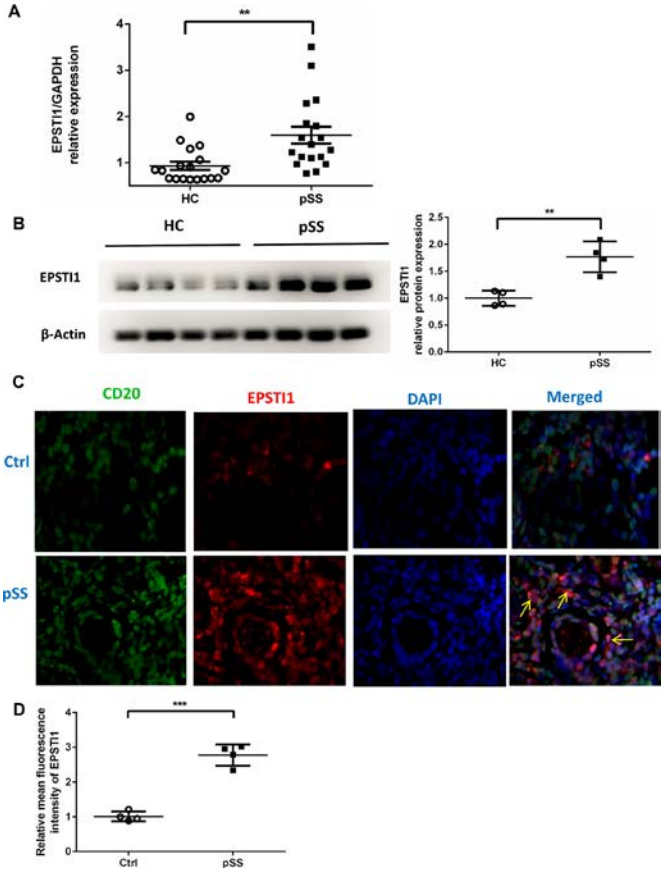

Figure 2 EPSTI1 is aberrantly upregulated in B cells from pSS patients. $CD19^+$ B cells were purified from peripheral blood samples of pSS and HC. (A) Relative EPSTI1 mRNA expression of B cells from pSS (n=18) and HC (n=18). (B) Representative western blot and summary statistics of EPSTI1 of B cells from pSS (n=4) and HC (n=4). (C) Representative immunohistological staining of CD20 (green), EPSTI1 (red) and DAPI (blue) of small labial gland from pSS (n=4) and controls (n=4). (D) Immunofluorescence quantification of EPSTI1 (red) in small labial gland from pSS (n=4) and controls (n=4). * p<0.05; **p<0.01; ***p<0.001. EPSTI1, epithelial mesenchymal interacting protein 1; HC, healthy controls; pSS, primary Sjögren's syndrome.

Epithelial mesenchymal interacting protein 1 (EPSTI1) are upregulated in B cells from pSS patients

We focused on epithelial mesenchymal interacting protein 1 (EPSTI1), which was highly expressed in B cells and was significantly overexpressed in B cells from pSS patients. We confirmed that mRNA expression of EPSTI1 was indeed upregulated in B cells from pSS patients using quantitative PCR (1.60±0.18 vs 0.93±0.09, p<0.01) (figure 2A). Furthermore, western blot showed that protein expression of EPSTI1 in B cells from pSS was significantly higher than that of HC (1.77±0.17 vs 1.00±0.01, p<0.01) (figure 2B). Given B cells are accumulated in small labial gland in pSS patients, we also examined EPSTI1 expression in small labial gland from pSS and HC. Immunohistochemistry of small labial gland revealed that EPSTI1 was expressed in salivary gland epithelial cells in pSS and controls, and highly expressed in lymphocytic infiltration regions in pSS (online supplementary figure S5). Consistently, the small labial gland-infiltrating $CD20^+$ B cells expressed higher level of EPSTI1 than those from HC (2.78±0.15 vs 1.01±0.07) (figure 2C–D). Furthermore, EPSTI1 was expressed in B cells in ectopic germinal centres in small labial glands from pSS patients (online supplementary figure S6). Taken together, peripheral and tissue-infiltrating B cells from pSS patients expressed higher level of EPSTI1.

EPSTI1 promotes B cells proliferation and immunoglobin production

To elucidate the potential function of EPSTI1 in B cells, we downregulated EPSTI1 expression in B cells from pSS patients via transfecting siRNA, which was confirmed by quantitative PCR and western blotting (online supplementary figure S2). We then stimulated the transfected B cells with CpG, and observed that EPSTI1-silenced B cells proliferated significantly lower than control B cells, which were examined by CCK8 (404,900±17 220 vs 652 400±23 560, p<0.0001) (figure 3A) and carboxyfluorescein succinimidyl ester (CFSE) dilution (42.3%±3.3% vs 26.7±2.9%, p<0.001) (figure 3B). Additionally, apoptosis of EPSTI1-silenced B cell and control B cells were comparable (figure 3C). Furthermore, EPSTI1-silenced B cells produced lower levels of IgG (611.4±46.8 vs 1026.0±79.4 ng/mL, p<0.01) and IgM (2058.4±124.9 vs 3122.0±148.6 ng/mL, p<0.01) (figure 3D). In contrast, no significant difference in proliferation, apoptosis or immunoglobulin productions was observed between EPSTI1-silenced and control B cells stimulated with anti-IgM and CD40L. Therefore, EPSTI1 promoted CpG-stimulated B cell proliferation and immunoglobin production, potentially through BCR-independent signalling.

EPSTI1 activates B cells through NF-κB signalling via degradation of IκBα

Given CpG stimulates B cells via TLR9 signalling, we first screened key molecules in the downstream of TLR9 signalling in EPSTI1-silenced B cells, including p65, p38 and JNK. EPSTI1-silencing significantly downregulated the phosphorylated p65, while expressions of total p65, phosphorylated p38 and phosphorylated JNK were stable (figure 4A and online supplementary figure S3), indicating that EPSTI1 facilitated p65 phosphorylation. Additionally, transforming growth factor activated kinase 1 (TAK1) and phosphorylated TAK1, which regulates IκBα, was not changed in EPSTI1-silencing B cells (online supplementary figure S4). Furthermore, IκBα expression in EPSTI1-silenced B cells was higher than that in control (figure 4B), suggesting that EPSTI1 promoted IκBα degradation. Consistently, we also confirmed the higher level of p-p65 and lower level of IκBα in B cells from pSS patients, compared with those from HC (figure 4C). VCP mediates EPSTI1-driven NF-κB activation in breast cancer cells;[14] however, VCP expression in B cells from pSS and HC, and the level of p-p65 and IκBα in CpG-stimulated Raji B cells were comparable (online supplementary fig S7).

We further overexpressed EPSTI1 in B cells from HC, and observed that phosphorylated p65 was upregulated and IkBα was downregulated (figure 4D). Finally, we pretreated B cells with DHE, an inhibitor of IκBα degradation, and observed that the upregulated phosphorylated p65 promoted by EPSTI1 was attenuated (figure 4E). Collectively, EPSTI1 promoted the degradation of IκBα, and subsequently facilitated the phosphorylation of p65 and activation of NF-κB signalling, which orchestrated with CpG-TLR signalling and finally promoted B cells activation.

DISCUSSION

In this study, we performed transcriptome analysis of B cells in pSS patients and found that type I IFN pathway and virus-responding pathway were activated. Transcriptome analysis also suggested that EPSTI1 was highly expressed in B cells from pSS patients. We further demonstrated that EPSTI1 promoted B

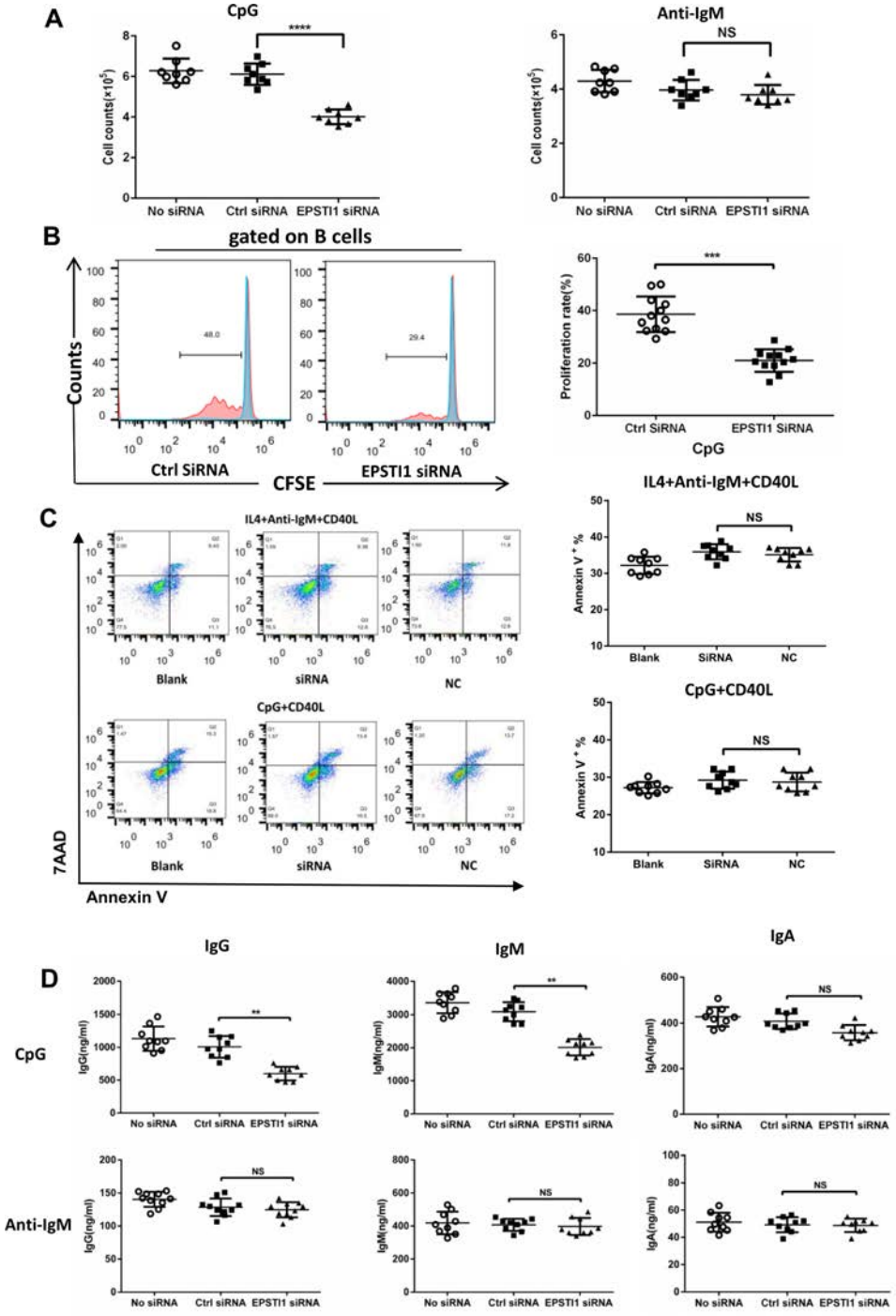

Figure 3 EPSTI1 promotes B cells proliferation and immunoglobin production. CD19$^+$ B cells from pSS were transfected with EPSTI1-siRNA or control-siRNA, and were stimulated with anti-IgM or CpG. (A) B cell proliferation stimulated by CpG (left) or anti-IgM (right) were quantified by CCK8 assay at 72 hours (n=8). (B) CFSE-labelled B cells (n=12) were stimulated with CpG for 72 hours and were analysed by flow cytometry. Representative FACS plots (left) and summary graph (right) demonstrating the proliferation of B cells. (C) Representative FACS plots (left) and summary graphs (right) demonstrating the B cell apoptosis (n=9). (D) EPSTI1-silencing or control B cells were stimulated with anti-IgM (n=9) or CpG (n=9) for 6 days. The supernatant IgG, IgM and IgA levels were quantified by ELISA. *p<0.05; **p<0.01; ***p<0.001. EPSTI1, epithelial mesenchymal interacting protein 1; pSS, primary Sjögren's syndrome.

cell proliferation and immunoglobulin production. Mechanistically, EPTSI1 facilitated IκBα degradation, then promoted p65 phosphorylation and finally stimulated TLR9-dependent B cell activation.

Type I IFN -induced genes are overexpressed in PBMC from pSS patients, which is defined as type I IFN signature.[15] Our transcriptome analysis identified several IFN-related genes in B cells from pSS patients, including IFI44L, IFI44, IFIT1, IFITM1, IFIT3, IFIT2, IRF7, IFI6 and ISG15.[16] IFN signalling is essential for host defence of virus. Consistently, GO and KEGG analysis revealed that the DEGs were enriched in microbial infection-related pathways, such as HCV, EBV, influenza A, measles and herpes simplex virus, supporting the concept of viruses as triggering factor of pSS.

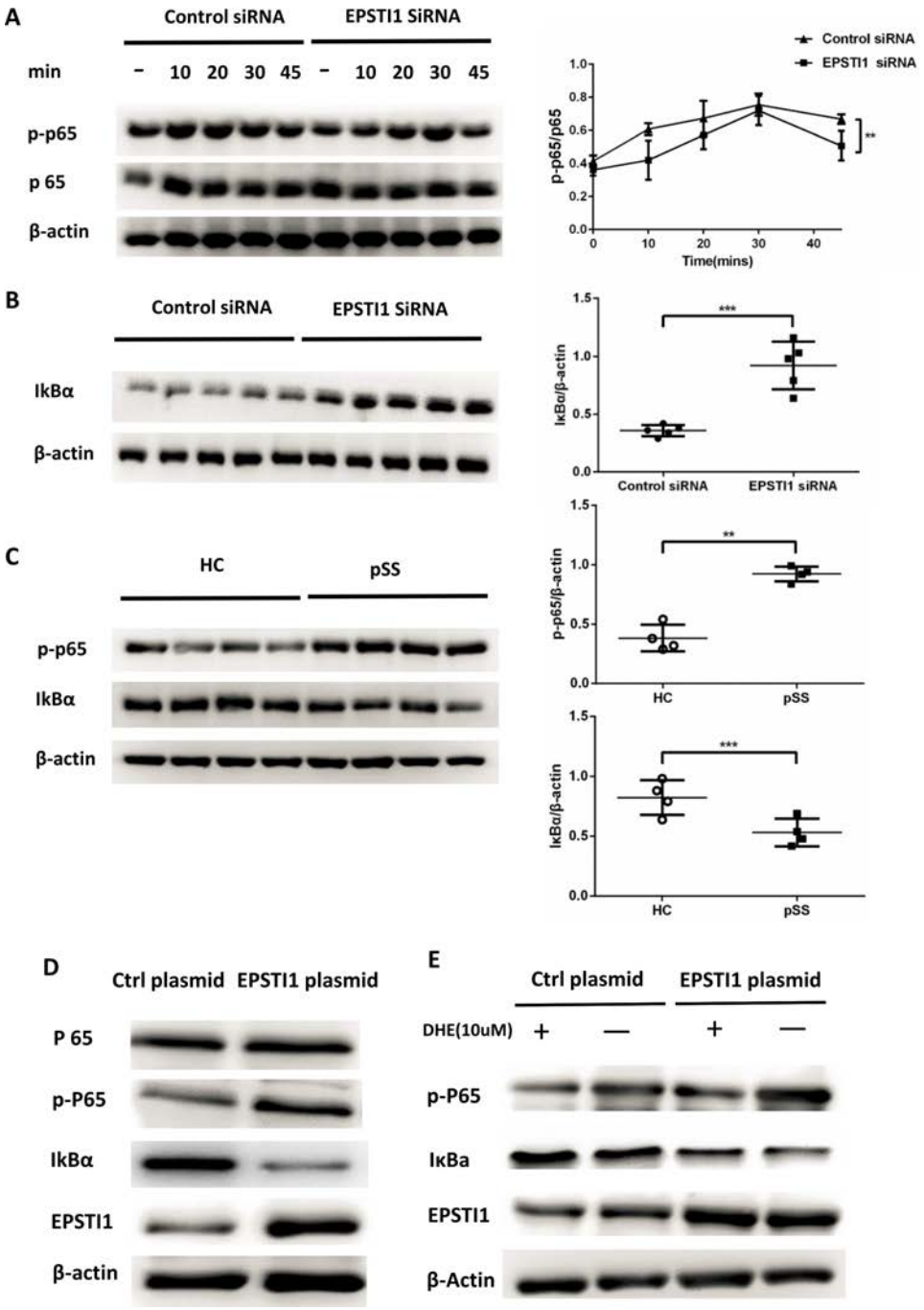

Figure 4 EPSTI1 activates B cells through NF-κb signalling. (A) Representative western blot (left) and summary graphs (right) of p65 and p-p65 of EPSTI1-silencing or control B cells stimulated with CpG for 0, 10, 20, 30 or 45 min. (B) Representative western blot (left) and summary graphs (right) of IκBα of EPSTI1-silencing or control B cells stimulated with CpG for 72 hours. (C) Representative western blot (left) and summary graphs (right) of p-p65, IκBα and β-actin of B cells from pSS (n=4) and HC (n=4). (D) Representative western blot of $CD19^+$ B cells from HC transfected with EPSTI1-expressing or control plasmids and stimulated with CpG for 60 hours. (E) Representative western blot of $CD19^+$ B cells from HC transfected with EPSTI1-expressing or control plasmids and stimulated with CpG for 60 hours in the presences or absence of IkBα inhibitor DHE (10 uM). Data were representative for three independent experiments. *p<0.05; **p<0.01; ***p<0.001. EPSTI1, epithelial mesenchymal interacting protein 1; HC, healthy controls; pSS, primary Sjögren's syndrome.

Beyond IFN-related genes, we found EPSTI1 was overexpressed in pSS patients. EPSTI1 is first reported in breast cancer cells, which facilitate growth and metastasis of cancer cells[14 17] by inhibiting apoptosis via interacting with caspase 8.[18] EPSTI1 plays an antiviral role in HCV infection by binding to protein kinase regulated by RNA promoter and promoting transcription of IFN-β, IFIT1, OAS1 and RNase L.[19] EPSTI1 also promote M1 macrophage polarisation. EPSTI1 expression is elevated in macrophages stimulated with IFN-γ and LPS.[20] In EPSTI1-knockout mice, peritoneal M1 macrophages are reduced, presumably through inhibiting phosphorylation and nuclear translocation of STAT1 and p65.[20]

Thus far, whether EPSTI1 regulate other immune cells such as B cells remains elusive. We found EPSTI1 indeed promoted B cell proliferation and immunoglobulin production through TLR signalling. Mechanistically, EPSTI1 promoted B cell activation

via NF-κB signalling. TLR9 signalling activation recruits MyD88 and forms TLR9 complex to activate IRAKs, TRAF6 and TAK1. TAK1 is an MAPK kinase that subsequently activates IKK, JNK and p38 MAPK, but TAK1 and phosphorylated TAK1 expression were not changed in EPSTI1-silencing B cells. EPSTI1-silencing significantly downregulated phosphorylated p65, but not phosphorylated p38 and phosphorylated JNK, indicating EPSTI1 regulated NF-κB signalling. Activated IKK ubiquitinates, phosphorylates and degrades IκBα, which phosphorylates NF-κB including p65. IκBα was upregulated in EPSTI1-silenced and downregulated in EPSTI1-expressing B cells, suggesting that EPSTI1 promoted IκBα degradation. Given TLR4 stimulated by LPS activates downstream pathway similar to TLR9 pathway in B cells, we did not exclude TLR4 pathway that was potentially regulated by EPSTI1 in B cells. Nevertheless, we demonstrated that elevated EPSTI1 potentially amplified the TLR signalling in B cells. EPSTI1 promoted B cell activation and potentially facilitated the B cell response to virus infection.

B cell hyperactivation is a hallmark of pSS. Given EPSTI1 promoting B cell activation, as well as B cells from pSS patients expressing higher level of EPSTI1, EPSTI1 might participate in the initiation of autoimmune humoral response to virus infection in pSS patients, and play a role in pSS pathogenesis. Thus, targeting EPSTI1 might be a potential approach to restore the B cell homeostasis in pSS patients.

In summary, we demonstrated that EPSTI1 promoted B cell activation by facilitating p65 phosphorylation and activation of NF-κB signalling via promoting IκBα degradation. EPSTI1 might be implicated in pSS pathogenesis and was a potential therapeutic target of pSS.

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Contributors HC and FZ conceptualised and designed the project and supervised the project; JS, SL and HZ performed all the experiments and JS wrote the manuscript with contributions from all authors; HC revised the manuscript. CL, ZC, TS, SZ, LZ, CH, MW and WZ participated in the sample collection and clinical analysis. All authors read and approved the manuscript.

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Competing interests None declared.

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

Patient consent for publication Obtained.

Ethics approval Institutional review board of Peking Union Medical College Hospital.

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CLINICAL SCIENCE

Intra-articular sprifermin reduces cartilage loss in addition to increasing cartilage gain independent of location in the femorotibial joint: post-hoc analysis of a randomised, placebo-controlled phase II clinical trial

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ABSTRACT

Objectives In the phase II FGF-18 Osteoarthritis Randomized Trial with Administration of Repeated Doses (FORWARD) study, sprifermin demonstrated cartilage modification in the total femorotibial joint and in both femorotibial compartments by MRI in patients with knee osteoarthritis. Here, we evaluate whether sprifermin reduces cartilage loss and increases cartilage thickness, independent of location.

Methods Patients were randomised 1:1:1:1:1 to three once-weekly intra-articular injections of 30 µg sprifermin every 6 months (q6mo); 30 µg sprifermin every 12 months (q12mo); 100 µg sprifermin q6mo; 100 µg sprifermin q12mo; or placebo. Post-hoc analysis using thinning/thickening scores and ordered values evaluated femorotibial cartilage thickness change from baseline to 24 months independent of location. Changes were indirectly compared with those of Osteoarthritis Initiative healthy subjects.

Results Thinning scores were significantly lower for sprifermin 100 µg q6mo versus placebo (mean (95% CI) difference: 334 µm (114 to 554)), with a cartilage thinning score similar to healthy subjects. Thickening scores were significantly greater for sprifermin 100 µg q6mo, 100 µg q12mo and 30 µg q6mo versus placebo (mean (95% CI) difference: 425 µm (267 to 584); 450 µm (305 to 594) and 139 µm (19 to 259), respectively) and more than doubled versus healthy subjects.

Conclusions Sprifermin increases cartilage thickness, and substantially reduces cartilage loss, expanding FORWARD primary results.

Trial registration number NCT01919164.

Key messages

What is already known about this subject?

- Clinical studies support the structure-modifying effects of sprifermin, a recombinant human fibroblast growth factor 18, in patients with knee osteoarthritis. The 2-year primary analysis of the phase II FORWARD study demonstrated statistically significant dose-dependent modification of change in cartilage thickness in the total femorotibial joint (TFTJ), medial and lateral femorotibial joints, and central medial and lateral TFTJ subregions with intra-articular sprifermin.
- MRI is commonly used for measuring cartilage thickness changes in specific femorotibial regions in clinical trials. However, region-specific analysis cannot elucidate whether cartilage loss is reduced, wherever it occurs in an individual joint. Application of location-independent analysis methodology can provide a more sensitive and informative analysis of cartilage loss and thickening independent of the location where it occurs.

What does this study add?

- This post-hoc exploratory analysis reports cartilage thickness change based on thinning/thickening scores and ordered values of subregional cartilage thickness change for patients with knee osteoarthritis enrolled in the FORWARD study between baseline and 24-month follow-up. It shows that treatment with sprifermin increases cartilage thickness and reduces cartilage loss. Corresponding results for healthy reference subjects from the Osteoarthritis Initiative were summarised to indirectly compare changes in thinning/thickening scores with FORWARD study patients. This comparison to the reference set indicates that thickening more than doubled, whereas thinning almost reduced to the level of healthy subjects, providing strong support for substantial cartilage modification by sprifermin.

INTRODUCTION

Osteoarthritis (OA) is characterised by loss of articular cartilage, which is associated with clinical outcomes including knee replacement.[1 2] Whereas current treatments alleviate symptoms without targeting structural progression,[3] disease-modifying OA drugs (DMOADs) aim to modify tissue structure, such as articular cartilage, ideally in conjunction with improving clinical outcomes.[4 5] No DMOADs have yet been approved in the USA or Europe.

Clinical studies support the structure-modifying effects of the recombinant human fibroblast growth factor 18, sprifermin, in knee OA.[6–8] The 2-year

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Key messages

How might this impact on clinical practice or future developments?

- There are currently no disease- (or structure-) modifying osteoarthritis drugs (DMOADs) approved for use in Europe or the USA.
- Primary results from the FORWARD study combined with findings from this post-hoc analysis suggest that sprifermin should be evaluated further in clinical trials as a potential DMOAD therapy for knee osteoarthritis that can substantially reduce cartilage loss.

primary analysis of the phase II FORWARD study demonstrated statistically significant dose-dependent modification of cartilage thickness change by quantitative MRI in the total femorotibial joint (TFTJ), and (central) medial and lateral femorotibial compartments with intra-articular (i.a.) sprifermin.[8]

MRI can measure cartilage thickness change in femorotibial subregions.[9] Yet, region-specific analysis cannot elucidate whether cartilage loss is reduced wherever it occurs in an individual joint. Location-independent analysis methodology, based on ordering subregional cartilage thickness change, provides a more sensitive and informative analysis of cartilage loss and thickening.[9–12] Location-independent methods were used in a 1-year, placebo-controlled, proof-of-concept phase Ib study,[7] which suggested that i.a. sprifermin reduced cartilage loss in addition to increasing cartilage thickness.[10] However, this used a small sample size, and the extent to which structure modification affected the cartilage thinning score compared with healthy subjects was not studied.

We conducted a post-hoc, exploratory analysis using thinning/thickening scores and ordered values (OVs) calculated from the larger FORWARD study, to evaluate whether sprifermin reduces cartilage loss independent of location in a given knee, in addition to the dose-dependent increase in mean cartilage thickness in the TFTJ.[8] Further, we indirectly compared changes in thinning/thickening scores[10] in FORWARD[8] with those in healthy reference subjects from the Osteoarthritis Initiative (OAI).[13–15]

METHODS

FORWARD study design

FORWARD is a multicentre, randomised, double-blind, placebo-controlled, dose-finding, phase II, 5-year study (NCT01919164). Study methods have been reported previously.[8] Briefly, patients aged 40–85 with symptomatic radiographic knee OA, Kellgren-Lawrence Grade 2 or 3, and medial minimum joint space width ≥2.5 mm in the target knee were randomised (1:1:1:1:1) to receive three once-weekly i.a. injections of: 30 µg sprifermin every 6 months (q6mo); 30 µg sprifermin every 12 months (q12mo); 100 µg sprifermin q6mo; 100 µg sprifermin q12mo; or placebo. The primary endpoint was change in total TFTJ cartilage thickness from baseline to 2 years, by quantitative MRI. See online supplementary file 1 for patient involvement information.

Structural change measurements

Clinical MRI scanners (1.5/3 Tesla (T)) obtained MRI acquisitions[8] for assessing cartilage thickness in 16 femorotibial subregions.[16]

Changes in subregional cartilage thickness between baseline and 24 months were ranked by magnitude to create 16 location-independent OVs, as described previously.[10–12] OV1 corresponded to the largest loss/smallest gain and OV16 to the smallest loss/largest gain in cartilage thickness in any subregion within each knee. Thinning and thickening scores for each knee were defined as the sum of each of the 16 subregions with negative and positive changes, respectively.[10 11] To determine the relationship between cartilage loss and gain, the ratio of the thickening to thinning score was calculated for each patient. Mean thinning/thickening scores were informally compared with measurements from an OAI reference group,[17] comprising 82 healthy subjects without radiographic knee OA, who were assessed at baseline and 24 months using the same image acquisition and analysis technology[16] as in the FORWARD study.[8] Healthy reference subjects from the OAI had no knee pain, no radiographic signs of knee OA and no risk factors for knee OA.[13]

Statistical analysis

In this exploratory, post-hoc analysis, differences between treatment groups were evaluated using a t-test, without adjusting for multiple comparisons. Patients in the modified intent-to-treat (mITT) population who had baseline and 24-month MRI data (thinning/thickening analysis set) were included. All endpoints were considered exploratory.

RESULTS

Patients

Baseline characteristics for the thinning/thickening score analysis set (online supplementary table 1) were similar to those previously reported for the mITT population.[8]

Cartilage thinning and thickening scores

Thinning scores were lower for all sprifermin doses versus placebo; statistically significantly less thinning was observed for the highest sprifermin dose (100 µg q6mo; table 1). Thinning scores with this dose approached those observed in OAI healthy reference subjects over the same 24-month observation period.

Thickening scores were substantially greater with sprifermin versus placebo; differences were statistically significant for the

Table 1 Mean (95% CI) thinning and thickening scores by FORWARD treatment group (modified intent-to-treat population) and in the Osteoarthritis Initiative healthy reference cohort over 24 months

		Sprifermin				
Mean (95% CI)	Placebo (n=83)	30 µg q12mo (n=92)	30 µg q6mo (n=83)	100 µg q12mo (n=90)	100 µg q6mo (n=86)	OAI healthy reference cohort (n=82)
Thinning, µm	−766 (−972 to −560)	−729 (−910 to −548)	−641 (−815 to −467)	−597 (−777 to −416)	−432 (−521 to −343)	−335 (−381 to −288)
Difference versus placebo	–	37 (−234 to 309)	125 (−143 to 393)	170 (−101 to 441)	334 (114 to 554)*	–
Thickening, µm	431 (358 to 505)	522 (447 to 596)	571 (475 to 666)	881 (759 to 1003)	856 (717 to 996)	356 (313 to 398)
Difference versus placebo	–	90 (−14 to 195)	139 (19 to 259)*	450 (305 to 594)*	425 (267 to 584)*	–
TFTJ cartilage thickness, µm	−21 (−36 to −5)	−12 (−26 to 2)	−5 (−20 to 10)	20 (4 to 37)	29 (15 to 43)	–
Difference versus placebo	–	9 (−12 to 30)	16 (−5 to 38)	41 (18 to 64)*	50 (30 to 71)*	–

*t-test p value<0.05.
OAI, osteoarthritis initiative; q6mo, every 6-month active cycles; q12mo, every 12-month active cycles; TFTJ, total femorotibial joint.

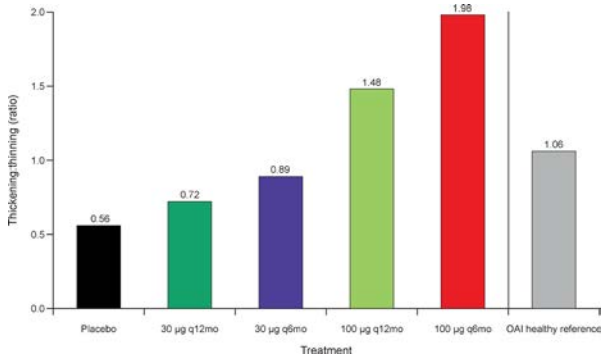

Figure 1 Ratio of thickening:thinning scores by FORWARD treatment group (modified intent-to-treat population) and in the Osteoarthritis Initiative healthy reference cohort over 24 months. q6mo, every 6-month active cycles; q12mo, every 12-month active cycles.

100 µg q6mo, 100 µg q12mo and 30 µg q6mo dose groups. The highest doses (100 µg q6mo and q12mo) of sprifermin approximately doubled the cartilage thickening score compared with placebo and OAI healthy reference subjects (table 1).

The thickening:thinning score ratio was 1.06 in OAI healthy reference subjects, indicating no net loss or gain of cartilage. For the sprifermin 100 µg q6mo and q12mo dose groups, the thickening:thinning score ratio was higher than that obtained for the OAI healthy reference subjects (1.98 and 1.48, respectively), indicating cartilage thickness gain. The thickening:thinning score ratio was lower in the placebo group (0.56) versus OAI healthy reference subjects, indicating cartilage thickness loss (figure 1).

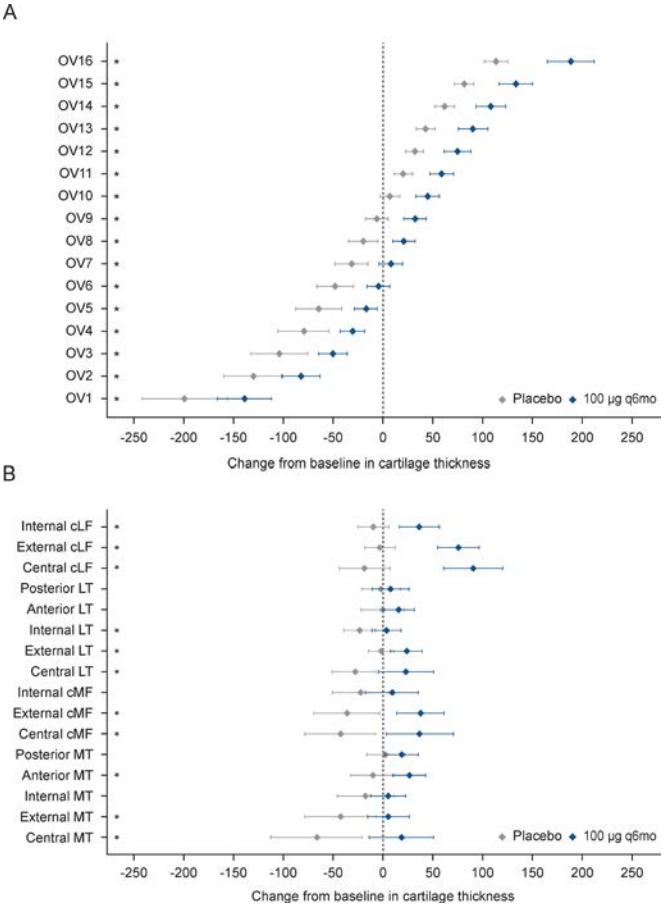

Figure 2 Mean change from baseline (95% CI) in cartilage thickness (µm) over 24 months for (a) 16 OVs and for (b) the 16 subregions in the sprifermin 100 µg q6mo and placebo groups (modified intent-to-treat population). CLF, condyle lateral femur; CMF, condyle medial femur; LT, lateral tibia; MT, medial tibia; OV, ordered value; q6mo, every 6-month active cycle. *Denotes statistically significant treatment effect: t-test p value<0.05.

OVs and subregion analysis

Sprifermin-treated patients (100 µg q6mo) gained more and lost less cartilage thickness across OVs versus placebo-treated patients over 24 months; the difference reached statistical significance in all 16 OVs (figure 2A; online supplementary table 2). The decrease from baseline was substantially lower in OV1 (the largest loss in cartilage thickness in any subregion within each knee) and the increase substantially greater in OV16 (the largest gain in any subregion within each knee) with sprifermin than placebo.

Change in cartilage thickness from baseline to 24 months was modified significantly with sprifermin 100 µg versus placebo in 11 of 16 femorotibial subregions. The greatest differences were observed in the central lateral femur and central medial tibia (figure 2B; online supplementary table 3).

DISCUSSION

This is the largest study, to date, to apply location-independent analysis of cartilage change in a DMOAD trial, and the first to compare cartilage thinning/thickening scores from treated patients versus healthy reference subjects. Location-independent analysis of thinning and thickening scores demonstrated efficacy with sprifermin over 24 months, whereby sprifermin increased cartilage thickness, and substantially reduced cartilage loss compared with placebo. Thinning scores with sprifermin 100 µg q6mo were approaching those observed in OAI healthy reference subjects, whereas thickening scores were more than doubled.

In a subset of the OAI progression cohort, the greatest per cent change and sensitivity to change in cartilage thickness were observed in the external and central medial tibia, and in the central medial femoral condyle.[17] These regions might be assumed to represent high load-bearing regions of the joint and regions with pre-existing cartilage damage. Sprifermin did not appear to be less effective in these subregions than in other subregions in the medial compartment or in the lateral tibia. Indeed, one of the regions with the greatest difference between sprifermin and placebo was the central medial tibia.

Limitations were potential for type 1 error due to multiple comparisons, and use of healthy reference subjects from a different cohort (the OAI[13]); consequently, caution must be applied when interpreting the data. However, subjects in the OAI study cohort were of a similar age to the FORWARD population and the studies used the same MR imaging sequences, orientation (coronal), parameters, spatial resolution and analysis technology.[18] Additionally, the current analysis did not evaluate the association of the modification of thinning/thickening scores with change in pain and/or inflammation (synovitis). Although a potential limitation, as 1.5 T MRI has a slightly lower signal-to-noise ratio than 3 T, precision errors have been shown to be only marginally greater at 1.5 T, and thickness measures were consistent between 1.5 T and 3 T.[19]

Key strengths of this study include the robust design and relatively large sample size of FORWARD, and the comparison of findings with healthy reference subjects. In knee OA, some knees show preferential changes in the medial compartment, while others show greater changes in the lateral compartment.[12] Clinical trials often do not account for differences in disease laterality, or restrict observations to those with only medial

disease, limiting generalisation to subjects with lateral OA. In contrast, FORWARD intentionally included patients with both medial and lateral disease. In this context, location-independent analysis is particularly advantageous, as it covers cartilage thinning and thickening wherever it occurs in a joint, independent of the compartment and location primarily affected. Thinning/thickening scores and OVs were shown to be sensitive and efficient methods for measuring independent changes in cartilage thickness in either direction.[9–12] In contrast, global or regional cartilage volume or thickness measurements may miss increases or decreases in cartilage thinning or thickening that occur simultaneously in a joint, although in different subregions.[11] Furthermore, such measurements cannot be used to discriminate between an increase in cartilage thickening (in some regions) in isolation, or in combination with modification of cartilage thinning at any given position within a joint.[9–12]

The current results support the concept that sprifermin increases cartilage thickness, and reduces cartilage loss. They expand the primary FORWARD results, showing structural modification of cartilage thickness with sprifermin.[8] Sprifermin should be evaluated further in clinical trials as a potential DMOAD for knee OA.

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Competing interests FE is CEO of Chondrometrics GmbH and has received consulting fees from Merck KGaA, Samumed LLC, Abbvie, Bioclinica, Kolon TissueGene, Servier, Galapagos, Novartis and Roche. JLK and AA are employees of EMD Serono Research & Development Institute, Inc. (a business of Merck KGaA, Darmstadt, Germany). WW and SM are co-owners and employees of Chondrometrics GmbH, and WW has received consulting fees from Galapagos. MCH has received consulting fees from Bristol Myers Squibb, Eli Lilly, EMD Serono Research & Development Institute, Inc., Flexion Therapeutics, Galapagos, Hofmann LaRoche, IBSA Biotechniq SA, Novartis Pharma AG, Pfizer, Plexxikon, Samumed LLC, Symic Bio, Theralogix LLC, TissueGene, TLC Biopharmaceuticals and Zynerba.

Patient consent for publication Not required.

Ethics approval The study was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation Guidelines for Good Clinical Practice and local regulations. The study protocol and all major amendments were approved by the relevant Institutional Review Boards or Independent Ethics Committees and by Health Authorities, according to country-specific laws.

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Data availability statement Data are available upon reasonable request. Any requests for data by qualified scientific and medical researchers for legitimate research purposes will be subject to Merck's Data Sharing Policy. All requests should be submitted in writing to Merck's data sharing portal (https://www.merckgroup.com/en/research/our-approach-to-research-and-development/healthcare/clinical-trials/commitment-responsible-data-sharing.html). When Merck has a co-research, co-development, or co-marketing or co-promotion agreement, or when the product has been out-licensed, the responsibility for disclosure might be dependent on the agreement between parties. Under these circumstances, Merck will endeavour to gain agreement to share data in response to requests.

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EPIDEMIOLOGICAL SCIENCE

Hypersensitivity reactions with allopurinol and febuxostat: a study using the Medicare claims data

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Handling editor Josef S Smolen

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ABSTRACT

Objective To assess the risk of hypersensitivity reactions (HSRs) with allopurinol and febuxostat in a population-based study.

Methods We used the 5% Medicare beneficiary sample (≥65 years) from 2006 to 2012 to identify people with a newly filled prescription for allopurinol, febuxostat or colchicine. We used multivariable-adjusted Cox regression analyses to compare the hazard ratio (HR) of incident HSRs with allopurinol or febuxostat use versus colchicine use; separate analyses were done in people exposed to allopurinol. Propensity-matched analyses (5:1) compared hazards with allopurinol versus febuxostat.

Results Crude incidence rates of HSRs were as follows: allopurinol, 23.7; febuxostat, 30.7; and colchicine, 25.6 per 1000 person-years. Compared with colchicine, allopurinol, febuxostat and febuxostat+colchicine were associated with significantly higher HRs of HSRs, 1.32 (95% CI: 1.10 to 1.60) and 1.54 (95% CI: 1.12 to 2.12) and 2.17 (95% CI: 1.18 to 3.99), respectively. In propensity-matched analyses, febuxostat did not significantly differ from allopurinol; HR for HSRs was 1.25 (95% CI: 0.93 to 1.67). Compared with allopurinol start dose <200 mg/day, allopurinol start dose ≥300 mg/day, diabetes and female sex were associated with significantly higher hazard of HSRs, 1.27 (95% CI: 1.12 to 1.44), 1.21 (95% CI: 1.00 to 1.45) and 1.32 (95% CI: 1.17 to 1.48), respectively. The majority (69%) of HSRs occurred in the outpatient setting.

Conclusions Compared with colchicine, allopurinol and febuxostat similarly increased the risk of HSRs. Allopurinol and febuxostat did not differ from each other. In allopurinol users, starting dose, female sex and diabetes increased this risk, findings that need further study.

Key messages

What is already known about this subject?

- In two previous studies, the risk factors for allopurinol hypersensitivity reactions (HSRs) were female sex, age ≥60 years, renal or cardiovascular comorbidities in a Taiwanese study, and a higher allopurinol start dose in both Taiwanese and New Zealand study. No population-level comparative risk data of HSRs associated with allopurinol and febuxostat are available in non-Taiwanese.

What does this study add?

- In propensity-matched analyses using the 5% US Medicare data, we found that compared with colchicine, both allopurinol and febuxostat were associated with significantly increased hazards of HSRs, and febuxostat did not significantly differ from allopurinol regarding the hazard of HSRs.
- In allopurinol users, allopurinol starting dose, diabetes and female sex increased the risk of HSRs.

How might this impact on clinical practice or future developments?

- Our study provides an estimate of the HSRs with urate-lowering therapies (ULTs) in general US population 65 years or older and shows that the two most commonly used ULTs are associated with a similar hazard of HSRs.
- Future studies need to examine as to why a higher allopurinol start dose, diabetes and female sex increase the risk of HSRs.

INTRODUCTION

A hypersensitivity reaction (HSR) is an important, but rare/uncommon adverse event associated with the use of allopurinol,[1] the most commonly used urate-lowering therapy (ULT) for the treatment of gout.[2 3] A recent study using the nationwide Taiwanese data showed that the annual incidence rate of allopurinol cutaneous reactions was 4.68 per 1000 new users.[4] Female sex, older age (≥60 years), renal or cardiovascular comorbidities and initial allopurinol dosage exceeding 100 mg/day were risk factors.[4] In a recent retrospective study from New Zealand, a higher allopurinol start dose was significantly associated with a higher risk of allopurinol hypersensitivity syndrome (AHS),[5] a severe form of HSR. Reviews indicated that HLA 58*01, Han Chinese/European ancestry and renal failure were other potential risk factors for AHS.[6 7] The same group of authors also showed that in people who tolerate allopurinol, allopurinol dose can be safely increased including patients with renal failure.[8] The findings of these studies have not been reproduced. Similar studies have not been conducted in populations from the USA or the European countries. Comparative studies of allopurinol versus febuxostat are limited to some ethnicities only.[9]

Our study objective was to use a nationally representative sample of older US adults to: (1) examine the crude incidence rates of HSRs with allopurinol, febuxostat or colchicine; (2) assess whether compared with colchicine, allopurinol or febuxostat use were independently associated with an increased risk of HSRs and whether allopurinol

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differed from febuxostat; and (3) evaluate whether starting allopurinol dose and comorbidity are significantly associated with a higher risk of HSRs in allopurinol users. We hypothesised that compared with febuxostat or colchicine, allopurinol use will significantly increase the risk of HSRs, this risk will be the highest in the initial few months of use and that initial allopurinol dose and the presence of renal failure will be risk factors.

METHODS

Study cohort

We used the 5% random sample of Medicare Beneficiaries (≥65 years) from 2006 to 2012 to perform this study, obtained from the Centers for Medicare and Medicaid Services Chronic Condition Data Warehouse, and widely used for epidemiological research by others[10 11] and us.[12 13]

We used the beneficiary summary file and Medicare Part D file to identify beneficiaries enrolled in Medicare fee-for-service with pharmacy coverage (parts A, B and D; files contain all insurance claims for each beneficiary) and not enrolled in a Medicare Advantage Plan, lived in the USA during the study period (2006–2012) and filled a new prescription of allopurinol or febuxostat or colchicine, three common medications used for gout and/or hyperuricaemia. We used an incident (or new) user design rather than a prevalent user design, which reduces bias by avoiding adjustment for characteristics that may be in the causal pathway and allows capture of both early and late events.[14]

We reported methods and results as recommended in the Strengthening of Reporting in Observational studies in Epidemiology (STROBE) statement.[15]

Exposure of interest: new treatment with allopurinol or febuxostat

All prescription claims, including drug name, dose and supply, were obtained from Medicare Part D file. A beneficiary began a new allopurinol (or febuxostat or colchicine) treatment episode by filling an allopurinol (or febuxostat or colchicine) prescription, provided they had not filled an allopurinol (or febuxostat or colchicine) prescription in the previous 365 days. This baseline 365-day clean period applied to all initial prescription episodes, whether they were the initial exposure or subsequent switching to another medication.

We assigned days of exposure for each allopurinol (or febuxostat or colchicine), calculated based on the days' supply variable provided in the Medicare Part D file and also included a 30-day residual/grace period. Continuous allopurinol (or febuxostat or colchicine) episode ended after 30 days of the end of allopurinol (or febuxostat or colchicine) exposure. If there were >30 days between prescription fills, new allopurinol (or febuxostat or colchicine) exposure started. Thus, a patient receiving a single 90-day prescription was considered exposed for 120 days, which included a 30-day residual period as medication adherence is usually imperfect, and the residual period accounts for any residual biological effects of medications. If a patient had prescriptions for more than one of these three drugs, then they were considered exposed to all prescribed drugs on a given day.

The main predictor of interest was new allopurinol or febuxostat use, with new colchicine use as the reference category, as colchicine is a common non-ULT medication (with a different mechanism of action) used by patients with gout. We assessed all allopurinol (<200, 200–299 and ≥300 mg/day), febuxostat (40 and 80 mg/day) and colchicine (0.6, 0.6-<1, 1-<2 and ≥2 mg/day) start doses, considering only the first prescription. We calculated the daily allopurinol (or febuxostat or colchicine) dose as the mean daily use for each continuous allopurinol, febuxostat or colchicine treatment episode. For each allopurinol, febuxostat or colchicine treatment episode, we categorised the duration of use by 30-day intervals.

Study outcome

The outcome of interest for our study was incident HSR, defined using a validated algorithm. The main analysis follows the definition of potential HSR similar to that by Wright *et al*[16] using eosinophilia (288.3), arthropathy associated with HSR (713.6), other anaphylactic reaction (995.0), unspecified adverse effect of unspecified drugs, medicinal and biological substance not elsewhere classified (995.2), or allergy, unspecified, not elsewhere classified (995.3). We added a baseline exclusion for codes E930 to E949 to increase the specificity of HSR. For sensitivity analyses, we included another definition of incident HSR, per Strom *et al*[17] based on the presence of codes 995.2, 995.0 or 995.3. Our outcome of overall HSRs is different than AHS that has been studied previously.

Covariates

Vital statistics (birthdate, date of death), and monthly entitlement indicators (A/B/C/D) were obtained from the Medicare claims data. We assessed several important covariates at baseline, including the patient demographics (age, gender, race/ethnicity), medical comorbidity and the use of medications for cardiovascular diseases from Medicare denominator and other claims files. We assessed the Charlson-Romano comorbidity index score, a validated weighted comorbidity index developed for claims data analysis[18] adapted from the Charlson index.[19] Cardiovascular medications (statins, beta-blockers, diuretics and angiotensin-converting enzyme (ACE)-inhibitors) served as markers of cardiovascular disease.

Patient and public involvement

The study question was informed by patients in our gout clinic who questioned whether there were any differences in the risk of HSRs with gout medications. There was no formal patient involvement in the study design.

Statistical analyses

We compared baseline characteristics of new medication exposure episodes with versus without incident HSRs and calculated crude incidence rates of HSRs for new allopurinol (or febuxostat or colchicine) episodes. As noted in the methods, we tested two different definitions to assess incidence rates, Wright *et al*[16] and Strom *et al*,[17] with and without the additional E codes for baseline exclusion. The Wright definition with E codes[16] was used for all other analyses.

For assessing comparative risk of HSR with allopurinol or febuxostat versus colchicine, we performed multivariable-adjusted Cox proportional hazard regression analyses adjusted for demographics (age, gender and race), cardiovascular medications (statins, beta-blockers, diuretics and ACE inhibitors) and Charlson comorbidity index to control for differences between patients exposed to allopurinol versus febuxostat versus colchicine. We used the Huber-White 'Sandwich' variance estimator to account for correlations between observations from the same patient.[20] We calculated the HR of new HSRs for new allopurinol or febuxostat use versus colchicine use (reference category); sensitivity analyses limited to people with gout were done.

We performed propensity-matched analyses comparing new allopurinol with new febuxostat use, in a 5:1 ratio. Propensity

Table 1 Demographic and clinical characteristics of incident HSRs in people with allopurinol or febuxostat or colchicine exposure

		HSR during the follow-up		
	All episodes	Yes	No	P value
Total, N (episodes)	66 178	1038	65 140	
Age, mean (SD)	76.49 (7.42)	76.3 (7.10)	76.5 (7.42)	0.42
Sex, N (%)				**0.001**
Male	33 132 (50.1%)	465 (44.8%)	32 667 (50.2%)	
Female	33 046 (49.9%)	573 (55.2%)	32 473 (49.9%)	
Race/Ethnicity, N (%)				0.4
White	51 043 (77.1%)	816 (78.6%)	50 227 (77.1%)	
Black	8981 (13.6%)	129 (12.4%)	8852 (13.6%)	
Hispanic	1150 (1.7%)	19 (1.8%)	1131 (1.7%)	
Asian	3441 (5.2%)	48 (4.6%)	3393 (5.2%)	
Native American	150 (0.2%)	0 (0.0%)	150 (0.2%)	
Other/unknown	1413 (2.1%)	26 (2.5%)	1387 (2.1%)	
Region, N (%)				0.18
Midwest	15 194 (23.0%)	265 (25.5%)	14 929 (22.9%)	
Northeast	11 663 (17.6%)	168 (16.2%)	11 495 (17.7%)	
South	27 503 (41.6%)	430 (41.4%)	27 073 (41.6%)	
West	11 818 (17.9%)	175 (16.9%)	11 643 (17.9%)	
Charlson-Romano* comorbidity score, mean (SD)	2.59 (3.09)	1.94 (2.94)	2.61 (3.10)	**<0.0001**
Select Charlson-Romano score comorbidities†				
Myocardial infarction	3157 (4.8%)	31 (3.0%)	3126 (4.8%)	**0.001**
Diabetes	19 727 (29.8%)	225 (21.7%)	19 502 (29.9%)	**<0.0001**
Malignancy	6009 (9.1%)	84 (8.1%)	5925 (9.1%)	0.26
Congestive heart failure	14 282 (21.6%)	177 (17.1%)	14 105 (21.7%)	**0.0003**
Moderate or severe liver disease	133 (0.2%)	0 (0%)	133 (0.2%)	0.15
Moderate or severe renal disease	14 983 (22.6%)	175 (16.9%)	14 808 (22.7%)	**<0.0001**

HSR, incident HSRs defined as per Wright *et al* with the occurrence of a new HSR with a clean baseline period of 365 days with no HSR. Bold font represents statistically significant comparisons with a p-value <0.05.
*Charlson-Romano comorbidity score was assessed for the baseline period for each episode. It is a validated measure of comorbidity that uses diagnosis codes for comorbidities for each inpatient and outpatient claim and dates from claims files. Comorbidities included diabetes, myocardial infarction, congestive heart failure, cerebrovascular disease, liver disease, pulmonary disease, peripheral vascular disease, rheumatic disease and so on.
†We selected six Charlson-Romano comorbidities a priori to assess their association with HSRs.
HSR, hypersensitivity reaction.

matching included age, gender, race, Charlson-Romano comorbidity score, region, each Charlson-Romano comorbidity, risk factors for coronary artery disease and current use of medications for cardiovascular diseases (statins, beta-blockers, diuretics and ACE inhibitors). We explored the association of daily dose and duration of use with the risk of HSRs in people with the same propensity of receiving either drug, allopurinol or febuxostat.

We also performed separate multivariable-adjusted analyses in allopurinol exposed people to assess the effect of allopurinol start dose and duration of use and whether specific comorbidities such as coronary artery disease, heart failure, renal failure, liver disease, diabetes and malignancy were associated. We assessed these comorbidities, as they may impact the metabolism of ULT drugs, and/or concomitant medications, which might have drug–drug interactions with ULT. We used SAS 9.0 (Cary, North Carolina, USA) to perform the analyses. A p-value <0.05 was considered statistically significant.

RESULTS

Study cohort characteristics

Of 39 261 people who contributed 66 178 new episodes of allopurinol/febuxostat/colchicine use, 1038 ended in a HSR; the majority (69%) occurred in an outpatient setting. The mean age was 77 years, 50% were men, 77% white and the mean Charlson-Romano score was 2.6 (table 1). Episodes with versus without HSRs had similar characteristics except higher comorbidity (table 1).

Crude incidence rates of HSRs

Crude incidence rates of HSRs were as follows: allopurinol, 23.9; febuxostat, 30.5; and colchicine, 25.7 per 1000 person-years (table 2). Crude incidence rates were higher in people using medication combinations, particularly febuxostat+colchicine and febuxostat+colchicine+allopurinol, 56.8 and 89.1 per 1000 person-years, respectively (table 2).

Crude incidence rates of HSRs were higher for allopurinol start dose ≥300 mg/day compared with lower doses, but not for higher febuxostat start dose (table 2). Rates were highest in the first 30 days of exposure to allopurinol, 73.9 and febuxostat, 60.6/1000 person-years, with a progressive reduction over time. However, HSR rates remained fairly stable for colchicine up to 120 days, with a slight reduction for use >120 days (table 2).

Incidence rates varied little, when we used alternate definitions for HSRs except for the Strom *et al* definition with no events for the simultaneous use of allopurinol and febuxostat (online supplementary table S1).

Multivariable-adjusted comparative risk of HSRs with allopurinol or febuxostat compared with colchicine

In multivariable-adjusted analyses, compared with colchicine, the use of allopurinol, febuxostat or febuxostat+colchicine combination was associated with significantly higher HRs of HSRs, 1.32 (95% CI: 1.10 to 1.60) and 1.54 (95% CI: 1.12 to 2.12) and 2.17 (95% CI: 1.18 to 3.99), respectively (table 3). Women had 1.28-fold higher HR of HSRs (table 3). When we

Table 2 Crude incidence rate of incident hypersensitivity reactions with allopurinol, febuxostat or colchicine exposure†

	Person-days of follow-up	#Cases	Incidence rate /100 000 person-days	Incidence rate /1000 person-years
Allopurinol	1 1167 791	731	6.5	23.91
Febuxostat	610 115	51	8.4	30.53
Colchicine	2 400 483	169	7.0	25.71
Allopurinol+colchicine	946 440	71	7.5	27.40
Allopurinol+febuxostat	34 789	3	8.6	31.50
Febuxostat+colchicine	70 719	11	15.6	56.81
Allopurinol+febuxostat+colchicine	8191	2	24.4	89.18
Allopurinol, days				
≤30	790 546	160	20.2	73.92
>30 to ≤60	744 433	75	10.1	36.80
>60–90	573 745	42	7.3	26.74
>90–120	524 781	47	9.0	32.71
>120	9 104 043	453	5.0	18.17
Febuxostat, days				
≤30	54 187	9	16.6	60.70
>30 to ≤60	57 372	8	13.9	50.93
>60–90	45 616	2	4.4	16.01
>90–120	43 816	4	9.1	33.34
>120	489 770	39	8.0	29.08
Colchicine, days				
≤30	555 612	53	9.5	34.84
>30 to ≤60	340 207	25	7.3	26.84
>60–90	156 794	13	8.3	30.28
>90–120	134 690	10	7.4	27.12
>120	1 622 913	98	6.0	22.06
Allopurinol starting dose, mg/day				
<200	5 695 415	342	6.0	21.93
200–299	2 110 108	119	5.6	20.60
≥300	3 932 025	316	8.0	29.35
Febuxostat starting dose, mg/day				
40	565 682	53	9.4	34.22
80	1 225 079	9	0.7	2.68
Colchicine starting dose, mg/day				
<0.6	380 142	18	4.7	17.29
0.6 to <1	1 090 327	83	7.6	27.80
1 to <2	1 151 515	90	7.8	28.55
≥2	188 142	8	4.3	15.53
Allopurinol average dose, mg/day*				
<200	5 884 009	353	6.0	21.91
200–299	2 216 864	127	5.7	20.92
≥300	4 056 335	327	8.1	29.44
Febuxostat average dose, mg/day*				
40	593 913	57	9.6	35.05
80	129 898	10	7.7	28.12
Colchicine average dose, mg/day*				
<0.6	477 550	19	4.0	14.53
0.6 to <1	1 348 965	111	8.2	30.05
1 to <2	1 382 330	108	7.8	28.54
≥2	216 898	15	6.9	25.26

*Totals over all dose combinations exceeds the total for the drug, as people could contribute to multiple categories.
†Drug exposure up to 30 days after last day of medication fill/refill; baseline period was 365 days, that is, each new exposure was defined as no previous exposure in the baseline.

limited the analyses to people with gout (n=883; fewer cases), all findings were replicated except that allopurinol exposure was no longer significantly associated (table 3; model 2).

Propensity-matched comparative risk of HSRs with febuxostat compared with allopurinol

Most differences were eliminated/reduced after propensity matching, and the mean propensity scores in 5:1 propensity-matched allopurinol to febuxostat exposures were the same (online supplementary table S2). In a propensity-matched analysis, febuxostat did not significantly differ from allopurinol, the HR of HSRs was 1.25 (95% CI: 0.93 to 1.67) (table 4).

In separate exploratory analyses, compared with allopurinol daily dose <200 mg/day, febuxostat 40 mg/day was associated with a significantly higher hazard of HSRs with HR of 1.43 (95% CI: 1.03 to 1.98) (online supplementary table S3); other allopurinol or febuxostat doses were not significantly associated.

Table 3 Multivariable-adjusted association of allopurinol or febuxostat use and other risk factors with incident hypersensitivity reactions

	Multivariable-adjusted (model 1)*		Multivariable-adjusted (model 2)†	
	Hr (95% CI)	P value	Hr (95% CI)	P value
Age (years)				
65 to <75	Ref		Ref	
75 to <85	0.98 (0.86 to 1.13)	0.82	1.02 (0.89 to 1.18)	0.76
≥85	0.92 (0.76 to 1.10)	0.36	0.90 (0.73 to 1.10)	0.29
Sex				
Male	Ref		Ref	
Female	**1.28 (1.13 to 1.45)**	**0.0001**	**1.28 (1.12 to 1.46)**	**0.0004**
Race				
White	Ref		Ref	
Black	0.99 (0.82 to 1.19)	0.88	1.02 (0.84 to 1.25)	0.81
Other	1.05 (0.85 to 1.30)	0.66	1.07 (0.84 to 1.35)	0.59
Charlson-Romano score, per unit change	1.01 (0.99 to 1.03)	0.36	1.03 (1.00 to 1.05)	0.037
Statins	0.94 (0.73 to 1.20)	0.61	0.99 (0.75 to 1.31)	0.94
Beta-blockers	1.06 (0.84 to 1.34)	0.63	1.10 (0.84 to 1.44)	0.51
Diuretics	1.10 (0.88 to 1.38)	0.39	1.16 (0.90 to 1.51)	0.26
ACE inhibitors	0.91 (0.69 to 1.20)	0.52	0.95 (0.70 to 1.30)	0.75
Colchicine	Ref		Ref	
Allopurinol	**1.32 (1.10 to 1.60)**	**0.003**	1.12 (0.92 to 1.36)	0.28
Febuxostat	**1.54 (1.12 to 2.12)**	**0.008**	**1.44 (1.04 to 2.00)**	**0.031**
Allopurinol+colchicine	1.05 (0.80 to 1.39)	0.73	1.07 (0.81 to 1.43)	0.62
Allopurinol+febuxostat	0.91 (0.29 to 2.85)	0.87	1.06 (0.34 to 3.34)	0.92
Febuxostat+colchicine	**2.17 (1.18 to 3.99)**	**0.013**	**2.23 (1.21 to 4.11)**	**0.01**
Allopurinol+febuxostat+colchicine	2.33 (0.58 to 9.39)	0.23	2.70 (0.67 to 10.91)	0.16

Bold font represents statistically significant comparisons with a p-value <0.05.
*Model 1=colchicine/allopurinol/febuxostat exposure+age+race+gender+Charlson-Romano index score as continuous+beta-blockers+diuretics+ACE inhibitors+statins; #episodes in model=1038.
†Model 2=model 1 limited to patients with gout; #episodes in model=883.
Ref, referent category.

Allopurinol start dose and duration versus the risk of HSRs

In multivariable-adjusted analyses limited to allopurinol users, compared with allopurinol start dose of <200 mg/day, allopurinol start dose of ≥300 mg/day was associated with a significantly higher hazard of HSRs, 1.27 (95% CI: 1.12 to 1.44) (table 5). Compared with the use duration of 1–30 days, longer use durations were associated with non-significantly HRs (table 5). Of the six prespecified comorbidities, only diabetes was significantly associated with a higher risk (table 5).

DISCUSSION

In this study of older Americans, we examined HSRs in patients newly exposed to allopurinol or febuxostat compared with colchicine. Our study examined all HSRs, not just AHS. Compared with colchicine, both allopurinol and febuxostat use were associated with significantly higher hazards of HSRs. In propensity-matched analyses, febuxostat was not significantly different from allopurinol regarding the risk of new HSRs. Female sex, diabetes and a higher allopurinol start dose were also risk factors. Several findings merit further discussion.

Table 4 Propensity-matched* association of allopurinol or febuxostat (5:1 match) with the hazard of incident hypersensitivity reactions

	HR (95% CI)	P value
Allopurinol	Ref	
Febuxostat	1.25 (0.93 to 1.67)	0.14

*Analysis comparing the hazards of incident HSR in allopurinol and febuxostat exposed groups that were propensity-matched on key characteristics (see Appendix 1 for pre-matching and post-matching differences in key characteristics.
. Ref, referent category.

Our study used a representative US national sample. It is among the first studies to describe HSR incidence rates with a new prescription of allopurinol or febuxostat. Notably, febuxostat combination with colchicine and triple combination (allopurinol, febuxostat and colchicine) were associated with much higher HSR rates. HSR incidence decreased 2 months after allopurinol or febuxostat initiation. This might represent the time course of HSRs that occur more commonly earlier, or the development of immune tolerance in some other people with time; this requires further study.

In a Taiwanese population-based cohort, cutaneous adverse reaction incidence rates were higher in allopurinol versus febuxostat, 15.37 vs 3.48 per 1000 person-years.[9] Although our HSR rates in allopurinol exposed were similar to those in the Taiwanese study at 24/1000 person-years, rates in febuxostat-exposed patients were much higher at 31/1000 person-years in our study. Important differences in studies were the country setting (USA vs Taiwan), population (older adults vs all ages) and the definition of outcome (all HSRs vs cutaneous adverse reactions). We caution the reader that despite a large sample size for the study, estimates for combinations are less precise and can change significantly with a difference of a few cases, which can easily happen by chance.

In adjusted analyses, compared with colchicine use, new allopurinol (HR, 1.32) or febuxostat use (HR, 1.54) were each independently associated with a higher risk of HSRs. Findings add significantly to the limited literature for febuxostat associated reactions.[21 22] The similarity of the risk of HSRs between allopurinol and febuxostat is novel.

A retrospective case–control study of 54 AHS cases (defined as the presence of two of the major criteria -worsening renal function, acute hepatocellular injury or rash; or one of the

Table 5 Association of various risk factors with incident hypersensitivity reactions in patients who received allopurinol

	Multivariable adjusted (allopurinol dose)		Multivariable adjusted (duration of allopurinol use)	
	HR (95% CI)	P value	HR (95% CI)	P value
Age (years)				
65 to <75	Ref		Ref	
75 to <85	1.09 (0.97 to 1.23)	0.16	1.08 (0.95 to 1.21)	0.24
≥85	0.93 (0.78 to 1.12)	0.44	0.90 (0.76 to 1.08)	0.26
Sex				
Male	Ref		Ref	
Female	**1.32 (1.17 to 1.48)**	**<0.0001**	**1.29 (1.15 to 1.45)**	**<0.0001**
Race				
White	Ref		Ref	
Black	0.95 (0.80 to 1.14)	0.59	0.94 (0.78 to 1.12)	0.46
Other	0.95 (0.77 to 1.16)	0.60	0.93 (0.76 to 1.14)	0.47
Select Charlson-Romano score comorbidities				
Myocardial infarction	1.06 (0.74 to 1.52)	0.76	1.04 (0.72 to 1.50)	0.83
Diabetes	**1.21 (1.00 to 1.45)**	**0.047**	**1.20 (1.00 to 1.44)**	**0.05**
Malignancy	0.97 (0.83 to 1.14)	0.73	0.97 (0.83 to 1.14)	0.74
Congestive heart failure	1.21 (0.95 to 1.54)	0.12	1.21 (0.95 to 1.53)	0.12
Moderate or severe liver disease	Not estimable	N/A	Not estimable	N/A
Moderate or severe renal disease	0.90 (0.75 to 1.09)	0.27	0.87 (0.72 to 1.05)	0.14
Statins	1.03 (0.80 to 1.33)	0.81	1.03 (0.80 to 1.32)	0.84
Beta-blockers	1.09 (0.85 to 1.40)	0.50	1.09 (0.85 to 1.39)	0.52
Diuretics	1.05 (0.83 to 1.34)	0.68	1.05 (0.82 to 1.33)	0.70
ACE inhibitor	0.98 (0.73 to 1.31)	0.87	0.98 (0.73 to 1.31)	0.87
Allopurinol start dose, mg/day				
Allopurinol <200	**Ref**		N/A	
Allopurinol 200–299	1.00 (0.84 to 1.19)	1.00		
Allopurinol ≥300	**1.27 (1.12 to 1.44)**	**0.0002**		
Allopurinol use duration, days	N/A			
1–30			**Ref**	
31–60			0.86 (0.53 to 1.40)	0.55
61–90			1.00 (0.61 to 1.62)	0.98
91–120			0.86 (0.53 to 1.40)	0.55
>120			0.79 (0.55 to 1.12)	0.19

Multivariable-adjusted dose and duration models were examined separately using different reference categories, as applicable.
Bold font represents statistically significant comparisons with a p-value <0.05.
*Based on person day count.
N/A, not applicable; Ref, reference category.

major criteria and one of the minor criteria (fever, eosinophilia or leukocytosis)) found that allopurinol start dose was higher in cases versus controls, 184 s. 112 mg/day.[5] Our findings add to the growing knowledge in this area.

We found in the overall analyses that compared with a starting allopurinol dose <200 mg/day, a higher allopurinol start dose of ≥300 mg/day was associated with a significantly higher hazard of HSRs in multivariable-adjusted analyses, 1.27 (95% CI: 1.12 to 1.44), but non-significantly associated in propensity-matched analyses, 1.25 (95% CI: 0.99 to 1.58). This is an interesting finding. We believe that the similarity of HRs in the two analyses indicates that the possibility of harm with a higher allopurinol start dose, but this possibility ranged from no harm, lower 95% CI of 0.99, to significant harm, higher 95% CI of 1.58 in the propensity-matched analyses. This has important clinical implications. Cautious clinicians may start allopurinol at a low dose and gradually escalate the dose to achieve target serum urate to potentially decrease the risk of HSRs and allow patients to achieve target sUA, an important disease target.

Our findings regarding the risk factors for allopurinol HSRs differ somewhat from a recent Taiwanese study, which found that female sex, age ≥60 years, initial allopurinol dose >100 mg/day, renal and cardiovascular comorbidity were significant risk factors for allopurinol-associated HSRs.[4] Our study found that allopurinol dose >300 mg/day, female sex and diabetes, but not malignancy, heart failure, myocardial infarction or renal disease were significantly associated with a higher risk of allopurinol HSRs. The independent association of diabetes with a higher risk of HSRs might be due to subclinical renal disease, and needs further confirmation. Thus, both studies independently found that female gender and higher allopurinol start dose were significant predictors, increasing confidence in these results. Differences in study findings may be due to the differences in study population (US adults≥65 years vs Taiwanese general population), medications studied (allopurinol, febuxostat and colchicine vs allopurinol only), the unit of analysis (episodes vs people) and analyses (Cox vs logistic regression).

Our study has several limitations, which must be considered while interpreting study findings. Despite the use of a validated algorithm to define HSR,[16] we are aware that the use of diagnostic codes may be associated with some misclassification bias, as well as underdocumentation or overdocumentation of HSR. The validation of Wright *et al* algorithm was in inpatient or emergency room setting[16] and of Strom *et al* included

all cases[17]; our study that used all settings and might have had lower PPV of the algorithm than that studied by Wright *et al*.[16] Due to observational study design, residual confounding is possible. However, it is unlikely that a large prospective cohort consisting of 50–100 000 patients using these medications will be performed; therefore, a retrospective database study is one of the most practical ways to examine rare events such as the HSR. These findings are not generalisable to younger people, as our study sample consists of older adults, ≥65 years. Our results only apply to 30-day or longer use of these medications; future studies can examine associations for a shorter duration of exposure, more common with colchicine versus other medications. Study strengths include the use of a representative US population, the availability of clinical and pharmacy fill data, the use of robust observational study design and analytic methods, and confirmation of study findings using several sensitivity analyses.

CONCLUSIONS

We described the crude incidence rates of HSRs in patients with new exposure to allopurinol, febuxostat or colchicine. In this study of a representative US population, we found that compared with colchicine, both allopurinol and febuxostat use was associated with a higher risk of HSRs. In propensity-matched analyses, febuxostat did not differ significantly from allopurinol regarding the risk of HSRs. In allopurinol users, start dose >300 mg/day was associated with a higher risk of HSRs, compared with lower doses. In allopurinol users, diabetes, but not a renal failure, and female sex were independent risk factors for incident HSRs. Our findings provide new knowledge regarding HSRs to these commonly used medications for the treatment of gout that can inform clinicians and patients alike starting these medications for the treatment of gout and other disorders.

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TRANSLATIONAL SCIENCE

Rare genetic variants in interleukin-37 link this anti-inflammatory cytokine to the pathogenesis and treatment of gout

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ABSTRACT

Objective Gout is characterised by severe interleukin (IL)-1-mediated joint inflammation induced by monosodium urate crystals. Since IL-37 is a pivotal anti-inflammatory cytokine suppressing the activity of IL-1, we conducted genetic and functional studies aimed at elucidating the role of IL-37 in the pathogenesis and treatment of gout.

Methods Variant identification was performed by DNA sequencing of all coding bases of *IL37* using molecular inversion probe-based resequencing (discovery cohort: gout n=675, controls n=520) and TaqMan genotyping (validation cohort: gout n=2202, controls n=2295). Predictive modelling of the effects of rare variants on protein structure was followed by in vitro experiments evaluating the impact on protein function. Treatment with recombinant IL-37 was evaluated in vitro and in vivo in a mouse model of gout.

Results We identified four rare variants in *IL37* in six of the discovery gout patients; p.(A144P), p.(G174Dfs*16), p.(C181*) and p.(N182S), whereas none emerged in healthy controls (Fisher's exact p-value=0.043). All variants clustered in the functional domain of IL-37 in exon 5 (p-value=5.71×10^{-5}). Predictive modelling and functional studies confirmed loss of anti-inflammatory functions and we substantiated the therapeutic potential of recombinant IL-37 in the treatment of gouty inflammation. Furthermore, the carrier status of p.(N182S)(rs752113534) was associated with increased risk (OR=1.81, p-value=0.031) of developing gout in hyperuricaemic individuals of Polynesian ancestry.

Conclusion Here, we provide genetic as well as mechanistic evidence for the role of IL-37 in the pathogenesis of gout, and highlight the therapeutic potential of recombinant IL-37 for the treatment of gouty arthritis.

Key messages

What is already known about this subject?

► Gout is characterised by severe interleukin (IL)–1-mediated joint inflammation induced by monosodium urate (MSU) crystals. An abundance of evidence has linked hyperuricaemia to gout; however, the genetic and mechanistic basis of progression from hyperuricaemia to inflammation and clinical gout remain undetermined. Defective regulation of IL-1-mediated inflammation following MSU crystal uptake by inflammatory cells is seemingly implicated in the pathogenesis of gout.

What does this study add?

► This study provides genetic, mechanistic and translational evidence that the anti-inflammatory cytokine IL-37 is implicated in the pathogenesis of gout. By sequencing all coding bases of *IL37*, we identified rare variants in gout patients, which result in loss of anti-inflammatory effects. In addition, we demonstrated that administration of functional, recombinant IL-37 is effective in dampening inflammation induced by MSU crystals.

How might this impact on clinical practice or future developments?

► By integrating ultrasensitive hypothesis-driven genetics with computational and functional data, this study illustrates the feasibility of personalised translational medicine. IL-37 treatment is being evaluated across a broad range of inflammatory diseases, and has the potential to translate into an effective therapeutic option. In the future, the approach described in this study may be used to unveil personalised treatment options across multiple inflammatory conditions.

INTRODUCTION

Gout is a disease characterised by recurrent episodes of acute joint inflammation induced by monosodium urate (MSU) crystals.[1] The prevalence of gout in Western countries is around 4% and rising.[2 3] In some individuals with hyperuricaemia,

MSU crystals deposit within joints and periarticular structures, triggering flares clinically characterised by severe joint pain and swelling.[1]

Inflammation in gout is centrally mediated by the proinflammatory cytokine interleukin (IL)−1β. Uptake of MSU crystals by macrophages activates the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome, which generates bioactive IL-1β for extracellular release.[4] IL-1β induces myriad secondary inflammatory mediators including prostaglandins, cytokines and chemokines. The subsequent recruitment of neutrophils, producing reactive oxygen species (ROS) and proteases, results in fulminant joint inflammation.[4 5]

Next to IL-1β, other IL-1 family cytokines contribute to the orchestration of innate inflammatory responses. IL-37 is a recently characterised IL-1 family member, which exerts potent anti-inflammatory effects by suppressing production of IL-1β and other proinflammatory cytokines.[6] Five transcripts for human *IL37* have been identified (*IL37a-e*), of which *IL37b* with the N-terminus at valine 46 is the most abundant and best characterised.[7] IL-37 acts through both intracellular and extracellular mechanisms: intracellular IL-37 translocates to the nucleus and suppresses transcription of proinflammatory genes;[8] extracellular IL-37 transduces anti-inflammatory signals via the IL-18Rα/IL-1R8 receptor complex.[9] The anti-inflammatory properties of IL-37 have emerged in various disease models, for example, LPS-induced shock, colitis, metabolic syndrome and inflammation-induced fatigue.[6 10–12] Notably, treatment with recombinant IL-37 proved effective in experimental arthritis,[13] and reduced MSU crystal-induced inflammation in vitro and in mouse peritonitis and foot pad models.[14 15]

Previous genome-wide association studies (GWAS) on gout revealed associations with serum urate-associated loci,[16 17] but the genetic basis of progression from hyperuricaemia to clinical gout remains undetermined.[18] Given the central role of IL-1β in gout flares, candidate gene studies identified associations with genes encoding TLRs, the NLRP3 inflammasome and IL-1β.[19–21] Notwithstanding, the role of other IL-1 family members in gout remains to be elucidated.

Supplementary to common polymorphisms, research from the past few decades has shown that rare and low-frequency variants are important contributors to genetic susceptibility in common diseases.[22] In some autoinflammatory diseases, the disease mechanism even appeared to be monogenic due to rare causal variants detected in a single gene.[23] For serum urate-associated loci and gout, it has also been shown that rare variants contribute to disease pathogenesis.[24 25] However, statistical confirmation of rare variants requires high-quality in-depth sequencing and large sample sizes, which is not the case for most GWAS performed to date in gout patients.[26–28] With recent advancements in sequencing technologies, the study of rare-frequency and low-frequency variants has remarkably improved. Molecular inversion probes (MIPs) are a cost-effective, targeted resequencing technology, which allows investigating specific regions of interest in the genome. With an appropriate functional hypothesis, this technique is ideally suited for the identification of common, low-frequency, rare and even private genetic variation in candidate genes.[29–31]

The anti-inflammatory IL-1 family member IL-37 is a highly promising candidate among possible regulatory mechanisms preventing gout flares. In this study, we investigate whether genetic variation in the *IL37* gene confers predisposition to gout, and evaluate the functional role of IL-37 in controlling gout flares. Specifically, we identify rare variants in *IL37* in gout patients, determine the effects on protein function and evaluate administration of recombinant IL-37 as a novel therapeutic strategy for gout flares.

METHODS

Study subjects

The discovery cohorts consisted of 675 patients with crystal-proven gout of self-reported European ancestry, and 520 healthy adults from the Human Functional Genomics project which were included in the analysis. The validation cohort comprised 2202 gout patients from New Zealand, Australia and Europe that met the 1977 American Rheumatism Association criteria for diagnosis,[32] and 2295 controls with no history of gout. For in vitro validation experiments, blood was drawn from healthy volunteers. For detailed information and ethical approvals, see online supplementary information (p1,2), figure S1 and table S1.

Aside from clinical sample contribution, patients were not involved in this study, which revolves around basic/translational science questions. However, patient associations will be instrumental to dissemination of research findings.

Sequencing and genotyping of *IL37*

We used MIPs for the targeted resequencing of human *IL37* in the discovery cohorts. Briefly, 15 MIPs were designed to cover the coding exon regions of *IL37*. Raw sequencing data were produced by the Illumina NextSeq500 system, aligned to the reference genome and after appropriate processing and filtering common and rare variant genotypes were determined. The average coverage depth for all individuals over all *IL37* MIPs was 1012x (online supplementary figure S2). Genotyping of p.(N182S) (rs752113534) in *IL37* was performed by TaqMan SNP Genotyping assay in validation cohorts, according to manufacturer instructions. For details, see online supplementary information (p2,3).

Predictive modelling

Tolerance to genetic variation of *IL37* based on homology calculations was studied using MetaDome.[33] Effects of *IL37* variants on protein structure were predicted using Yasara View,[34] by introducing the amino acid changes resulting from detected rare variants. For details, see online supplementary information (p4).

Experiments and measurements

Plasma levels of IL-1β were measured with Ella (ProteinSimple). For in vitro experiments, peripheral blood mononuclear cells (PBMCs) and polymorphonuclear cells (PMNs) were isolated by Ficoll gradient centrifugation. Cells were stimulated with (opsonised) MSU crystals alone for PMNs or in combination with palmitic acid (C16.0) (MSU/C16.0) for PBMCs to mimic induced arthritis,[35] and treated with the naturally occurring form, IL-37b (46-218) or a shortened form based on the p.(C181*) variant, IL-37b (46-180). Production of IL-1β, IL-8 and ROS was determined in supernatants with ELISA and chemiluminescence, respectively. Numbers of participants for each experiment are described in online supplementary figure S1, the 'Results' and each corresponding figure legend. For specific details on reagents, concentrations, timing and determinations, see online supplementary information (p4,5).

Animal studies

Male C57BL/6 mice (10–12 weeks) received treatment with recombinant human IL-37 (n=5) or vehicle (n=5), followed by induction of gouty arthritis through direct intra-articular injection of C16.0

and MSU crystals in both knee joints. Joint swelling and histology were evaluated, and synovial IL-1β and IL-6 were measured by ELISA. For details on procedures and IACUC approval, see online supplementary information (p5,6).

Statistical analyses

Detailed information on statistical analyses can be found in the online supplementary information (p6,7), as well as in each figure legend.

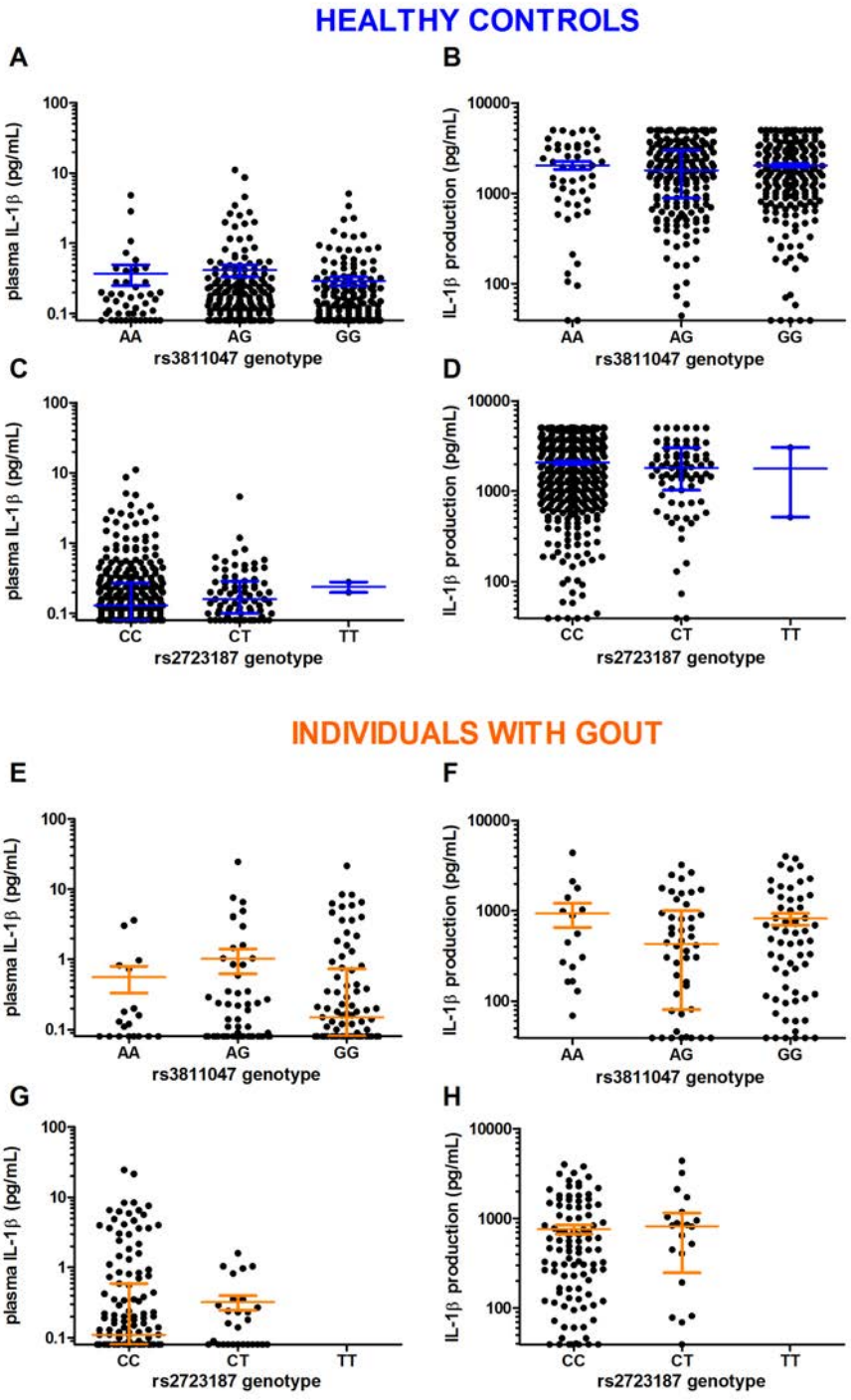

Figure 1 Common variants in *IL37* are not associated with plasma IL-1β levels or IL-1β production by PBMCs stimulated with C16.0 and MSU in a cohort of healthy controls and a cohort of individuals with gout. MIP-based resequencing of *IL37* identified seven common variants in 520 controls and 675 individuals with crystal-proven gout. IL-1β levels (A, C, E, G) were determined in plasma from a subset of the controls (n=437) and individuals with gout (n=194). PBMCs from a subset of the controls (n=472) and gout patients (n=171) were isolated, stimulated with a combination of C16.0 (50µM) and MSU crystals (300 µg/mL) for 24 hours, and IL-1β production was measured in the supernatant by ELISA. The genotypes of the two common haplogroups tagged by rs3811047 (A, B, E, F) and rs2723187 (C, D, G, H) in the *IL37* gene were tested versus plasma IL-1β levels and IL-1β production by PBMCs after stimulation (B, D, F, H). Two-sided Kruskal-Wallis tests demonstrated no significant differences. Blue and orange lines represent medians plus IQR. IL, interleukin; MIP, molecular inversion probe; MSU, monosodium urate; PBMCs, peripheral blood mononuclear cells.

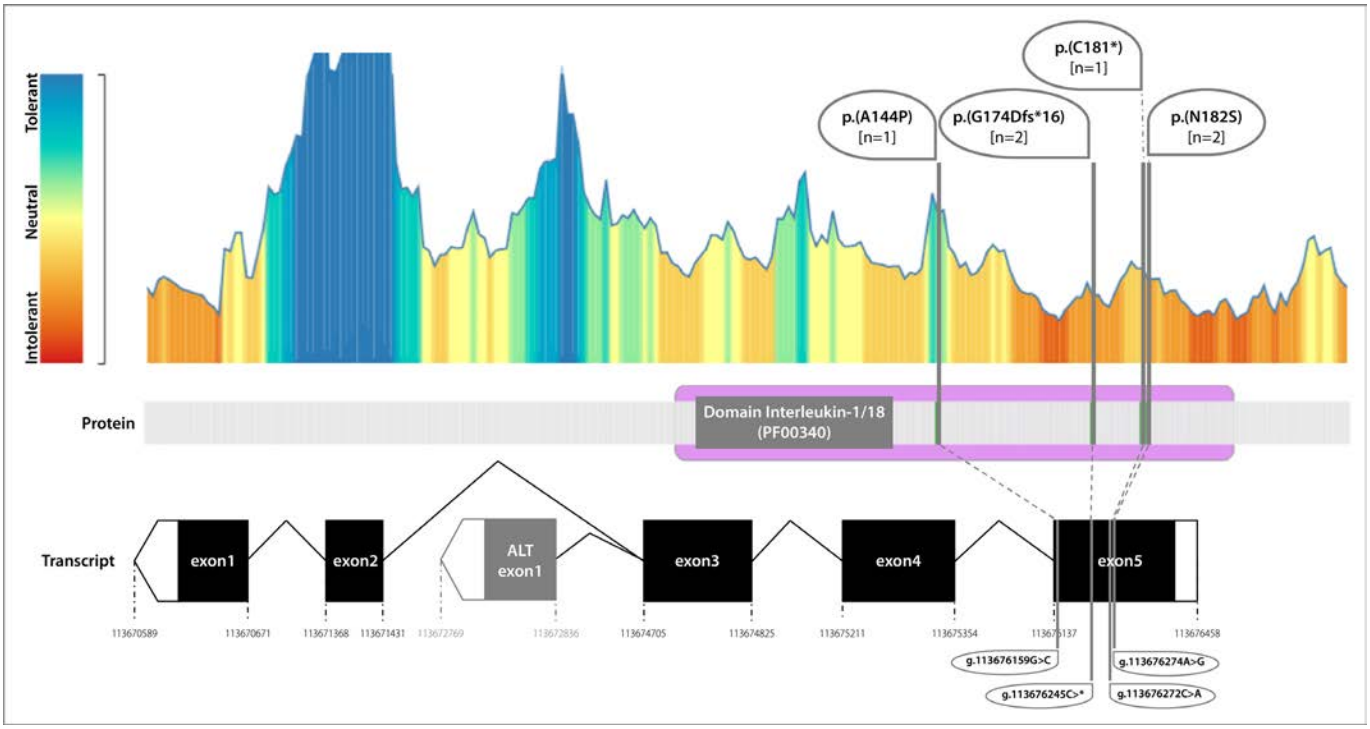

Figure 2 Rare variants in the *IL37* gene identified in patients with gout. The detected rare variants are plotted on a landscape that represents the mutational tolerance in the human *IL37* transcript ENST00000263326.3 (GRCh37/hg19) using MetaDome.[33] These four variants are located in exon 5 (transcript below tolerance plot), which partly encodes the functional domain (PF00340). This domain has 10 homologous occurrences throughout the genome and all these domains collectively are characterised by lower tolerance to mutations.

RESULTS

Common variants in *IL37* are not associated with gout, circulating IL-1β and IL-1β production

We first investigated potential associations between common *IL37* gene variants and gout, by comparing the allele frequencies (AFs) of representative *IL37* haplogroups, rs3811047 and rs2723187,[36] as identified by the MIP-sequencing in 675 gout patients and 520 controls (discovery cohort). We did not detect significant differences between gout patients and controls (online supplementary table S2). We then evaluated potential correlations of these haplogroups with circulating IL-1β levels or IL-1β production by PBMCs stimulated with MSU/C16.0. Again, there were no significant associations between *IL37* haplogroups and IL-1β levels, either in gout patients or controls (figure 1).

Four distinct rare variants in *IL37* in individuals with gout

Using MIPs in the discovery cohorts, we identified four distinct, heterozygous, non-synonymous rare (AF <0.1 in general population) variants in six individuals with gout (figure 2). We did not detect any *IL37* rare variants in the control cohort (Fisher's exact p-value=0.043). Interestingly, all variants were located close to each other in exon 5, which contains the functional domain of the IL-37 protein. This clustering of variants is not observed in large population control databases and is therefore unlikely to be a chance finding (p-value=5.71×10^{-5}) (for details, see online supplementary information p6,7).

The variant p.(G174Dfs*16), detected in two gout patients, was reported with AF=4.49×10^{-4} in the Genome Aggregation Database European Non-Finnish (gnomAD-ENF) reference population.[37] The variant p.(N182S), detected in two different patients, was only detected in a very small number of East Asian individuals (AF=2.2×10^{-4}) and has AF=0 in gnomAD-ENF. The variant p.(A144P), detected in one patient, was only reported in filtered gnomAD whole-genome sequencing data (not in exomes), with AF=6.48×10^{-5} in gnomAD-ENF. The remaining variant, p.(C181*), likewise detected in one patient, has not been reported in any database to date.

A flowchart with information on the discovery and validation of rare variants can be found in online supplementary figure S1.

IL37 rare variants result in aberrant protein structure and function

To determine the effects of *IL37* rare variants on protein function, we first inspected the genomic positions and their tolerance to variation using MetaDome.[33] As shown in the tolerance landscape of *IL37* (figure 2), all four rare variants are located in the functional PF00340 domain. Variant p.(A144P) is located in a tolerant region to genetic variation, whereas variants p.(G174Dfs*16), p.(N182S) and p.(C181*) are located in regions with less to no tolerance to genetic variation.

We then investigated the functional consequences of these rare variants on IL-37 protein structure, using the predictive modelling tool Yasara View.[34] As shown in figure 3, our identified rare variants are predicted to result in different protein structures. Specifically, variant p.(N182S) has a minor effect on the amino acid residue; variant p.(A144P) results in a residue change affecting the α-helix of the protein and variants p.(G174Dfs*16) and p.(C181*) are deleterious and result in truncated IL-37 protein. In detail, variant p.(C181*) introduces a premature stop-codon at p.181, whereas p.(G174Dfs*16) is a frameshift variation altering the amino acid sequence from p.174 and resulting in a premature stop-codon at p.188.

Variant p.(N182S) contributes to genetic susceptibility to gout in New Zealand Māori and Pacific Island populations

In the validation cohort, the rare variant p.(N182S) was absent in gout patients of European ancestry (n=1011), but exhibited

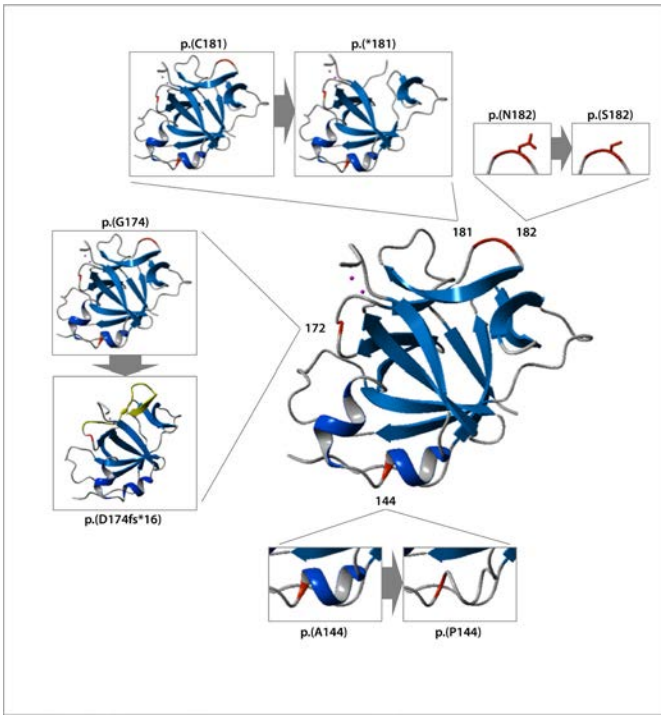

Figure 3 Protein prediction of rare variants in *IL37* identified in patients with gout. The 3D protein structure was modelled in Yasara View.[34] The figure shows the wild-type IL-37b protein in the centre, with four identified rare variants highlighted in red and zoomed in on their alterations on protein structure. The variant in p.H172 causes a frameshift which results in an altered amino acid sequence from p.174 on (highlighted in yellow), which results in a premature stop-codon.

a frequency of 0.054 in gout patients of the total Polynesian ancestry group (n=1191) (table 1). Comparing gout cases to all controls, the p.(N182S) minor allele (G) did not exhibit significant association in either Polynesian group; East Polynesian (EP) (n=739 cases; n=850 controls; OR=1.26; p-value=0.250) and West Polynesian (WP) (n=452 cases; n=412 controls; OR=1.07; p-value=0.850) (data not shown).

However, comparing gout cases to hyperuricaemic controls (serum urate levels ≥0.41 mmol/L), we detected evidence of association in a meta-analysis of the EP and WP sample sets (n=1191 cases; n=310 controls; OR=1.81; 95% CI 1.05 to 3.12; p-value=0.031) (see online supplemental information p7 and figure S3).

Variant p.(C181*) results in a loss of anti-inflammatory properties in vitro and increased cytokine production ex vivo

We next investigated the functional impact of rare genetic variants on the anti-inflammatory properties of IL-37. PMNs from healthy volunteers were stimulated with opsonised MSU crystals, and ROS and IL-8 production was evaluated in the presence or absence of different IL-37 protein forms (n=12 and n=19, respectively). The recombinant form of the naturally occurring IL-37b (46-218) significantly reduced ROS and IL-8 production (figure 4A,B). Conversely, a recombinant form of IL-37b (46-180) based on the stop-variant p.(C181*) was significantly less effective in reducing ROS and IL-8 production (figure 4A,B). Moreover, in vivo p.(C181*) was demonstrated to be dysfunctional as PBMCs from the gout patient carrying this variant exhibited increased IL-1β production on stimulation with MSU/C16.0 compared with 11 matched, non-carrier gout patients (figure 4C).

Treatment with recombinant IL-37 suppresses MSU crystal-induced inflammation

To evaluate the therapeutic potential of IL-37 in gout, mice were subjected to a model of MSU-induced arthritis. Treatment with recombinant IL-37b (46-218) significantly decreased joint swelling (figure 5A) (n=10), a finding paralleled by reduced synovial concentrations of proinflammatory cytokines IL-1β and IL-6 (figure 5B,C) (n=5). Histology studies confirmed marked reductions in synovial inflammation and cell influx (figure 5D,E) (n=5). In PMNs isolated from the gout patient carrying p.(N182S), opsonised MSU crystal-induced ROS and IL-8 production were, regardless of the presence of p.(N182S), accordingly reduced on treatment with recombinant IL-37b (46-218) (figure 5F,G).

Clinical characteristics of individuals carrying rare variants in *IL37*

In the discovery cohort, gout patients carrying rare *IL37* variants either had a young age of disease onset, or exhibited multiple inflammatory comorbidities (ie, metabolic syndrome, myocardial infarction, kidney failure and vasculitis). Polynesian patients with p.(N182S) developed gout at an earlier age compared with non-carriers (in EP (carriers n=76; non-carriers n=640; p-value=0.014; Beta=−4.309) and in WP (carriers n=47; non-carriers n=394; p-value=0.058; Beta=−3.655)). All clinical data regarding age at gout onset and comorbidities are summarised in online supplementary tables S3 and S4.

DISCUSSION

This study shows that rare genetic variants in *IL37* confer predisposition to gout, provides clinical and translational evidence supporting the role of IL-37 in the pathogenesis of gout and points at IL-37 as a possible therapeutic strategy for MSU-driven inflammation.

Previous studies have shown that common genetic variation in *IL37* has clinically relevant effects in inflammatory diseases. For example, rs3811047 is associated with the development of coronary artery disease and susceptibility to *Mycobacterium tuberculosis* infection.[38 39] Additionally, rs2723187 has been

Table 1 Frequency of p.(N182S) variant in *IL37* in gout cases and hyperuricaemic controls of the validation cohort

	Gout cases				HU controls without gout			
p.(N182S)	AA N (Freq)	AG N (Freq)	GG N (Freq)	G-allele freq	AA N (Freq)	AG N (Freq)	GG N (Freq)	G-allele freq
European	1011 (1.000)	0 (0.000)	0 (0.000)	0.000	114 (1.000)	0 (0.000)	0 (0.000)	0.000
EP	662 (0.895)	73 (0.098)	4 (0.005)	0.054	163 (0.915)	15 (0.084)	0 (0.000)	0.042
WP	404 (0.893)	47 (0.103)	1 (0.002)	0.054	122 (0.924)	10 (0.075)	0 (0.000)	0.037

Allele frequencies are shown for gout cases and hyperuricaemic (HU) controls (serum urate ≥0.41 mmol/L) in our validation cohort, comprising European population (n=1011 cases; n=114 controls), East-Polynesian (EP)-(n=739 cases; n=178 controls) and West-Polynesian (WP)(n=452 cases; n=132 controls) populations.

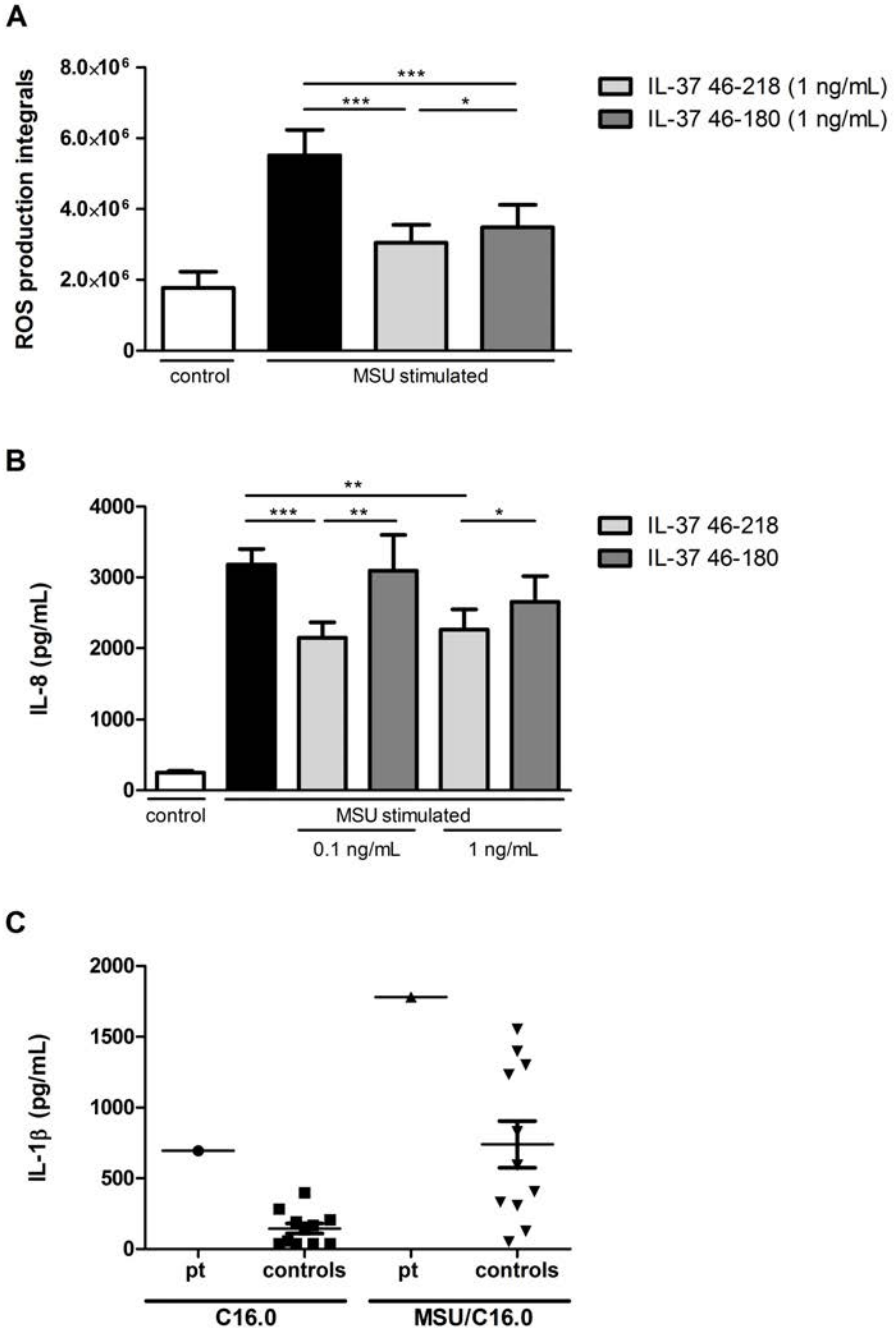

Figure 4 p.(C181*) results in a loss of protein function in vitro and in vivo. PMNs of healthy volunteers were isolated and pretreated with RPMI (black bar), 1 ng/mL of recombinant full-length IL-37 (amino acids 46–218) or 1 ng/mL of recombinant IL-37 based on the p.(C181*) variant (amino acids 46–180) for 1 hour. PMNs were subsequently stimulated with RPMI (control) or opsonised MSU crystals (5 mg/mL). ROS production was measured over the following 1 hour (A); IL-8 production was measured with ELISA in supernatant after overnight incubation (B). Two-sided Wilcoxon matched test; n=12 for experiment A and n=19 for experiment B; bars represent mean±SEM. PBMCs were isolated from individuals with crystal-proven gout and stimulated with C16.0 (50 µM) or a combination of C16.0 and MSU crystals (300 µg/mL) for 24 hours. IL-1β levels were measured in supernatant by ELISA. IL-1β levels after stimulation were compared between a patient with gout harbouring the p.(C181*) variant in *IL37* (male, >1 year gout, 74 years old, SUL 0.24 mmol/mL) and 11 matched gout patients without the mutation from the discovery cohort (males, >1 year gout, 73 years old (mean) and SUL 0.24 mmol/mL (mean)) (C). IL, interleukin; MSU, monosodium urate; PBMCs, peripheral blood mononuclear cells; PMNs, polymorphonuclear cells; ROS, reactive oxygen species; SUL, serum urate levels.

associated with rapid degradation and significantly lower levels of IL-37 protein, with consequently reduced anti-inflammatory properties.[40] For gout specifically, no associations with common variants in *IL37* have been described to date. In this study, we also did not find a significant association between *IL37* common variants and gout, nor with IL-1β levels (figure 1).

The 'Common Disease, Rare Variant' hypothesis argues that genetic susceptibility to common diseases does not reside in

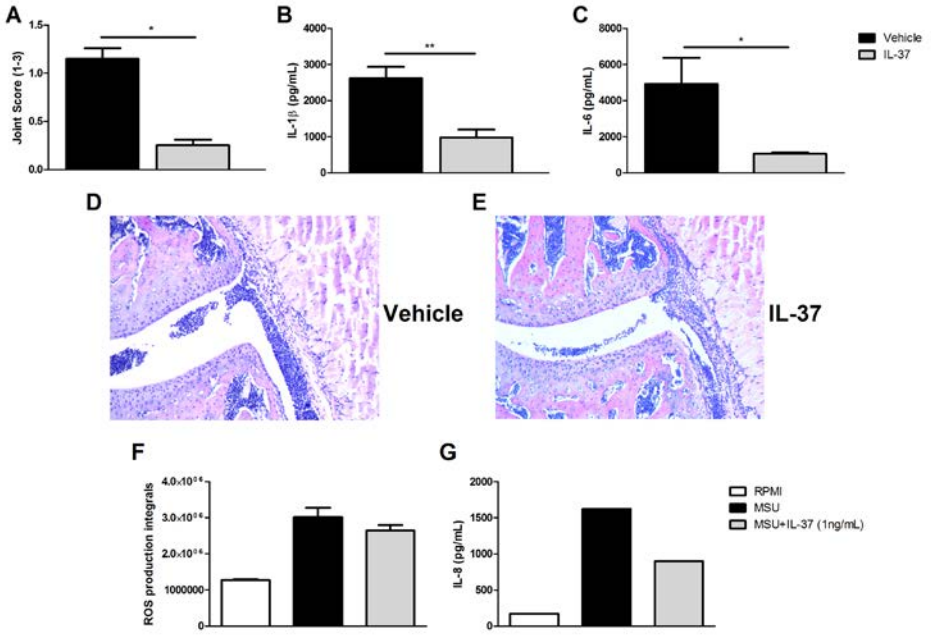

Figure 5 Recombinant IL-37 inhibits MSU crystal-induced joint inflammation in wild type mice and ex vivo in a patient with p.(N182S). Male C57Bl/6 wild-type mice were treated with intraperitoneal injections of vehicle or recombinant human IL-37 (1 µg/mouse) 2 hours, 24 hours and 48 hours prior to induction of a gout flare by intra-articular injection of C16.0 (200 µM) mixed with 300 µg/L MSU crystals in 10 µL PBS into both knee joints. Macroscopic joint inflammation was scored after 4 hours on a scale ranging from 0 to 3 in all 10 joints. Scores for each mouse were combined and compared between groups (A). Patellae of right knee joints were isolated and cultured for 1 hour. Levels of IL-1β (B) and IL-6 (C) were measured by ELISA (A, B and C: n=5 per group, two-sided Mann-Whitney tests). Left knee joints were removed, fixed and H&E stained. Two representative pictures are shown for induced arthritis in vehicle treated wild-type mice (D) and IL-37 pretreated wild-type mice (E). PMNs were isolated from the patient with p.(N182S) from the discovery cohort, preincubated with 1 ng/mL of recombinant IL-37 for 1 hour and stimulated with opsonised MSU crystals for 24 hours. ROS production was measured in quadruplicate during the subsequent hour (F) and IL-8 production in supernatant was measured after overnight incubation (G). A–C, F: bars represent mean±SEM; G: bars represent a single value. IL, interleukin; MSU, monosodium urate; PMNs, polymorphonuclear cells; ROS, reactive oxygen species.

common genetic variants, but rather in a multiplicity of individually rare genetic variations, each with relatively high penetrance.[22] MIP-based resequencing is a novel, sensitive and affordable method for targeted sequencing, which enables the identification of all coding variants in candidate genes,[23 31 41 42] and as such allows for the identification of rare variants in an unbiased way. Using this technology, we identified four distinct heterozygous rare variants in *IL37* in six gout patients, all clustering in exon 5 encoding for the functional domain of IL-37.[9]

To predict the functional consequences of our identified rare variants, we first used MetaDome, which revealed various regions with constraint against variation. Characteristically, these regions were located at the start of the protein and in the functional domain, where our identified rare variants also clustered, which is in accordance with the critical importance of this region for protein function.[9] Subsequent in silico modelling confirmed that these variants impact protein structure and function. Particularly, the two nonsense mutations, which introduce premature stop-codons in the last exon of *IL37* (and therefore do not undergo nonsense-mediated decay[43]), result in truncated proteins. Above all else, since tertiary protein structures are built on interactions between amino acid residues, which are affected by our rare variants, it is likely that the resulting IL-37 structures do not remain intact and collapse, with major consequences on protein function.[44] Consistent with this, recombinant IL-37b (46-180) based on p.(C181*), did not exert anti-inflammatory effects in an in vitro model of gouty arthritis. Moreover, the patient carrying p.(C181*) exhibited higher IL-1β production on stimulation compared with matched non-carrier gout patients (figure 4C), demonstrating a loss of anti-inflammatory function due to protein structure change as a result of the terminal loss of 38 amino acids.

Our in vitro and in vivo studies further confirmed a relevant role for IL-37 in the development of gouty inflammation. Treatment with a recombinant form of the naturally occurring IL-37b (46-218) reduced IL-8 and ROS production in human PMNs stimulated with MSU, both in PMNs from healthy volunteers as in PMNs from the patient carrying p.(N182S). Furthermore, treatment with recombinant IL-37 markedly reduced joint inflammation in vivo in a model of MSU crystal-induced arthritis.

The variant p.(N182S) had a relatively high prevalence of 0.054 in the New Zealand EP and WP population from our validation cohort. Accordingly, genome-wide principal component analysis showed that European patients carrying this variant clustered with individuals of Polynesian ancestry (online supplementary figure S4). In hyperuricaemic individuals from the Polynesian cohort, the carrier status of p.(N182S) conferred an increased risk of developing gout. The p.(N182S) AF is also in line with the high prevalence of gout in the New Zealand Māori and Pacific population, with 8%–13% of adults affected.[45]

We also show that rare variants in *IL37* are linked with either increased disease severity or an earlier onset of gout. In our discovery cohort, the mean age of gout onset was 29 for patients carrying a rare *IL37* variant, compared with 51 for non-carrier patients.[46] Likewise, EP patients carrying p.(N182S) developed

gout at a significantly earlier age compared with non-carrier patients. Notably, some individuals carrying a rare variant in *IL37* exhibited a strikingly severe clinical phenotype or multiple inflammatory comorbidities. Their clinical phenotype was remarkably severe, even considering the already high incidence of cardiovascular, metabolic and renal comorbidities in gout patients.[47–49] However, due to the limited number of individuals carrying rare variants, larger follow-up studies are needed for statistical confirmation.

This study has some limitations, as functional validation of some identified variants was limited by either the intrinsic rarity of the variant, or the unavailability of biological samples from carrier individuals for in vitro/vivo assays. Furthermore, we did not generate a recombinant IL-37 protein for each identified rare variant. Instead, we focused on p.(C181*), which was predicted to detrimentally impact protein structure and function based on available information and in silico modelling. Nevertheless, collective evidence generated in this study indicates that different *IL37* rare variants can predispose to the development of gouty inflammation.

The correlations between circulating levels of IL-37, CRP and uric acid shown by recent studies[50] already suggested a role for IL-37 in gout. Our in-depth approach provides genetic as well as mechanistic evidence to the role of IL-37 in the pathogenesis of gout. Furthermore, we provide clinical evidence that a carrier status for *IL37* rare variants may act as strong disease-predisposing factor. Previous studies also showed that recombinant IL-37 inhibits MSU crystal-induced inflammation in vitro and in mouse peritonitis and footpad models.[14 15] Our studies confirm and expand these findings, and point at IL-37 as a potential therapeutic agent for gout. A fusion protein with IL-37 and the Fc-domain of human IgG1 might result in increased plasma half-life and be ideally suited for therapeutic administration.[51]

In conclusion, this study provides genetic, mechanistic, clinical and translational evidence that the anti-inflammatory cytokine IL-37 is a relevant mediator in the pathogenesis of gout, and that exogenous administration of this molecule has therapeutic potential for the treatment of this condition.

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Patient consent for publication Not required.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. Data sets from the cohort of healthy volunteers are available on https://hfgp.bbmri.nl/. Data sets from gout cohorts and code are available upon reasonable request.

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Not only synovitis but also tenosynovitis needs to be considered: why it is time to update textbook images of rheumatoid arthritis

Rheumatoid arthritis (RA) is typically represented as synovitis and bone erosions of small joints. This classic picture resulted from comparing patients with RA with other rheumatic joint diseases for clinical and radiographic characteristics. Although different classification criteria for RA have been developed over time, this classic picture has not changed since the mid-20th century. During the last decennium, advanced imaging modalities, such as MRI and musculoskeletal ultrasound (US), have refined our understanding of tissues involved in RA. We will argue that tenosynovitis at the level of the hand and feet joints is a feature that deserves to be added as the third classic trait of RA.

A feature can be considered as a disease trait when it occurs frequently and is specific, and when a new trait is considered its connection with the disease is not a substitute of an already acknowledged classic feature. We will study the occurrence of tenosynovitis in RA in the light of these principles.

Many, but not all, tendons at the hand and feet joints are surrounded by a sheath.[1 2] Tendon sheaths have a cell composition similar to the synovial lining of joints.[3]

Fiona McQueen was the first to describe tenosynovitis in early RA using MRI.[4] The reported prevalence of tenosynovitis depends on the number of tendon sheaths studied (wrist, metacarpophalangeal (MCP) and/or metatarsophalangeal (MTP) joints, unilateral or bilateral). A prevalence of ~50% is described,[5 6] but most were higher (~80%).[7–11] MRI studies in consecutive early RA showed a sensitivity of tenosynovitis of 75%–87%.[7–9] Figure 1A–C presents imaging examples (MRI, US) in early RA. Imaging studies in persons from the general population repetitively showed a prevalence of tenosynovitis at small joints ranging from 0% to 3%,[12–14] corresponding with a specificity of 97%–100%. The specificity in patients with other arthritides as reference is also high. A study at the tendon level of the wrist and MCP joints, comparing consecutive patients with RA and other early arthritis (including psoriatic arthritis), reported a specificity ranging from 82% to 99%.[8] Thus, tenosynovitis at the level of small joints (MCPs, wrist, MTPs) has high sensitivity and specificity for RA.

Studies in an experimental mouse model showed that tenosynovitis was the first sign of inflammation.[15] Infiltration of the tendon sheaths by granulocytes and macrophages was the first pathological event in the preclinical phase; only few T cells were present and B cells were initially absent (figure 1D). Hyperplasia of the joint synovial lining was observed at the onset of clinical arthritis but not in the preclinical disease.[15] The question if tenosynovitis is also the initiating feature of arthritis in humans with RA is still unsolved. However, a serial MRI study in pre-RA revealed that tenosynovitis and synovitis occurred very early, before the development of clinical arthritis and erosions.[16] The notion that tenosynovitis is a very early feature of RA is further supported by the consistent finding that tenosynovitis is an independent predictor for developing RA in patients with clinically suspect arthralgia and undifferentiated arthritis, whereas synovitis is not constantly predictive in multivariate analysis (online supplementary table).

Finally we explored whether tenosynovitis contributes to symptoms and signs that are characteristic of RA. A summary of currently available data reveals that tenosynovitis is related to the presence of joint swelling, joint tenderness, morning stiffness and functional impairments in RA and in earlier disease phases (online supplementary table). Associations were independent of possible concomitant imaging-detected synovitis.

To summarise, tenosynovitis at the level of small joints has high sensitivity and specificity for early RA. Tenosynovitis occurs early during RA development. It underlies symptoms and signs that are characteristic of RA, both in preclinical stages and in clinical RA. Based on this we propose that, in addition to synovitis and structural damage, future textbook images from now on also depict tenosynovitis as a classic trait of RA, as portrayed in figure 1E. In addition, if classification criteria for the earliest phases of RA were to be derived or modified, tenosynovitis could be included.

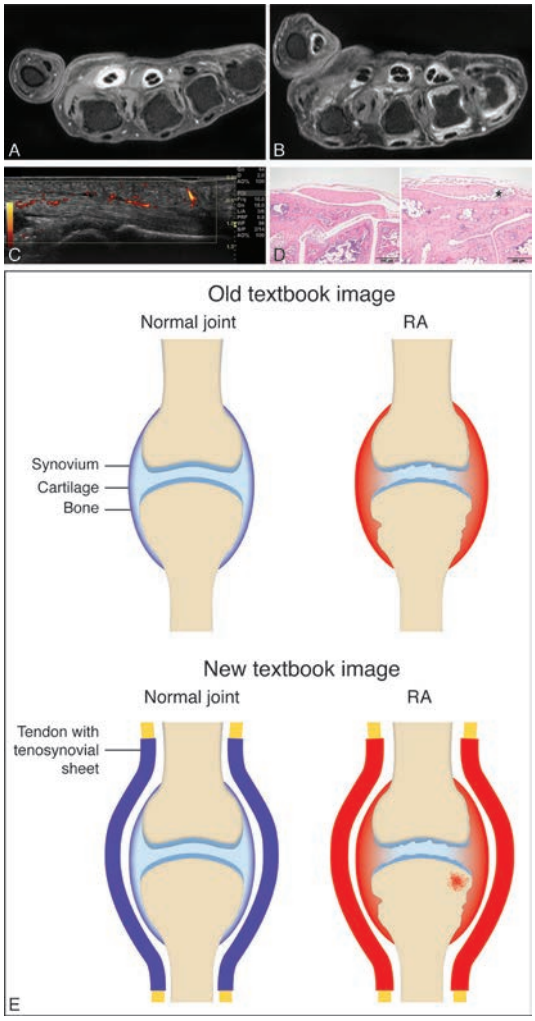

Figure 1 Tenosynovitis as an early trait in RA (A–C) and experimental arthritis (D), and the proposed new textbook image of RA including tenosynovitis (E). (A–B) MRI (axial plane after contrast enhancement, 1.5T MRI) of two patients with early RA with flexor tenosynovitis at MCP 2 and 3 (A) and flexor tenosynovitis at MCP 1, 3 and 4, and synovitis at MCP 4 and 5 (B). (C) Ultrasound (longitudinal plane) in a patient with early RA showing flexor tenosynovitis at MCP 2. (D) H&E–stained transverse section planes of the hind paw of 4-week-old wild-type (left) and hTNFtg (right) arthritis mice with tenosynovitis (*infiltration of lymphocytes and inflammation of the tendon sheath) in the preclinical phase of arthritis (magnification 100×). (E) Proposed new textbook image with tenosynovitis and osteitis. MCP, metacarpophalangeal; RA, rheumatoid arthritis.

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Retinal vasculometric characteristics and their associations with polymyalgia rheumatica and giant cell arteritis in a prospective cohort: EPIC-Norfolk Eye Study

Both polymyalgia rheumatica (PMR) and giant cell arteritis (GCA) have been associated with an increased future risk of cardiovascular disease (CVD).[1] However, it remains uncertain whether this is a consequence of inflammatory disease or relates to a common underlying mechanism. Retinal vascular images are a sensitive measures of vascular health, which are emerging as important biomarkers of future cardiovascular risk with changes affecting arterioles and venules.[2] In this study, we assess whether vasculometric features associated with CVD are detectable prior to the onset of PMR and GCA.

We analysed data from initially healthy subjects enrolled in the EPIC-Norfolk Study, a prospective population-based cohort which enrolled participants between the years 1993 and 1997.[3 4] Digital photographs of the retinal fundus were taken of 8112 participants between 2004 and 2011 using a TRC-NW6S non-mydriatic retinal camera and IMAGEnet Telemedicine System (Topcon Corporation, Tokyo, Japan) with a 10 MP Nikon D80 camera (Nikon Corporation, Tokyo, Japan). Retinal vessel widths were measured using the QUARTZ (QUantitative Analysis of Retinal vessel Topology and siZe) programme.[5] The fully automated algorithm uses an ensemble classifier of bagged decision trees to allocate vessels into arterioles and venules at 80% probability and calculates summary measures for each participant with an averaged measure between right and left eyes.

Cases of PMR and GCA were identified by three methods: (1) free text questionnaire responses at enrolment, and thereafter, 18 months, 3, 10 and 13 years; (2) linkage to hospital electronic discharge summaries containing International Classification of Diseases (ICD) codes (3) linkage to keyword searches (*polymyalgia or rheumatica or giant or arteritis*) of out-patient clinic letters. To be identified as PMR or GCA, participants were required to have received at least two prescriptions for oral glucocorticoids within 6 months following their diagnosis.

Table 1 Retinal vasculometric characteristics and their association to diagnoses of PMR and GCA

	Incident cases*	Incident cases meeting classification sets	Control n=5477
PMR (n)	30	24	
Venular width (µm) and SD	96.6 SD 12.5	100.0 SD 11.3	91.1 SD 10.6 from 5036 controls
Difference in venular width (µm) (95% CI) 80% probability†	5.5 (1.7 to 9.3) p=0.005	8.9 (4.7 to 13.2) p=<0.001	
Adjusted for age at time of retinal photograph capture and sex	4.4 (0.7 to 8.2) p=0.021	7.8 (3.6 to 12.0) p=<0.001	
Arteriolar width (µm) and SD	75.6 SD 7.6	76.9 SD 7.8	75.0 SD 6.3 from 5037 controls
Difference in arteriolar width (µm) (95% CI) 80% probability†	0.7 (–1.6 to 2.9) p=0.575	0.6 (–1.6 to 2.9) p=0.57	
Adjusted for age at time of retinal photograph capture and sex	1.0 (–1.2 to 3.3) p=0.366	1.1 (–1.1 to 3.4) p=0.32	
GCA (n)	16	13	
Venular width (µm) and SD	93.0 SD 9.4	93.7 SD 10.3	91.1 SD 10.6 from 5036 controls
Difference in venular width (µm) (95% CI) 80% probability†	1.9 (–3.3 to 7.1) p=0.47	2.6 (–3.2 to 8.4) p=0.38	
Adjusted for age at time of retinal photograph capture and sex	1.1 (–4.1 to 6.2) p=0.68	1.5 (–4.2 to 7.2) p=0.60	
Arteriolar width (µm) and SD	74.4 SD 5.9	73.8 SD 6.0	75.0 SD 6.3 from 5037 controls
Differences in arteriolar width (µm) (95% CI) 80% probability†	–0.6 (–3.6 to 2.5) p=0.73	–1.2 (–4.7 to 2.2) p=0.48	
Adjusted for age at time of retinal photograph capture and sex	–0.03 (–3.1 to 3.0) p=0.98	–0.7 (–4.1 to 2.7) p=0.70	

*Incident cases—median time period 2.9 years between retinal image capture and subsequent diagnosis with >75% having an interval of >1 year.
†Probability of vascular segment type (arteriole or venule) weighted by segment length, 95% CI.
GCA, giant cell arteritis; PMR, polymyalgia rheumatica.

This approach follows classification 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. Case assignment was carried out independently by two rheumatologists (MY, RW). Only incident cases with retinal images captured before their PMR or GCA diagnosis were included.

Among 5532 participants who had retinal images analysable by QUARTZ, we identified 30 cases of incident PMR (median age at diagnosis: 74.8 years, range (60.5, 87.0); mean erythrocyte sedimentation rate (ESR) at diagnosis: 48 mm/hour; 70.0% female) and an additional 16 cases of GCA (median age at diagnosis: 75.0 years, range (62.1, 84.0); mean ESR at diagnosis: 80 mm/hour; 81.3% female). Vasculometric measures of those subsequently developing PMR (table 1), showed wider venules compared with controls (5.5 µm increased width 95% CI 1.7 to 9.3, p=0.004), which remained significant after adjustment for age at time of retinal image capture, and sex (4.4 µm wider, 95% CI 0.7 to 8.2, p=0.021). Some who were diagnosed with disease did not meet the classification criteria. A stronger association was present when the analysis was limited to those cases which fulfilled current classification criteria sets. Although, on average those subsequently developing GCA had wider venules compared with controls (93 vs 91.1 µm) the difference failed to reach statistical difference. There was no association between arteriolar measures for either PMR or GCA.

Using a novel retinal marker in a longitudinal population-based setting, this analysis shows that participants who developed PMR already had wider retinal venules prior to the onset of their inflammatory disease. The data are limited by the relatively small number of cases with incident disease and need to be replicated in other settings. They nevertheless lend weight to the hypothesis that vascular changes precede the onset of PMR.

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Contributors MY: study design, data curation, analysis, drafting the manuscript. RoW: computer programmer for the QUARTZ group. AR: data analyst for generating vasculometric measures from QUARTZ derived measures of pixel width. TP: data controller of retinal images. AJM: input to study design and supervision of MY during his doctoral thesis. AK: ophthalomologist with input in to the design and execution of the third health check during which retinal images were taken. RAW: input to study design and secondary supervision of MY during his doctoral thesis. DB: ophthalomologist with input in to the design and execution of the third health check during which retinal images were taken. SH: research associate for EPIC with responsibility for the runing of the third health check. RL: research data manager

responsibile for EPIC-Norfolk. SB: PI and lead of QUARTZ group. CO: data analyst for generating vasculometric measures from QUARTZ derived measures of pixel width. K-TK: PI of EPIC-Norfolk and custodian of the data. PF: PI of the third health check for EPIC. All PIs had input into the conception, design and undertaking of the study. All authors commented and edited previous drafts of the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study was approved by the Norfolk Local Research Ethics Committee. Participants consented to the study and access to their records was granted. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. 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 availability statement All data requests and collaborations are reviewed and assessed by the EPIC-Norfolk Management Committee.

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Dupilumab as a novel steroid-sparing treatment for IgG4-related disease

IgG4-related disease (IgG4-RD) is a rare fibroinflammatory, multisystemic condition with a relapsing-remitting progression.[1] The level of serum IgG4 correlates with inflammatory activity and organ involvement.[1] Glucocorticoids are first line for IgG4-RD, but there are numerous adverse effects with chronic use.[2] Dupilumab is a monoclonal antibody that acts on the interleukin 4 (IL-4) receptor alpha, shared by the IL-4 and IL-13 receptors.[1] IL-4 causes isotype switching from IgM to IgG4 and IL-13 is implicated in fibrosis.[3] Thus, it was postulated by the authors to investigate dupilumab as a novel steroid-sparing treatment for IgG4-RD.

A 67-year-old man with no known allergies and a history of sensory neural hearing loss, recurrent bronchitis, spinal stenosis, moderate positional obstructive sleep apnoea, asthma, atopic dermatitis (which caused swelling around his eyes) and allergic rhinoconjunctivitis underwent extensive investigations over the past 2 years due to suspected IgG4-RD.

The patient's initial complaint was pruritic erythematous lesions on the legs, arms, chest and palms. Further investigations revealed parotitis, sinusitis, normocytic anaemia and eosinophilia. An MRI showed retroperitoneal and genitourinary fibrosis (figure 1A). Total IgG and IgG_4 levels were found to be 32.40 g/L and 20.60 g/L, respectively. The patient had a prostate biopsy which revealed 50 IgG4 cells per high power field and an IgG4+/IgG+ cell ratio of 40%. This result is exactly borderline as per the IgG4-RD comprehensive diagnostic criteria,[4] making the result of the biopsy probable for IgG4-RD. Interventional radiologists determined the retroperitoneal fibrosis to be inaccessible for biopsy and the patient declined a repeat prostate biopsy. Although the biopsy was borderline, given that the imaging, clinical features and laboratory investigations fulfilled the remainder of the comprehensive diagnostic criteria (1 to 3a), IgG4-RD was the consensus diagnosis.[4]

A treatment plan of a 40 mg daily dose of prednisone was suggested by rheumatology, with the option of adding the adjuvant immunosuppressant azathioprine. The patient was on 40 mg prednisone daily but declined other agents due to the risk of adverse effects.

Laboratory investigations revealed haemoglobin counts of 131 g/L (normal range 135–175 g/L), haematocrit levels of 0.391 L/L (normal range 0.4–0.5 L/L), eosinophil levels of 1.4×10^9 cells/L (normal range $0.0–0.5\times10^9$/L) and alkaline phosphatase serum levels of 34 U/L (normal range 40–129 U/L). On examination, atopic dermatitis was present with 50% body surface area (BSA) involvement with an Investigator Global Assessment (IGA) score of 4, indicating severe disease. An initial 600 mg subcutaneous injection of dupilumab, followed by a 300 mg subcutaneous injection every other week for 12 months was given to treat atopic dermatitis, asthma and potentially IgG4-RD.

After 3 months on dupilumab, the patient's eye swelling resolved, and his skin and asthma noticeably improved to IGA1 and <10% BSA. Both total IgG and IgG4 levels reduced substantially to 19.41 g/L and 11.43 g/L, respectively. After 12 months on dupilumab, the patient's retroperitoneal fibrosis improved dramatically corresponding with the decreased IgG4 levels (figure 1B). It is noted that dupilumab is in itself an IgG4 monoclonal antibody.

Current treatments for IgG4-RD are associated with many long-term adverse effects. The first-line treatments are glucocorticoids, second-line treatments are chemotherapeutic immunosuppressants and the third-line treatment is B-cell depleting rituximab, an anti-CD20 monoclonal antibody. The adverse effects associated with these therapies include increased risks of infection and potentially lasting immune deficiency.[5]

Dupilumab has been observed to be safe with long-term use across multiple indications.[6] In this patient, IgG4-RD was

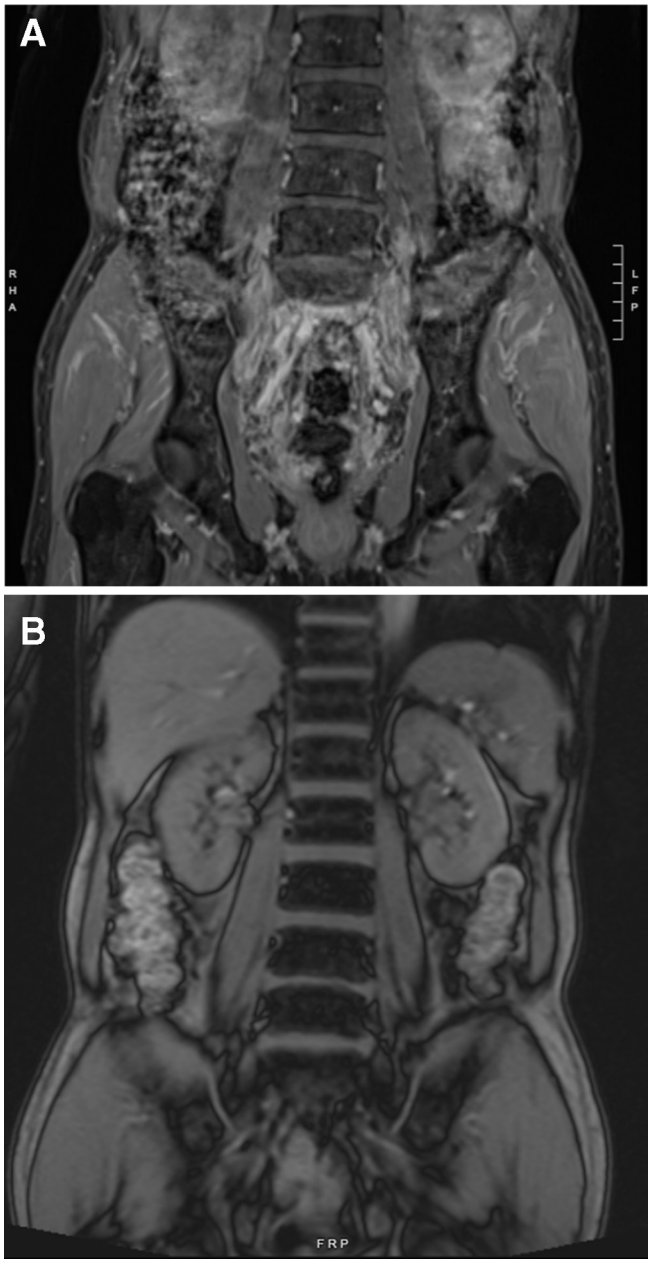

Figure 1 (A) Patient's initial MRI findings before dupilumab treatment showed extensive retroperitoneal and extraperitoneal fibrosis. (B) MRI taken approximately 1 year after dupilumab treatment showed dramatic resolution of fibrosis.

controlled with no further relapses across all affected organ systems with no significant long-term adverse events and prednisone withdrawal within 2 months. Dupilumab's efficacy in the treatment of IgG4-RD also highlights the importance of IL-4 and IL-13 in the pathological mechanisms of this condition.

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responsibile for EPIC-Norfolk. SB: PI and lead of QUARTZ group. CO: data analyst for generating vasculometric measures from QUARTZ derived measures of pixel width. K-TK: PI of EPIC-Norfolk and custodian of the data. PF: PI of the third health check for EPIC. All PIs had input into the conception, design and undertaking of the study. All authors commented and edited previous drafts of the manuscript.

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Dupilumab as a novel steroid-sparing treatment for IgG4-related disease

IgG4-related disease (IgG4-RD) is a rare fibroinflammatory, multisystemic condition with a relapsing-remitting progression.[1] The level of serum IgG4 correlates with inflammatory activity and organ involvement.[1] Glucocorticoids are first line for IgG4-RD, but there are numerous adverse effects with chronic use.[2] Dupilumab is a monoclonal antibody that acts on the interleukin 4 (IL-4) receptor alpha, shared by the IL-4 and IL-13 receptors.[1] IL-4 causes isotype switching from IgM to IgG4 and IL-13 is implicated in fibrosis.[3] Thus, it was postulated by the authors to investigate dupilumab as a novel steroid-sparing treatment for IgG4-RD.

A 67-year-old man with no known allergies and a history of sensory neural hearing loss, recurrent bronchitis, spinal stenosis, moderate positional obstructive sleep apnoea, asthma, atopic dermatitis (which caused swelling around his eyes) and allergic rhinoconjunctivitis underwent extensive investigations over the past 2 years due to suspected IgG4-RD.

The patient's initial complaint was pruritic erythematous lesions on the legs, arms, chest and palms. Further investigations revealed parotitis, sinusitis, normocytic anaemia and eosinophilia. An MRI showed retroperitoneal and genitourinary fibrosis (figure 1A). Total IgG and IgG_4 levels were found to be 32.40 g/L and 20.60 g/L, respectively. The patient had a prostate biopsy which revealed 50 IgG4 cells per high power field and an IgG4+/IgG+ cell ratio of 40%. This result is exactly borderline as per the IgG4-RD comprehensive diagnostic criteria,[4] making the result of the biopsy probable for IgG4-RD. Interventional radiologists determined the retroperitoneal fibrosis to be inaccessible for biopsy and the patient declined a repeat prostate biopsy. Although the biopsy was borderline, given that the imaging, clinical features and laboratory investigations fulfilled the remainder of the comprehensive diagnostic criteria (1 to 3a), IgG4-RD was the consensus diagnosis.[4]

A treatment plan of a 40 mg daily dose of prednisone was suggested by rheumatology, with the option of adding the adjuvant immunosuppressant azathioprine. The patient was on 40 mg prednisone daily but declined other agents due to the risk of adverse effects.

Laboratory investigations revealed haemoglobin counts of 131 g/L (normal range 135–175 g/L), haematocrit levels of 0.391 L/L (normal range 0.4–0.5 L/L), eosinophil levels of 1.4×10^9 cells/L (normal range $0.0–0.5\times10^9$/L) and alkaline phosphatase serum levels of 34 U/L (normal range 40–129 U/L). On examination, atopic dermatitis was present with 50% body surface area (BSA) involvement with an Investigator Global Assessment (IGA) score of 4, indicating severe disease. An initial 600 mg subcutaneous injection of dupilumab, followed by a 300 mg subcutaneous injection every other week for 12 months was given to treat atopic dermatitis, asthma and potentially IgG4-RD.

After 3 months on dupilumab, the patient's eye swelling resolved, and his skin and asthma noticeably improved to IGA1 and <10% BSA. Both total IgG and IgG4 levels reduced substantially to 19.41 g/L and 11.43 g/L, respectively. After 12 months on dupilumab, the patient's retroperitoneal fibrosis improved dramatically corresponding with the decreased IgG4 levels (figure 1B). It is noted that dupilumab is in itself an IgG4 monoclonal antibody.

Current treatments for IgG4-RD are associated with many long-term adverse effects. The first-line treatments are glucocorticoids, second-line treatments are chemotherapeutic immunosuppressants and the third-line treatment is B-cell depleting rituximab, an anti-CD20 monoclonal antibody. The adverse effects associated with these therapies include increased risks of infection and potentially lasting immune deficiency.[5]

Dupilumab has been observed to be safe with long-term use across multiple indications.[6] In this patient, IgG4-RD was

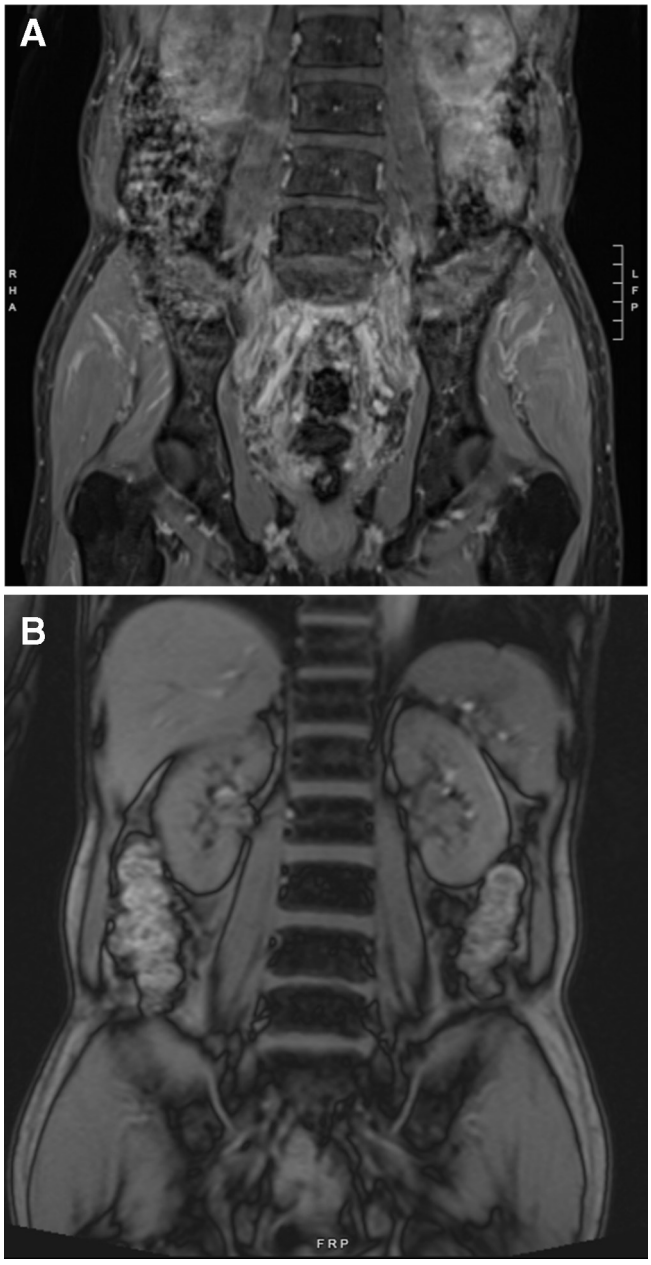

Figure 1 (A) Patient's initial MRI findings before dupilumab treatment showed extensive retroperitoneal and extraperitoneal fibrosis. (B) MRI taken approximately 1 year after dupilumab treatment showed dramatic resolution of fibrosis.

controlled with no further relapses across all affected organ systems with no significant long-term adverse events and prednisone withdrawal within 2 months. Dupilumab's efficacy in the treatment of IgG4-RD also highlights the importance of IL-4 and IL-13 in the pathological mechanisms of this condition.

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'To switch or not to switch': the missing piece in the puzzle of biosimilar literature?

We read with great interest the paper from Glintborg and coauthors 'To switch or not to switch' reporting biosimilar etanercept switching in Denmark.[1]

In the Danish Biologic (DANBIO) cohort, patient treated with originator etanercept (Enbrel) were informed of a mandatory switch to SB4. However, the 25 mg pen or a powder-based preparation of original etanercept (OE) were still available. At 1 year after this decision, the authors reported that only 79% of patients had switched to SB4 and 21% remained treated with the originator biologic.

In westernised countries, biologics therapies take a high toll on healthcare systems. With their 20%–50% lower costs, biosimilars' wide diffusion are therefore a necessity in order to provide sustainable healthcare to patients with chronic inflammatory rheumatic diseases. While the use of biosimilar in patient initiating a treatment is a simple subject, switching from originator to biosimilar and the strategy to do it (shared decision vs mandatory switch) has been a hot topic of debate in the rheumatology community.[2–5]

Two strategies for the use of biosimilars can be differentiated in patients already treated with an originator: mandatory switch or physician–patient shared decision. Physician–patient shared decision has been favored by rheumatology scientific societies, by an international consensus group and by patients association.[6–8] Indeed, real-life studies reporting the acceptance of the switch from OE to SB4 in case of shared decision together with an optimised communication strategy have reported acceptance rates of 92%–99%.[9 10] Outside an improved acceptance rate of physician–patient decision, there are reasonable evidence suggesting that forcing the switch on a patient is likely to increase the risk of *nocebo* effect, with negative effect on the patient and on physician–patient relationship (reviewed by Kravvaviti).[11] This *nocebo* effect might, at best, mandate a reswitch to the originator, therefore, a failure of the switching strategy. In the worst case (if the originator is not available anymore), the patient will be switched to another (possibly originator) biologic, leading to an avoidable exhaustion of therapeutic options, a weakening of the patient–physician relationship and increased healthcare costs. Considering this body of evidence, we believe that Glintborg's study was the missing piece in the puzzle of the biosimilars literature, demonstrating that a mandatory switch is probably not the most efficient strategy for the wide diffusion of biosimilar in chronic rheumatic diseases and reinforcing the evidence of the necessity of a shared physician–patient decision as recommended by many.

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Response to "To switch or not to switch': the missing piece in the puzzle of biosimilar literature?' by Scherlinger *et al*

Thank you for the interest[1] in our recent publication, in which we explored treatment outcomes following a Danish mandatory switch from originator to biosimilar etanercept (SB4, 50 mg) in routine care.[2] We showed that of the 2061 patients who were receiving originator etanercept and thus were eligible for the switch, as many as four of five (79%) switched to the biosimilar, despite the continued availability of the originator drug (as 25 mg pen or 50 mg powder solution). Among the patients who switched, we observed high retention rates of the biosimilar. The 6-month retention rate after switch (88%) was very similar to results of a recent Dutch study (90%), which reported outcomes of a non-mandatory switch following a specifically designed communication strategy.[3] Furthermore, we found that the disease activity and flare rates 3 months prior to versus 3 months after the switch were similar at the level of the individual patients. Thus, we agree with Scherlinger and Schaeverbeke that biosimilars hold the potential to provide sustainable healthcare in inflammatory rheumatic diseases at reduced costs.[1]

The question raised by Scherlinger *et al* is whether the outcome of a shared patient-physician decision (=non-mandatory) is more favourable than a mandatory switch. In previous studies that explored non-mandatory switching, the shared patient-physician decision-making included training of personnel and use of specific questionnaires or communication techniques.[3 4] For the Danish mandatory switch, no extra resources were allocated to conduct the switch procedure and no specific education of the healthcare personnel was provided. Furthermore, it was beyond the scope of our study to explore the practical aspects of the switch procedure including communication strategy with the patients. However, we have previously demonstrated that a mandatory switch from originator to biosimilar infliximab did not lead to a detectable increase in the use of healthcare resources.[5]

To determine whether shared patient-physician decision is superior to a mandatory switch in terms of lower nocebo effect, increased treatment efficacy and reduced healthcare costs, large-scale studies which are designed to explore these specific aspects are necessary—and highly needed. Such studies must also include evaluation of the extra healthcare resources allocated to and arising from the strategies investigated.

In conclusion, our paper adds important evidence to the use of biosimilars in routine care—however, some pieces are still missing in the puzzle.

Bente Glintborg ,[1,2] **Anne Gitte Loft**,[3,4] **Emina Omerovic**,[5] **Oliver Hendricks**,[6] **Asta Linauskas**,[7] **Jakob Espesen**,[8] **Kamilla Danebod**,[2] **Dorte Vendelbo Jensen**,[2] **Henrik Nordin**,[9] **Emil Barner Dalgaard**,[10] **Stavros Chrysidis**,[11] **Salome Kristensen**,[12] **Johnny Lillelund Raun**,[13] **Hanne Lindegaard**,[14] **Natalia Manilo**,[15] **Susanne Højmark Jakobsen**,[16] **Inger Marie Jensen Hansen**,[16] **Dorte Dalsgaard Pedersen**,[17] **Inge Juul Sørensen**,[1,18] **Lis Smedegaard Andersen**,[19] **Jolanta Grydehøj**,[20] **Frank Mehnert**,[21] **Niels Steen Krogh**,[22] **Merete Lund Hetland**[18]

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When binary and continuous responses disagree

In the observational TOCERRA study by Lauper *et al*,[1] the authors showed that tocilizumab (TOC; either as monotherapy or combination therapy) had superior drug retention than tumour necrosis factor inhibitors (TNFi; as monotherapy or combination therapy), in patients with rheumatoid arthritis with prior exposure to at least one biologic disease-modifying antirheumatic drug (bDMARD). Yet, efficacy (measured by Clinical Disease Activity Index (CDAI) change over time) was the same! The authors offered the following astute explanations: (1) CDAI does not comprehensively assess drug efficacy; (2) different tolerance between TOC and TNFi groups; or (3) retention captures something that is not evaluated by CDAI. I would like to expand on these explanations, since this phenomenon has previously appeared in this journal.

When a patient starts any treatment, it is generally not continued if it is not effective. More so with expensive bDMARDs. In fact, many countries enforce bDMARD discontinuation unless response is demonstrated. Such a patient would typically stop contributing data to his treatment episode in the registry. The analyst cannot compare responses that she does not have. This essentially means that she is comparing response among responders of both treatment arms—unsurprising, then, that their responses were the same. Of course, not all non-responders discontinued treatment; we can see this from the data. Some evidence to support my point is that 24% of TOC monotherapy stopped due to inefficacy, far more than 14% in the TNFi combination group.[1] How is this possible if efficacy were truly no different? (There should be no reason to believe that TNFi prescribers systematically under-recorded inefficacy as a reason for discontinuation.) A similar inconsistency was reported in the study by Ciurea *et al*, where current smoking did not (meaningfully) influence Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) change over time, yet led to 45% reduced odds of BASDAI50 response, compared with never smokers.[2 3]

What is the solution? If the data are 'Missing Not at Random' (ie, missingness is determined by unmeasured values, as is likely the case here) then solutions can be complex.[4] The LUNDEX method[5] is one simple yet elegant option when binary outcome variables are used. But, in observational studies, binary variables are themselves problematic.[2] Validity of binary responses depends on (1) no baseline differences between exposure groups (which was not the case in either studies[1 2]) and (2) how it is defined. Binary response variables can work with the LUNDEX if the denominator is defined as *patients adhering to the drug*, but not if it is *all patients* (ie, assuming that patients who discontinued were non-responders—a popular approach) (figure 1).

I would be interested to see the change in Disease Activity Score 28 joints (DAS28) over time, which was specified in methods but not reported, to see whether results were consistent with the greater TOC (monotherapy and combination therapy) response using binary derivatives of DAS28.

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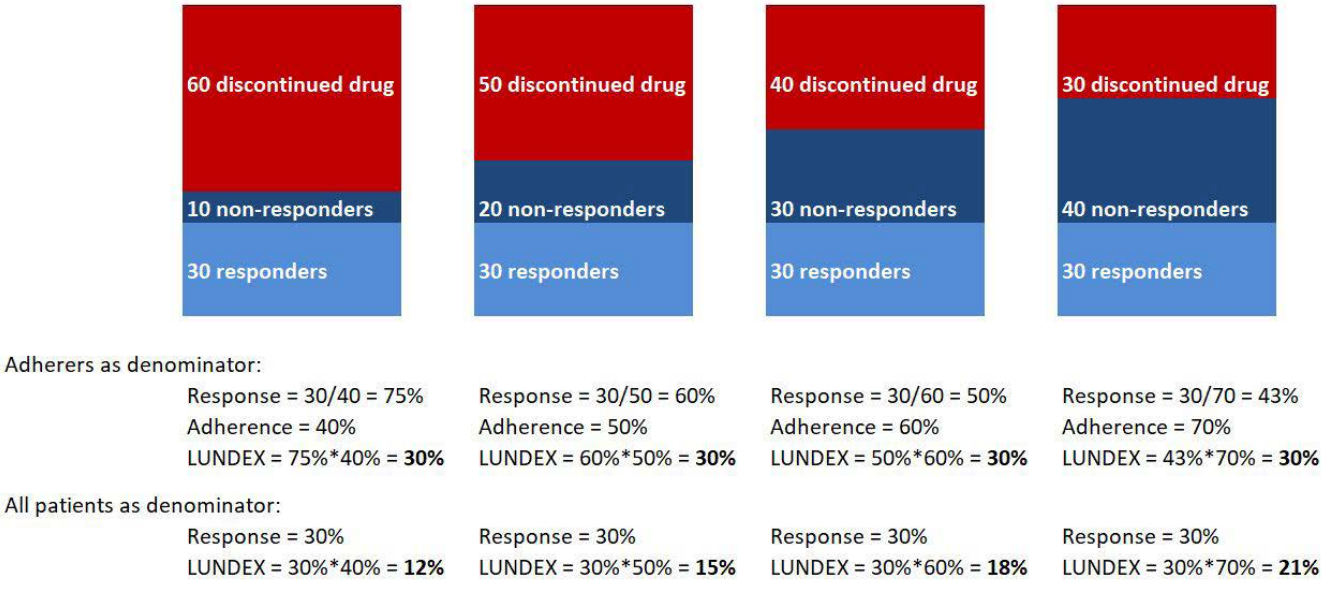

Figure 1 Non-responders commonly, but variably, discontinue drug. The LUNDEX method multiplies the proportion of responders with proportion of adherers, at a fixed time point. It is an elegant solution to variable non-responder discontinuation when response is defined using adherers as the denominator, but not all patients. The latter is a common approach to define response in observational studies, but is not compatible with the LUNDEX. Readers should also note that patients who would have otherwise responded may discontinue for other reasons (eg, adverse events), which the LUNDEX does not account for.

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Response to: 'When binary and continuous responses disagree' by Dr Ouyang

We thank Dr Ouyang for his comment on our work.[1]

We fully agree that patients stop treatment based, in part, on a lack of satisfactory clinical response. Thus, examining disease activity during follow-up, for instance at 1 year, will be influenced by attrition bias. If patients mostly stop the drug due to inefficacy, comparing only the patients who remain on treatment may lead to the tautological finding that for the people remaining on treatment, the drug is effective.

Another potential explanation for the dissociation between drug retention and other measures of effectiveness could be related to the fact that patients treated with tocilizumab had more previous therapies than patients treated with tumour necrosis factor (TNF) inhibitors.[2] It is therefore plausible that the lack of other treatment alternatives may influence patients and physicians to keep a less effective treatment.

In addition to ineffectiveness, patients stop their treatment for many other reasons, which are unfortunately not always well documented. In our study, half of the patients in each group stopped for 'other reasons' than effectiveness or adverse events, which may include a combination of reasons. We thus cannot assert the exact motive of drug discontinuation for most patients, which prevented us to draw any conclusion regarding differences between groups. Moreover, one treatment could be more effective than the other, and even if we were to consider only patients who remained under therapy, we could still detect some differences in degree of efficacy.

Though the LUNDEX[3] is a solution to account in part for attrition bias, we agree with Dr Ouyang that it is incomplete because it supposes that all patients stopped for ineffectiveness, which may underestimate true effectiveness, and because it does not take into account difference in baseline characteristics. In addition, it does not allow directly statistical hypothesis testing to determine whether a difference is significant or not. In our opinion, new methods and recommendations are thus dearly needed for comparative effectiveness research. Points to consider on this particular subject are currently being developed by a European League Against Rheumatism task force, in which we are actively taking part. We hope that the results of this initiative will help researchers to navigate between the different methods available and improve the quality of future studies.

Regarding Disease Activity Score 28 joints (DAS28) evolution, we found that DAS28 decreased more with tocilizumab as monotherapy and in combination with conventional synthetic disease-modifying antirheumatic drugs (csDMARD) than with TNF inhibitors, but this was statistically significant only for tocilizumab in combination with csDMARDs compared with TNF inhibitors in combination with csDMARDs (coefficient 0.44, p=0.04). As discussed in our article, this is consistent with tocilizumab's greater effect on acute-phase reactants.

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Risk of severe infection following rituximab and the efficacy of antimicrobial prophylaxis

We read with interest the article 'Trimethoprim–sulfamethoxazole prophylaxis prevents severe/life-threatening infections following rituximab in antineutrophil cytoplasm antibody-associated vasculitis' by Kronbichler *et al*.[1] Severe infections continue to be a significant cause of morbidity and mortality in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV), and we commend the authors for pursuing this study. However, we have several concerns regarding their methodologies.

First, more than 90% of patients with AAV analysed were prevalent cases. This may limit the generalisability of their findings to incident cases in whom there has been no prior treatment with cyclophosphamide or other immunosuppressive agents. As the authors note, there was an average delay of 4 years between initial diagnosis and rituximab initiation, during which time the patients received a variety of immunosuppressive medications, including large quantities of glucocorticoids. These treatments likely contributed to the risk of infection observed by the authors.

Second, it is unclear whether the authors accounted for the fact that exposure to trimethoprim-sulfamethoxazole was time-varying. In other words, were time and outcomes (eg, infections) after antibiotic discontinuation attributed to the unexposed arm (eg, no antibiotic)? If antibiotic prophylaxis was discontinued during follow-up, subsequent infections should have been attributed to the unexposed arm.

Third, the authors did not account for confounding by indication or contraindication. They identified certain patients, particularly those with head and neck disease, as those more likely to receive prophylaxis. This greater likelihood of prophylaxis stems, no doubt, from the belief by some investigators that therapy with trimethoprim-sulfamethoxazole diminishes the risk of disease flares in the upper respiratory tract.[2] Moreover, patients with head and neck disease may be more likely to have 'limited' disease and less likely to have disease that is 'disseminated'.[3] Such patients are less likely to receive intensive immunosuppressive therapy with drugs such as cyclophosphamide or high-dose glucocorticoids for prolonged periods of time and inherently less likely to suffer from infectious complications of treatment. Answering the question evaluated by the authors therefore requires an approach to account for this potential confounding.

Finally, the potential adverse effects associated with sulfa medications should not be minimised. In the trial by Stegeman *et al*,[2] 20% of patients had to discontinue trimethoprim-sulfamethoxazole because of side effects. Life-threatening hypersensitivity reactions including Stevens-Johnson syndrome, interstitial nephritis, thrombocytopaenia, liver function test abnormalities and drug-resistant infections can all occur with chronic antimicrobial prophylaxis.[4] The risks of adverse effects for patients receiving immunosuppressive treatment for AAV are not uniform and likely lower than in earlier eras, when cyclophosphamide and high-dose glucocorticoid regimens dominated the approaches to treatment. Further studies are necessary to quantify these risks in contemporary cohorts.

Although we also believe that trimethoprim-sulfamethoxazole prophylaxis has a role in the prevention of infectious adverse events in AAV, the full range of risks as well as potential benefits need to be more clearly understood.

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Response to: 'Risk of severe infection following rituximab and the efficacy of antimicrobial prophylaxis' by Wallace *et al*

We thank Dr Wallace *et al* for their response to our recently published article 'Trimethoprim–sulfamethoxazole prophylaxis prevents severe/life-threatening infections following rituximab in antineutrophil cytoplasm antibody-associated vasculitis', highlighting some methodological limitations of our study.[1 2]

One of the limitations mentioned by Wallace and colleagues is the inclusion of incident and prevalent cases and since only 15 out of 192 patients were incident cases, generalisability of our findings for this subset of patients may not be possible. We agree that the use of immunosuppression prior to initiation of rituximab likely confers a risk to develop infectious complications after rituximab administration. Cyclophosphamide was used to control disease in 62 patients the year before rituximab was initiated. Among these, 53 patients had no severe infection (median cyclophosphamide exposure 7 g, range 0.66–45 g), while 9 patients receiving cyclophosphamide the index year before had a severe infection following rituximab (median cyclophosphamide exposure 4.8 g, range 0.8–10 g). While this argues against an immediate impact of cyclophosphamide before rituximab on the risk of severe infections (53/143 with no severe infection against 9/49 with infection received cyclophosphamide), we also believe there exists a carry-over effect of diverse immunosuppressive measures on the risk to develop infectious complications. A recent analysis of non-antineutrophil cytoplasm antibody (ANCA)–associated vasculitis cases using The Health Improvement Network found that glucocorticoid exposure confers a high risk for lower respiratory tract infections.[3] Since a majority of infections observed in our analysis were related to the lower respiratory tract (63/95), the 'true influence' of concomitant glucocorticoid treatment may have been underestimated in our study. Moreover, we did not calculate the damage attributable to active vasculitis in this study. It is likely that patients with a higher Vasculitis Damage Index score are in particular prone to infectious complications.

We acknowledged the fact that prescription of trimethoprim–sulfamethoxazole (TMP-SMX) was time-varying, which means that if patients stopped TMP-SMX and developed their first severe infection afterwards, they were assigned to the unexposed group. Since analyses were performed with first infectious complication as the primary outcome, no such adjustments were undertaken for subsequent infectious complications.

Confounding by indication or contraindication is a limitation of observational studies. Novel approaches such as active-comparator design and new-user design could be helpful to mitigate such biases.[4] We did not perform an active-comparator study, which would comprise a cohort of patients receiving cyclophosphamide as part of their induction treatment. Moreover, a new-user study was not feasible since recently diagnosed or relapsing patients were unlikely to receive a cyclophosphamide-based induction treatment after results of the Rituximab in ANCA-Associated Vasculitis (RAVE) trial have been published.[5] As stated by Wallace *et al*, there were differences in the prescription of TMP-SMX in our study. Patients with ear, nose and throat (ENT) involvement were more likely to receive TMP-SMX prophylaxis (online supplementary table S5).[2] We agree that patients with predominant ENT involvement are less likely to receive 'intensive' immunosuppressive therapy; however, our patients with TMP-SMX prescription had a non-significantly higher BVAS (p=0.093) and similar rates of kidney and lung involvement. Patients receiving TMP-SMX prophylaxis had lower CD4 T-cell count and were exposed to more cyclophosphamide the year before rituximab and had a higher concomitant glucocorticoid exposure (online supplementary table S5).[2] Thus, we conclude that the subset of patients receiving prophylaxis in our study had at least a comparable disease severity compared with those not receiving TMP-SMX.

Despite its proven efficacy to reduce the occurrence of infectious complications, TMP-SMX exhibits a variety of severe and non-severe side effects. The study by Stegeman and colleagues used a therapeutic dosage of TMP-SMX (twice daily, 800/160 mg) for a period of 2 years and found side effects in 8/41 patients.[6] Of note, only a minority of patients received a similar high dose of TMP-SMX in our cohort, while prophylaxis consisted of 480 mg or 960 mg every other day in most cases (online supplementary table S4).[2] TMP-SMX was stopped in 5 out of 73 patients in our cohort due to adverse events.[2] A recent study investigated the role of TMP-SMX in patients with prolonged exposure to high-dose glucocorticoids and found a reduction in the occurrence of infections with *Pneumocystis jirovecii*. Among 262 patients receiving prophylaxis, two cases with serious adverse events attributable to TMP-SMX were reported (pancytopenia and Stevens-Johnson syndrome), while mild side effects were found in another 34 cases.[7] A recent randomised controlled trial tested the role of TMP-SMX (800/160 mg twice daily for 1 week) against placebo for the management of uncomplicated skin abscess. Among 630 subjects receiving TMP-SMX, only one case with pancytopenia was noted and in general, no difference in the frequency of side effects was reported.[8] No such investigations including cases with ANCA-associated vasculitis have been performed so far, but larger studies in other entities and as an anti-infective measure point towards an acceptable safety profile of TMP-SMX.

In clinical practice, we sometimes see patients with severe side effects attributable to TMP-SMX prophylaxis. There is a need to balance risks and benefits of such prophylaxis, but in the absence of prospective studies (observational or ideally a randomised controlled trial), we recommend prophylaxis in those patients with comorbidities or receiving background immunosuppression (mainly glucocorticoids) or with an exposure to a variety of immunosuppressive agents in the past.

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Potential roles for tenascin in (very) early diagnosis and treatment of rheumatoid arthritis

We read the interesting article by Aungier *et al* suggesting that targeting proinflammatory signals from the C-terminal fibrinogen-like globe domain (FBG) of tenascin-C (TNC) might provide a viable strategy to treat rheumatoid arthritis (RA).[1]

The present story and possible developments are really interesting and might link to other recent evidences concerning the same argument.

We described for the first time in 1992 the distribution of TNC in normal and pathological synovial tissues from patients with RA and osteoarthritis (OA) by indirect immunofluorescence using specific monoclonal antibodies.[2]

Tenascin was found in normal synovium just beneath the whole lining cell layer; however, a higher density and spreading pattern of distribution was observed in RA and OA sections, but the possible meaning was unclear at that time.

Soon after, these early data were confirmed by others, and several investigations added that TNC levels were elevated in both RA cartilage and synovium and the T-C soluble form was detectable in synovial fluids of patients with RA.[3] Additionally, serum TNC levels were found to correlate with joint erosions in patients with RA.[4]

Interestingly, TNC stimulates inflammation by inducing de novo cytokine synthesis via activation of toll-like receptor 4 (TLR4), modulating cytokine synthesis post-transcriptionally via induction of microRNAs and regulating adaptive immunity by driving Th17 cell polarisation.[5 6] In murine models of arthritis, TNC expression is required to maintain chronic joint inflammation and, of note, the FBG of TNC is arthritogenic following its intra-articular injection.[7]

Here, the story offers important aspects.

Generally, anticitrullinated protein antibodies (ACPAs) are well-established markers for the diagnosis of RA, appearing before evident clinical symptoms and correlating with a poor prognosis and progressive joint destruction.[8]

However, very few molecules recognised by ACPA have been demonstrated in the joint, epitope-mapped, antigen specificity confirmed and evaluated in independent large cohorts.[8]

Among these, the most important recent TNC-related discovery was that a citrullinated peptide from the FBG domain of TNC (cTNC5) was detected in RA synovial fluids, and surprisingly antibodies to cyclic peptides containing citrullinated sites again from the FBG domain were found in both pre-RA and RA sera.[9]

In particular, the autoantibody response to the FBG immunodominant cTNC5 peptide was analysed in 101 pre-RA sera (median 7 years before disease onset) and two large independent RA cohorts. Interestingly, 18% of pre-RA sera, and in 47% and 51% of RA cohorts were found positive with a specificity of 98% compared with healthy controls and patients with OA.

In addition, FBG domain cTNC5 antibody levels were found the highest in the whole anti-CCP2 antibody-positive subgroup and even 4.9% of the patients with RA within the anti-CCP2 antibody-negative group were also anti-cTNC5 ACPA-positive. Therefore, the study suggested that the FBG domain of TNC may be important in both the aetiology and pathogenesis of RA.

The actual study of Aungier *et al* shows that monoclonal antibodies recognising the FBG of TNC neutralise the FBG activation of TLR4 and therefore inhibit cytokine release by RA synovial cells and prevent disease progression and tissue destruction during collagen-induced arthritis.

These results might really represent a new approach for (very) early RA therapy, by targeting early changes in the synovial microenvironment, especially in ACPA-positive patients.

In conclusion, testing the presence of anti-FBG cTNC in the sera of patients with early synovitis might help in discovering patients potentially developing RA, and might offer the chance of therapeutically targeting from the beginning the same FBG TNC domain with specific monoclonal antibodies.

This approach might block proinflammatory/immune signals from the extracellular matrix proteins (ie, tenascin) inside the synovial tissue, and from the beginning, as in a previous paper also some authors of the present study already recently tested and discussed.[10]

We agree with the authors that, on the light of these recent achievements, further explorations about potential roles of TNC in clinical practice for (very) early diagnosis and treatment of RA.

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Response to: 'Potential roles for tenascin in (very) early diagnosis and treatment of rheumatoid arthritis' by Cutolo *et al*

We thank the authors for their commentary[1] on our article which was recently published in the *Annals of Rheumatic Diseases*.[2] Cutolo *et al* write an extended discussion of the study, in which we describe the development of therapeutic monoclonal antibodies that block the pro-inflammatory activity of the fibrinogen-like globe (FBG) domain of tenascin-C, and the efficacy of these antibodies in preventing disease progression in preclinical models of rheumatoid arthritis (RA). The commentary includes a detailed summary of the autoantibody response to a citrullinated epitope (cTNC5) within the FBG domain of tenascin-C which arises very early during the development of RA, and which can also be detected in around one in five people at risk of developing RA. As highlighted by the authors, the questions around how the response to modified components of the extracellular matrix evolves during the development of RA, and whether or not this autoantibody response contributes to disease pathogenesis, are an area of ongoing research. We also agree that detection of anticitrullinated peptide antibodies recognising cTNC5 in people with RA, or who will go on to develop RA, should be explored as a potential companion diagnostic with which to identify individuals who may benefit from treatment with therapies directed against the FBG domain of tenascin-C. If this hypothesis holds true, then we may well be able to stratify patients in whom we can intervene to stop disease progression from an extremely early stage. Following the seminal paper by Cutolo *et al* in 1992,[3] there has been enormous progress worldwide in the field of tenascin-C and joint pathology. Although there remains much work still to be done, not least in assessing the potential benefits of targeting tenascin-C as a means to treat people with RA in the clinic, as well as discovering more about whether a direct link exists between the pathogenic action of the FBG domain as a modulator of Toll-like receptor 4-mediated inflammation with its role in adaptive immunity in this disease, these are challenges that we look forward to facing.

Susan Aungier,[1] Alison J Cartwright,[1] Anja Schwenzer,[1] Jennifer Marshall,[2] Mike R Dyson,[3] Peter Slavny,[3] Kothai Parthiban,[3] Aneesh Karatt-Vellatt,[3] Ilfita Sahbudin,[4,5] Eric Culbert,[6] Patrick Hextall,[6] Felix IL Clanchy,[1] Richard Williams,[1] Brian D Marsden,[1,7] Karim Raza,[2,8] Andrew Filer,[2] Christopher D Buckley,[1,2] John McCafferty,[3] Kim S Midwood [1]

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Clinical trial and 'real-world' data support switching from a bio-originator to its biosimilar

In their correspondence, Cantini and Benucci[1] voice concern regarding the recommendation of our international multidisciplinary task force on biosimilars that 'a single switch from a bio-originator to one of its biosimilars is safe and effective.'[2] This recommendation was based on consistent evidence from randomised controlled trials comparing biosimilars to their respective reference products in patients with rheumatologic diseases, in which subjects treated with a reference product were subsequently transitioned to treatment with its biosimilar. In all such studies that have been published to date, there has been no significant loss of efficacy or increase in the incidence of adverse events or of antidrug antibodies following such a change. This has been demonstrated not only for biosimilars of infliximab[3–6] and etanercept,[7] but also for biosimilars of adalimumab.[8 9]

The NOR-SWITCH study met its primary endpoint at 52 weeks, thereby demonstrating non-inferiority of changing treatment from bio-originator infliximab to biosimilar infliximab CT-P13 (infliximab-dyyb) to continued treatment with bio-originator infliximab in patients with any of the six inflammatory diseases for which infliximab is indicated who had exhibited stable disease activity over the previous 6 months.[10] It is important to recognise that this prospective, double-blind, randomised controlled trial was powered to demonstrate non-inferiority of changing to the biosimilar to continued treatment with the bio-originator in the aggregated population of patients with the six inflammatory diseases; it was not designed to assess non-inferiority of this treatment strategy in any individual disease. As Cantini and Benucci point out, 248 (51.6%) of the 481 subjects enrolled in NOR-SWITCH had inflammatory bowel disease and 35 (7.3%) had psoriasis. However, the other 198 (41.2%) had an inflammatory rheumatologic disease and, although not powered to do so, this study demonstrated non-inferiority of changing to biosimilar infliximab for the subgroup of patients with spondyloarthritis. Thus, the results of the NOR-SWITCH study support changing treatment from bio-originator to biosimilar infliximab in patients with inflammatory rheumatologic diseases.

Ample published 'real-world' experience supports the efficacy and safety of switching from bio-originator infliximab to biosimilar infliximab CT-P13 in patients with inflammatory rheumatologic diseases. Avouac and coworkers observed no change in objective disease activity measures or infliximab trough levels among 260 patients with chronic inflammatory diseases, who were maintained on bio-originator infliximab and systematically transitioned to treatment with biosimilar infliximab CT-P13, of whom 31 (11.9%) had rheumatoid arthritis and 131 (50.4%) had axial spondyloarthritis.[11] After the third infusion of biosimilar infliximab CT-P13, 148 (91.4%) of these 162 patients remained on treatment with the biosimilar; the majority of those who discontinued treatment did so because of perceived inefficacy and not because of adverse events. Germain and colleagues observed similar treatment retention rates, after a median follow-up of 120 weeks, among 50 patients with 'stable rheumatic diseases' who had transitioned from bio-originator infliximab to biosimilar infliximab CT-P13, as compared with a historical cohort of patients treated with the bio-originator.[12] Benucci and collaborators reported no statistically significant differences in efficacy, safety or immunogenicity among 41 patients with spondyloarthritis who had been treated for at least 6 months with bio-originator infliximab and were changed to treatment with biosimilar infliximab CT-P13 for economic reasons.[13] Nikiphorou and colleagues observed similar patient-reported disease activity and symptoms after transitioning to biosimilar infliximab CT-P13, among 39 consecutive patients with inflammatory rheumatologic diseases that had been well controlled or in remission on treatment with bio-originator infliximab.[14] Six (54.5%) of the 11 patients in this cohort who discontinued biosimilar infliximab did so for subjective reasons without evidence of increased disease activity. Smaller 'real-world' observational studies also have confirmed comparable efficacy and safety of transitioning from bio-originator infliximab to biosimilar infliximab CT-P13 to that of continuing treatment with bio-originator infliximab.[15]

Data from registries also support the safety and efficacy of changing from a bio-originator to its biosimilar. Although the adjusted absolute retention rate after a mandated change in treatment to biosimilar infliximab CT-P13, among the 802 patients with rheumatoid arthritis, psoriatic arthritis or axial spondyloarthritis in the DANBIO registry, was slightly but statistically significantly lower than that in a historical cohort of patients treated with bio-originator infliximab, the 1-year crude retention rate (84.1%) on biosimilar infliximab CT-P13 did not differ significantly from that on the bio-originator (86.2%) in the historical cohort.[16] Likewise, among the 1621 patients with rheumatoid arthritis, psoriatic arthritis or axial spondyloarthritis in the DANBIO registry who changed from bio-originator etanercept to biosimilar etanercept SB4, the 1-year adjusted retention rate (83%) was higher than that (77%) of the 440 patients who remained on treatment with the bio-originator.[17]

Cantini and Benucci also suggest that our recommendation that 'multiple switching between biosimilars and their bio-originators or other biosimilars should be assessed in registries'[2] 'may be misleading for clinicians' because of 'the paucity of data from real-life and the absence of controlled trials.'[1] The double-blind, randomised, controlled EGALITY trial demonstrated no loss of efficacy after three switches back and forth between bio-originator etanercept and biosimilar etanercept GP2015 in patients with moderate-to-severe chronic plaque psoriasis.[18] Although switching between different biosimilars and their bio-originators has not yet been studied in a clinical trial, available clinical trial and 'real-world' data do not suggest that this will result in significant loss of efficacy or increase in adverse events or immunogenicity. Bio-originators have undergone multiple manufacturing process changes after marketing approval,[19] which have brought about batch-to-batch variation in molecular characteristics and occasionally in functional properties.[20 21] Batches of a bio-originator sourced in the European Union may differ in various product attributes even from batches of the same drug sourced in the USA.[22] Thus, for years, patients already have been switched unwittingly between variants of the same bio-originator that may differ as much or as little as do biosimilars from their reference products and from one another. Careful postmarketing pharmacovigilance should be conducted for all biopharmaceuticals, both bio-originators and biosimilars, and the information obtained through this process should be maintained in registries. These accumulated data will provide additional evidence to inform the practice of switching among multiple biosimilars and their reference products.

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'Everything we see is a perspective, not the truth'

We read with interest the article on 'Low incidence of vertebral fractures in early spondyloarthritis: 5-year prospective data of the DESIR cohort' by Julie Sahuguet *et al*.[1] The results are exciting regarding the significantly lower prevalence of vertebral fracture (VF) among patients with 'early SpA' and the crucial aspect of recognising vertebral defects as confounding factors. However, certain aspects of this study require further clarifications.

First, the external validity of the data remains questionable. The current study population has a higher number of female patients compared with the previous studies[2 3] where male:female ratio was 4:1. The proportion of patients who were HLA B27 positive is also substantially lower in the current cohort compared with the previous one.[3] So, the present cohort may not be the true representative of the spondyloarthropathy (SpA) population.

Second, the diagnostic utility of the low back pain criteria is much lower than expected as shown in a study by Poddubnyy *et al*. Specificity of the Calin criteria for diagnosing axial SpA is as low as 25%, and specificity for the Berlin criteria is 32%–44.8% depending on whether the physician was blinded or not. The specificity further reduced to 20% and 18%, respectively, if the patients applied the criteria.[4] It will be interesting to know how many of the patients in the DEvenir des Spondylarthopathies Indifférenciées Récentes (DESIR) cohort subsequently satisfied the classification criteria for SpA.

Third, the authors have selected patients having low back pain for a duration of 3 months to 3 years. It will be interesting to know the total number of patients screened to achieve these number of patients to be included, as in general, the available data suggests that the median delay to diagnosis has remained stable at 5 years for the patients diagnosed between 1999–2003, 2004–2008 and 2009–2013.[5] Could these patients be different from the usual axial SpA? Like having a higher incidence of peripheral arthritis that may be confounding factor for early case pickup.[5]

Fourth, the higher percentage of the female population in the DESIR cohort may be due to higher proportion having non-radiographic SpA, that may behave differently from radiographic SpA like accruing damage or osteopenia and VFs.

These informations would be crucial to the external validity—extrapolating the findings of the study.

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Response to 'Everything we see is a perspective, not the truth' by Chattopadhyay *et al*

We would like to thank Chattopadhyay *et al* for their interest in our article presenting the low incident rate of vertebral fractures in an early axial spondyloarthritis (axSpA) population. We have read with interest their comments regarding the external validity of the data we are presenting.[1 2]

We would like to highlight that the manuscripts the authors are referring to in their letter were focusing only in patients with either very long-standing disease (22.5 years in the Montala study[3]) or with radiographic involvement (ie, radiographic axSpA, also referred as ankylosing spondylitis) in both studies.[4]

We would like to emphasise that Devenir des Spondylarthropathies Indifférenciées Récentes (DESIR) is an early axSpA cohorts and to be included, patients could not have axial symptoms for more than 3 years. Furthermore, the presence of radiographic abnormalities was not an inclusion criteria.[5] Other early onset axSpA cohorts, such as SPondyloArthritis Caught Early (SPACE) or German Spondyloarthritis Inception cohort (GESPIC) have shown comparable populations. Male gender was 46.6% in the DESIR cohort, 44.6% in the SPACE cohort[6] and 51% patients in the GESPIC cohort,[7] human leukocyte antigen-B27 was positive in 57.8 %, 67.7% and 79.0% in DESIR, SPACE and GESPIC cohorts, respectively. This phenomenon (early disease presentation being slightly different from long-standing disease) is not unique in axSpA and has also been reported in other diseases such as rheumatoid arthritis (RA). The percentage of anti-citrullinated protein antibody (ACPA)-positive patients included in randomised phase III clinical trials with established disease is usually >75%, whereas the percentage is around 30% in the early RA cohorts.[8]

Concerning the comment on the diagnostic utility of the low back pain as a criteria for axSpA, we would like also to emphasise that in order to be included in DESIR, patients had to present with inflammatory back pain (and not just low back pain) according to the Calin[9] or the Berlin[10] criteria for inflammatory back pain for more than 3 months and less than 3 years. But they also have an axSpA diagnosis confidence >5/10 according to the rheumatologist.[5] Furthermore, at inclusion, 92.1% patients fulfilled at least one classification for axSpA.

Finally, the authors suggest that perhaps our results are different from the literature due to the inclusion of both nonradiographic and radiographic forms of axSpA. This seems difficult to confirm, since in our analysis, the prevalence of vertebral fracture was not different in both groups, but the incidence was so low overall that it could not be tested.

Anna Molto ,[1,2] **Julie Sahuguet**,[1] **Jacques Fechtenbaum**,[1] **Adrien Etcheto**,[1] **Clementina López-Medina**,[1,2] **Pascal Richette** ,[3] **Maxime Dougados**,[1,2,4] **Christian Roux**,[1,2,4] **Karine Briot** [1,2]

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IgA vasculitis in adults: few certainties and many uncertainties

We read with great interest the article on cardiovascular, thromboembolic and renal outcomes in patients with immunoglobulin A vasculitis (IgAV), published recently online in *Annals of the Rheumatic Diseases*.[1]

Tracy *et al*, estimated both a childhood and an adult onset of IgAV incidence rates and reported an increased risk of hypertension and chronic kidney disease in patients with IgAV, compared with age-matched and sex-matched controls based on retrospective data over a 12-year period extracted from a primary care database in the UK. They estimated the incidence rate of adult IgAV at 2.2 per 100 000 person-years,**1** which is close to the historic belief that IgAV rarely affects adults but was 2.3 times lower than the incidence rate of adult IgAV estimated at our secondary/tertiary medical centre in Slovenia at 5.1 (95% CI 3.4 to 7.4) cases per 100 000 persons per year.[2] And we believed our estimation was rather conservative as we prospectively, over 3 years, included only histologically proven adult IgAV cases. Moreover, our patient cohort was considerably older (mean age 62.4 (18.8) vs. 43.3 (18.8) years) and suggested, in line with other epidemiological studies,[3] a distinct male preponderance (63% vs. 48.4% males), compared with the UK cohort of adult patients with IgAV.[2] Although these differences may reflect the true differences between the two cohorts, they probably rather reflect the different methods of case ascertainment. Tracy *et al*, addressed some of the limitations and uncertainty regarding the classification of adult IgAV in their study.[1] A French group demonstrated a very low positive predictive value of the D69.0 code of the 10th revision of the International Statistical Classification of Disease for an ascertainment of IgAV cases from electronic medical records.[4]

The current analysis of baseline clinical features and comorbidities of our prospective adult IgAV cohort over a 9-year period, consisting of 262 patients (median age (IQR) 64.6 (46.1–77.1) years, 59.5% males, with kidney, gastrointestinal and articular involvement in 45.0%, 30.2% and 38.5%, respectively) had a positive history of arterial hypertension, diabetes, hyperlipidaemia, ischaemic heart disease, stroke and chronic kidney disease in 48.9%, 19.9%, 19.1%, 5.0%,3.4% and 17.9%, respectively. Moreover, arterial hypertension and acute kidney injury were each diagnosed concurrently with IgAV in an additional 10% of patients. Our patients with IgAV more commonly had a history of arterial hypertension and diabetes mellitus than age matched controls in the Slovenian population in general (source: the National Institute of Public Health;[5] Table 1). In addition, obesity was more prevalent in younger adults with IgAV than the age matched general Slovenian population. Regardless of, in our opinion, an over-conservative estimation of the incidence rate of IgAV, and an unexpected age and gender distribution in the reported UK cohort the associations of IgAV with hypertension, diabetes and obesity were noticed in both cohorts. It would be of a great interest to know whether these prevalent conditions contribute, if at all, to the risk of developing IgAV.

Hopefully, further studies of this oft-neglected, and contrary to common belief, not at all uncommon vasculitis in adults will improve our insight.

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Table 1 The comparison between patients with IgAV and general population

Age (years)	18–24		25–34		35–44		45–54		55–64		65–74		≥75	
Characteristics	IgAV	Pop	IgAV	Pop	IgAV	Pop	IgAV	Pop	IgAV	Pop	IgAV	Pop	IgAV	Pop
Hypertension (%)	0.0	3.5	0.0	3.6	7.7	10.4	25.8	22.5	53.5	39.2	71.7	50.7	77.5	59.9
Diabetes (%)	0.0	0.9	0.0	1.4	3.8	1.2	16.1	4.5	30.2	11.6	23.9	16.6	22.5	18.2
Ischaemic heart disease (%)	0.0	0.7	0.0	0.3	0.0	0.3	0.0	1.9	4.7	4.3	6.5	7.5	10.0	12.5
Stroke (%)	0.0	0.1	0.0	0.0	0.0	0.0	0.0	1.0	0.0	2.0	6.5	2.9	7.5	6.9
COPD (%)	0.0	1.6	0.0	2.3	7.7	2.3	0.0	3.5	16.3	5.7	17.4	6.9	7.5	8.8
Current smokers (%)	23.1	24.0	21.7	33.2	30.8	29.7	32.3	31.2	27.9	24.3	19.6	9.4	3.8	4.1
BMI 25.0–29.9 (%)	38.5	18.4	47.8	30.4	23.1	40.1	19.4	37.4	14.0	41.9	26.1	42.7	28.8	43.4
BMI ≥30 (%)	15.4	5.1	0.0	8.1	38.5	16.2	48.4	24.3	44.2	28.8	32.6	25.9	31.3	20.8

BMI, body mass index (kg/m^2); COPD, chronic obstructive lung disease; IgAV, immunoglobulin A vasculitis; Pop, population.

Response to 'IgA vasculitis in adults: few certainties and many uncertainties' by Hočevar *et al*

We thank Hočevar and colleagues for their comments on our recently published work on the epidemiology of IgA vasculitis (IgAV) in adults and children.[1 2] We agree that there is some uncertainty regarding the incidence of adult-onset IgAV as this has not previously been widely studied.

Our calculated incidence of 2.20 (95% CI 2.08 to 2.37) per 100 000 person-years in adults is slightly higher than most previous work has suggested, but is lower than that reported by Hočevar *et al* in a Slovenian tertiary centre study with no clearly demarcated denominator population.[2–4] This disparity may be related to inter-population differences, for example, in exposure to environmental risk factors such as viral infections.[5] Alternatively, the difference may be due to contrasting strategies used to identify cases of IgAV. Hočevar and colleagues used both clinical and pathological records to identify cases, thus reducing the risk of missing patients who had not been appropriately coded. A strength of our estimate is that it derives from a nationally representative population-based study. Nevertheless, the absolute risk is very small (2–5 per 100 000 person-years) and therefore the dissimilarity should be interpreted with caution.

We agree that some patients may receive clinical codes inappropriately despite not having a diagnosis of IgAV, and conversely that some individuals with IgAV may not have the diagnosis documented. Rather than relying on a single code, we used a comprehensive list to identify individuals with IgAV and reduce the risk of missing cases. Nevertheless, it is likely that some cases were not captured by this method. The cited work by Deshayes *et al* highlights potential pitfalls of using clinical codes.[6] However, this was from a single-centre study evaluating a different coding structure in a separate healthcare system to ours and is therefore not directly applicable.

It is notable that Hočevar and colleagues' cohort had a much higher age at IgAV diagnosis than our cohort. However, our cohort's mean age of diagnosis was closer to previous reports.[7–9] The adult-onset IgAV cohort did have an unexpectedly equal gender distribution, although our data show a trend towards higher incidence of hypertension (adjusted HR (aHR) 1.51, 95% CI 1.19 to 1.91 vs aHR 1.30, 95% CI 0.99 to 1.71) and chronic kidney disease (CKD; aHR 2.04, 95% CI 1.43 to 2.92 vs aHR 1.23, 95% CI 0.91 to 1.66) in men compared with women. It is possible that men tend to present with more severe disease and have therefore been over-represented in hospital-based IgAV cohorts.

We note with interest the baseline characteristics reported by Hočevar *et al* for their cohort of patients with IgAV, which reinforces some patterns we observed in our own data. The higher burden of baseline comorbidities for individuals who develop IgAV is intriguing, and the cause of this association is unclear.

Notably, our analyses were adjusted for baseline comorbidities including obesity, diabetes and dyslipidaemia, so these do not explain the increased incidence of hypertension and CKD observed in patients with IgAV. In addition, the observed associations were robust when our analysis was restricted to CKD or hypertension recorded at least 1 year after each participant's index date. This suggests that renal impairment and hypertension are not solely features of the acute illness. The mechanism underlying this association may involve secondary factors such as intraglomerular hypertension, glomerular hypertrophy and proteinuria. However, investigation of this was beyond the scope of our study.

There are many opportunities for further study of IgAV in adults. The incidence of this disease is not well characterised, and further population-based research will be required to clarify this. The high baseline prevalence of hypertension and diabetes mellitus in the described adult IgAV cohorts is currently unexplained. Explanation of this association may offer opportunities for interventions that improve long-term health outcomes in these patients.

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