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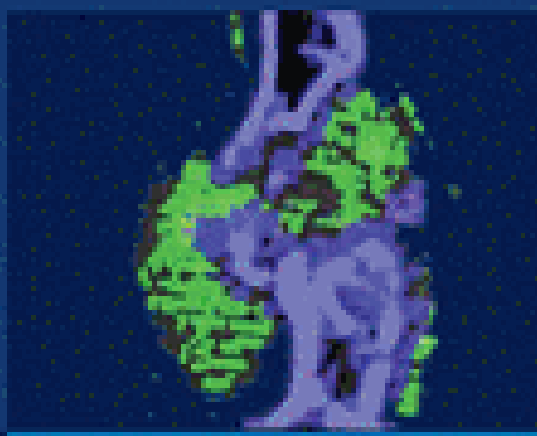

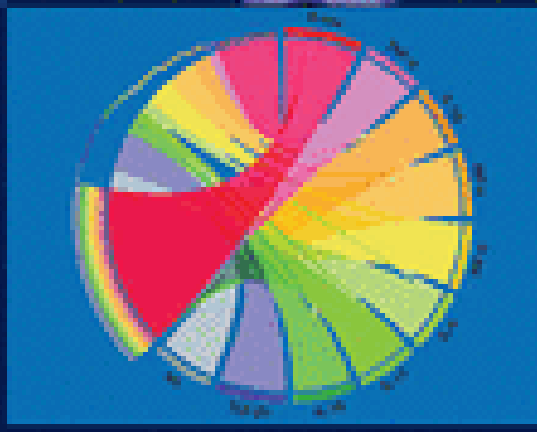

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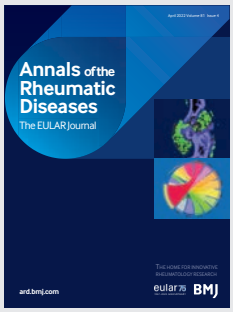

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What evidence is needed to demonstrate the beneficial effects of exercise for osteoarthritis?

Margreet Kloppenburg ,[1] François Rannou,[2] Francis Berenbaum [3]

Osteoarthritis (OA) of the lower limb is highly prevalent and result in a high disease burden. One of the key symptoms is pain, which is the result of various underlying peripheral and central pain mechanisms. Further, patients experience other impairments like stiffness with decreased range of motion, muscle atrophy and loss of muscle strength, loss of joint stability, all leading to difficulty in performing activities of daily living and diminished quality of life. Recently, also an increased risk of cardiovascular disease and mortality is reported in patients with knee or hip OA with walking disability.[1] Within the arsenal of treatments non-pharmacological therapies as exercise is one of the options. The opinion about the value of exercise for OA has considerably changed over time. Before the 1990s not exercise but rest, next to medication, was advocated for patients with acute exacerbations of arthritis, including for osteoarthritic joints.[2] From the end of the 1980s more and more evidence became available about beneficial effects of exercise in rheumatic and musculoskeletal diseases, including OA.

Benefits of exercise comprise joint specific effects as decrease in joint pain, increase in muscle strength and in proprioceptive acuity, increase in joint range of motion and flexibility leading to an improvement of physical functions like mobility. Furthermore, increasing physical activity and aerobic capacity lead to less cardiovascular diseases, hypertension, non-insulin dependent diabetes mellitus, osteoporosis and obesity. Also beneficial effects have been reported on psychological well-being.[3 4] In accordance, guidelines are in favour of exercise for the management of OA of the knee and hip since the 1990s. In the most recent recommendations of the American College of Rheumatology it is strongly recommended,[5] and in the guidelines of the Osteoarthritis Research Society International it is considered as the core treatment.[6] In many countries now reimbursed programmes are set up, such as GLA:D (Good Life with osteoArthritis in Denmark), Better management of patients with OsteoArthritis in Sweden and OsteoArthritis Chronic Care Programme in Australia, for patients with OA in primary care, where education, exercise and physical therapy form the core.

However, already from the start of recommending education and exercise, concerns about the evidence for efficacy were brought up.[7 8] This is due to the challenge to design clinical trials to investigate these treatments.[5 7–9] One difficulty in performing randomised clinical trials to investigate exercise is the blinding, which lowers the GRADE scores and feeds scepticism about the validity of non-pharmacological clinical trials. Through the way exercise is delivered it is impossible to blind the intervention for the patient or the health professional. Which is quite different from pharmaceutical interventions. What can be done is blinding for the outcome assessment by a blinded assessor,[10] however, often primary outcomes are subjective measures as pain obtained via a questionnaire, so a blinded assessor is not helpful in that respect.

Another difficulty and reason for scepticism is the control group. In the ideal trial design the control group receives everything except the 'active element of the intervention'. Education and exercise programmes consist of many components incorporating not only the exercise in itself, but also advice and education about self-management and physical function, and reassurance and sociopsychological support, in fact it is a package of care delivery or complex intervention.,[7 8 10 11] So, what should be controlled? Mostly, as control, usual care or no treatment while on a waiting list, is used, which enables an evaluation of the whole package. Supervised-exercise as part of physical therapy is depending on contact with a health professional. Some, bring up that in the study design also the contact and attention time should be taken in account through a control group.[7] However, it could also be argued that attention time is part of the package of care. Others bring up, that a placebo intervention should be considered.[12] But then the question is what the best placebo is for a complex intervention delivered as package, such as an education and exercise programme?

Lastly, it can be discussed what the appropriate outcome measures should be to assess efficacy for clinical trials investigating education and exercise. In clinical trials evaluating pharmacotherapeutic therapies 'pain' is mostly used as the primary outcome measure, with eventually 'function' as coprimary outcome, and for rehabilitation interventions this is also advised.[10] However, it is the question whether the benefits of multimodal management that integrates also potential cross-cutting benefits on other pathologies as well as potential sociopsychological benefits can be demonstrated with the usual primary outcomes.

Bandak *et al* perform an open-label randomised controlled equivalence trial including a placebo intervention.[13] A total of 206 patients (with a mean age of 68.4 years, 54% male) were equally randomised to an education and supervised exercise programme or intra-articular saline injections, under the presumption that both interventions are equally beneficial and as such communicated with the patients. The programme was an 8-week group-based programme, following the GLA;D protocol, and consisted of two 1.5-hour educational sessions and twelve 1-hour exercise sessions; an average of 11.1 sessions were attended (79.3%). The control group received an average of 3.4 ultrasound guided 5 mL saline injections (out of possible 4; 84.9%) in the knee over 8 weeks, after aspiration when appropriate. After 8 weeks, the education and exercise group improved more than the injection group (mean (SD) improvement of 10.0 (1.5) and 7.3 (1.5), respectively) on the Knee Injury and Osteoarthritis Outcome Score pain scale (primary outcome; range 0 (worst) to 100 (best)), a subjective pain scale assessed with a questionnaire. This difference was not statistically different between the groups (2.7 (95% CI −0.6 to 6.0), so the authors concluded that the interventions are equally effective. Patient global assessment improved statistically significantly more in the education

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and exercise group, than in the injection group, but other secondary outcomes did not, including performance tests. The authors conclude that an 8-week exercise and education programme provides beneficial effects on symptoms and function, that are equal to four intra-articular open-label saline injections over 8 weeks. What does this mean and what does it learn us?

In the trial by Bandak *et al*, the interventions are not blinded and the primary outcome measure is subjective as is mostly the case in education and exercise trials, which could lead to bias in the results. Semiobjective tests as walk test were also performed and assessed by a blinded observer, but also these are biased in a single blinded setting.[14] Tests as muscle strength are less biased, but not performed in this trial. In the control group patients are treated with four open-label intra-articular saline injections to control for the placebo response including placebo or contextual effect. In studies investigating OA placebo responses are generally high. Partly, this can be explained by regression to the mean and natural fluctuations in the disease course,[9] which affect both the intervention and control arm of a randomised clinical trial. On top of that a placebo or contextual effect can be seen, which can be ascribed to effects such as expectations of patients, patient–health professional interaction, meaning response, Hawthorne effect (behaviour change through being observed), and relief of anxiety.[14] Several studies have been done to investigate the factors that influence the size of the placebo effect, and these showed that the way of delivery is of considerable importance.[15] Bannuru *et al* showed in a systematic review with network meta-analyses that intra-articular placebo had an effect size of 0.58, which was statistically significantly higher than for oral placebo (standardised mean difference (SMD) 0.20, 95% CI 0.09 to 0.49).[16] In analyses estimating SMDs between different types of active treatments and placebo, for instance active intra-articular injections versus oral placebo, these SMDs could increase or decrease considerably.[16] No network analyses are available comparing education and exercise programmes with pharmaceutical treatments. So, the size of these two placebo effects cannot be compared, but we do know that intra-articular injections have a high placebo effect, which could be even higher for repeated injections in a short time frame. When the placebo effects between two groups are different, the true effect of a treatment cannot be estimated, which make a trial result hard to interpret.

An education and exercise programme is considered a complex intervention,[11] because of the properties of the intervention itself, but also because of the context in which it is delivered, and the interaction between the two. Education and exercise treatment consists of several components, which interacts, depends on behaviours, expertise and skills of those delivering and those receiving it, delivered by different healthcare organisations, and is flexible and tailored to the needs of the patients.[17] Research evaluating complex interventions encounter many challenges and difficulties as acknowledged by the UK Medical Research Council.[11 18] Therefor a framework was published, which was recently updated, for the development and evaluation of complex interventions.[17] Although it was acknowledged that no novel design exists that caters for 'complex interventions', it was emphasised that more options exist to design a study than randomised controlled trials, and guidance was given on the function of the intervention and usefulness of evidence.[17] Use of pragmatic randomised clinical trials investigating effectiveness or real world data could be helpful. Six core elements have been described that are crucial in development and evaluation: context, programme theory, stakeholders, uncertainty, intervention refinement and economic considerations.

Exercise, one of the components of the education and exercise programmes have been investigated extensively, especially in patients with knee OA. A recent systematic review and meta-analysis included 42 studies with in total 6863 patients evaluating 'pain immediately post-treatment'.[19] The estimated SMD was 0.5 (95% CI 0.37 to 0.63), where the control intervention was 'usual care', no treatment (eg, waiting list), a minimal intervention (eg, medication) or non-supervised exercise therapy (eg, home-based exercise treatment). For trials with low risk of bias it was somewhat higher, with less heterogeneity. Individually supervised exercises showed somewhat more efficacy (SMD 0.61) compared with group exercises (SMD 0.37), which was used in the trial by Bandak and colleagues.[13] The authors concluded that exercise is effective and clinically worthwhile. They also showed that based on an analysis using an extended funnel plot that an additional study will have very limited impact to change the current effect estimate to 'unclear if worthwhile'. Trials comparing two different effective treatments for OA could further increase our understanding of relative efficacy of different treatment options. In that light a recent trial comparing a physical therapy with intra-articular glucocorticoid injections is of interest.[20] In a 1-year clinical trial, 156 patients with symptomatic knee OA were randomised. Intra-articular triamcinolone acetonide (40 mg) up to three times over the 1-year trial period (mean 2.6 injections, range 1–4) was compared with physical therapy sessions: eight over the initial 4–6 weeks period and additionally one to three sessions at 4 months and 9 months reassessments (mean of 11.8 (range 4–22) attended visits). The personalised physical therapy sessions included instructions and images for exercises, joint mobilisations, and the clinical reasoning underlying the priorities, dosing and progression of treatment. Both intervention groups improved over 1 year, but the physical therapy showed to be more beneficial than intra-articular glucocorticoid injections, (mean between group difference 18.8; 95% CI 5.0 to 32.6) on the Western Ontario and McMaster University Osteoarthritis Index total score (range 0–240, higher scores indicating worse pain, function and stiffness). This study support the evidence for effectiveness of education and exercise treatment, and its role as core treatment.

All together there is compelling evidence to support the use of exercise therapy as treatment for OA, especially of the knee and hip. Still many studies are undertaken.[21] Performing studies to evaluate the efficacy of non-pharmacological programmes is challenging due to their complexity, and asking for different trial designs than in pharmacological treatments. Especially blinding of the participants for the treatment they receive is difficult. Many aspects needs further research.[21] Which (combinations of) modalities of education or exercise are most effective? What is most effective and safe in certain subpopulations, such as in patients with comorbidities? What is the minimal therapeutic dose required for beneficial effects? How can evidence-proven education and exercise programmes best be implemented? How can effects be maintained on the long term? And how can activity behaviour be changed? These are all important questions, which can be investigated in randomised clinical trials, but also in observational studies, using real-world data.

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Cutaneous signs and mechanisms of inflammasomopathies

Carina Borst , Dörte Symmank , Mathias Drach, Wolfgang Weninger

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ABSTRACT

The emerging group of autoinflammatory diseases (AIDs) is caused by a dysregulation of the innate immune system while lacking the typical footprint of adaptive immunity. A prominent subgroup of AIDs are inflammasomopathies, which are characterised by periodic flares of cutaneous signs as well as systemic organ involvement and fever. The range of possible skin lesions is vast, ranging from urticarial, erysipelas-like and pustular rashes to erythematous patches, violaceous plaques and eventual necrosis and ulceration. This review provides a structured overview of the pathogenesis and the clinical picture with a focus on dermatological aspects of inflammasomopathies. Current treatment options for these conditions are also discussed.

INTRODUCTION

The skin is not only our outermost protection layer against pathogens and environmental factors; it also mirrors the health status of internal organs. Therefore, examination of the skin helps physicians in the assessment of patients with systemic diseases. Autoinflammatory syndromes are defined by a hyperactive innate immune system and commonly reveal cutaneous lesions early in the disease course. While certain signs and symptoms may direct experienced physicians towards the correct diagnosis, the large pattern variability and overlap with other conditions present a significant challenge. It is important for dermatologists and physicians of other specialties dealing with autoinflammatory syndromes to be aware of the possible skin manifestations. This comprehensive review discusses the range of cutaneous signs visible in inflammasomopathies with reference to the causative molecular mechanisms.

CUTANEOUS SIGNS OBSERVED IN AIDS

Autoinflammatory diseases (AIDs) were initially introduced in 1990.[1] These conditions show signs of harmful inflammatory processes although lacking the typical footprint of the adaptive immune system seen in autoimmune diseases. Manifold genetic aberrations have been described as underlying factors leading to overshooting innate immune responses that potentially damage cells in many tissues. Frequently, signs of this hyperactivated immune system appear on the skin accompanied by systemic symptoms such as fever or fatigue. Careful clinical evaluation of the morphology, pattern, localisation, onset, duration and the possible triggers of the cutaneous lesions may steer physicians to a specific diagnosis of autoinflammation. A wide range of lesions has been described in autoinflammatory syndromes; an effort of classifying cutaneous signs of monogenic AID resulted into nine distinct categories, including non-specific maculopapular rashes, neutrophilic urticaria, pustular skin rashes and panniculitis, as well as different vasculopathies, disorders with hyperkeratotic and pigmented skin lesions, and lastly also bullous and aphthous lesions (online supplemental table 1).[2] The various differences in the histopathological evaluation are summarised in online supplemental table 2.

INFLAMMASOMOPATHIES

The inflammasome is considered a central player in the regulation of immune responses.[3] As one of the main defenses against intracellular danger signals, distinct types of inflammasomes with various activators and mediators have been identified (figure 1). The main building blocks of the different forms of inflammasomes are largely similar. Sensors, often pattern recognition receptors, initially detect potential pathogenic signals. They connect with or without an adaptor, for example apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), to the effector molecule, mainly caspase-1. The activated effector molecule then splices procytokines, such as interleukin-1β (IL-1β) and IL-18, into their active forms.[4 5] Furthermore, a lytic programmed cell death called pyroptosis can be triggered by the inflammasome.[5–7]

IL-1β is central to the innate immune system as it induces further expression of proinflammatory cytokines, drives the fever response, prostaglandin synthesis, tissue infiltration and activation of various immune cells as well as Th17 differentiation.[8 9] IL-18 induces the expression of interferon-gamma, thus influencing the adaptive immune system without inducing fever.[10] When caspase-1 cleaves gasdermin D, a molecule involved in pyroptosis, rapid rupture of the plasma membrane commences, releasing the intracellular contents of the cell, which in turn stimulate neighbouring cells.[11] These actions secure clearance of pathogens, making the inflammasome a critical regulator of the inflammatory reaction against potentially pathogenic endogenous and exogenous stressors such as microbes, viruses, fungi or parasites. It is obvious that alteration of this delicate process, for example through the chronic activation of the inflammasome, can result in an inflammatory state with distinct clinical signs and symptoms, culminating in organ damage.

THE NLRP3 INFLAMMASOME-ASSOCIATED AUTOINFLAMMATORY DISEASE

NLRP3 (NOD-containing, LRR-containing and pyrin domain-containing protein 3 or Nod-like receptor protein 3) acts as the sensor of the prototypic

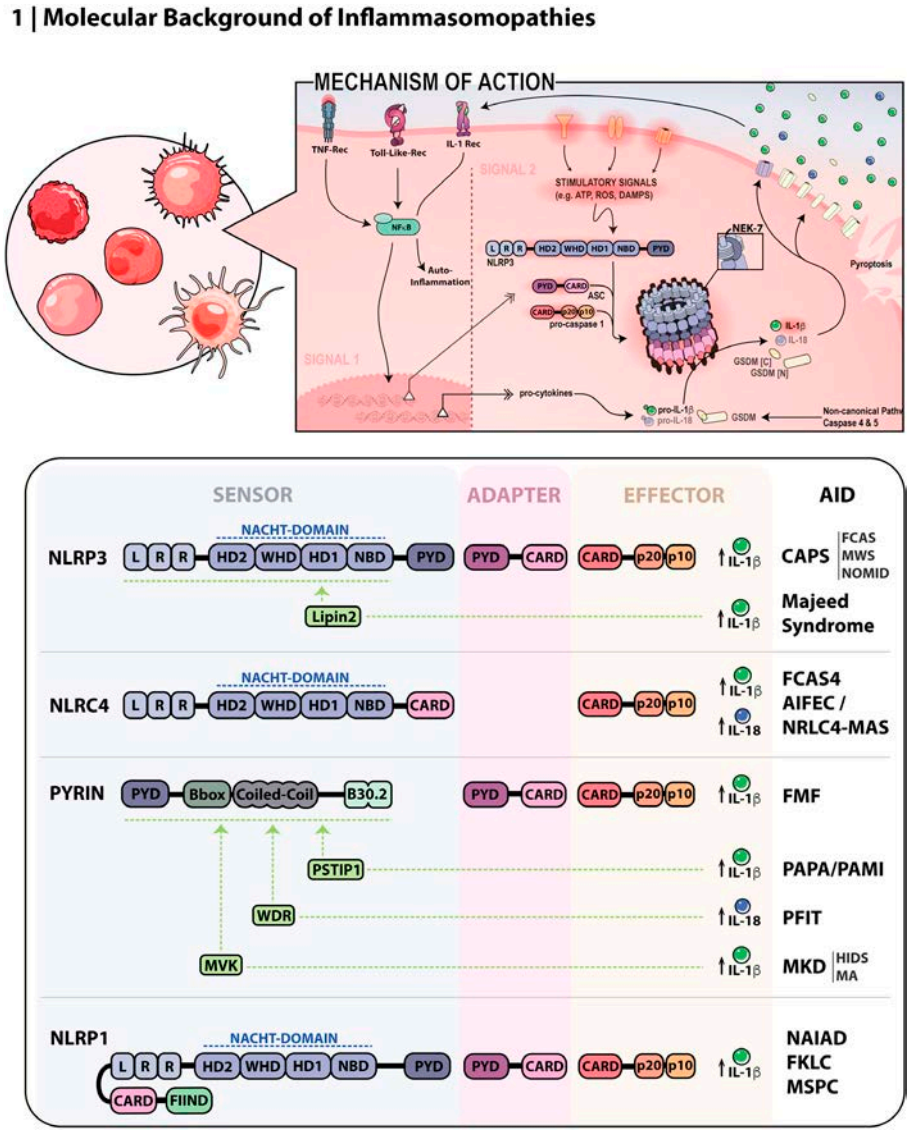

Figure 1 Inflammasomopathies. The inflammasome is a defensive mechanism of many innate immune cells (macrophages, monocytes, neutrophils, basophils and dendritic cells are shown in the upper left panel). The differences in the inflammasome's building blocks and the main product (interleukin) of each AID are listed in the midsection of the figure. NRLP3 forms the canonical inflammasomes by interacting with the adapter molecule ASC and the effector molecule pro-caspase-1. Exemplary mechanism of NLRP3 is shown in the upper right panel. The priming signal (signal 1) can be delivered by various receptors, such as members of the toll-like receptors (mostly known via TLR4), the TNF receptor family and the IL-1 receptor family. Priming of the NLRP3 through other means (eg, via post-translational modulation) is not shown. Signal 1 induces the cellular upregulation of NLRP3, de novo protein synthesis, as well as the transcription of procytokines (especially pro-IL-1β) by NF-κB-mediated signalling pathways. Signal 2 includes various stimuli-like PAMPs or DAMPs but can also include ROS, mitochondrial dysfunction, ATP or ion influx, sensed by NLRP3. This activates the assembly of the proteins needed for an active inflammasome. Nek-7 bridges the gap between the NLRP3s and mediates the oligomerisation process. After ASCs connects to the card domain of the procaspases, it auto-proteolyzes into the p10 and p20 subunits. The active caspase-1 then cleaves procytokines of the IL-1 family (mainly pro-IL-1β, pro-IL-18) in their active form and GSDM in GSDM [C] and GSDM [N]. GSDM [N] oligomerises and forms pores in the membrane. IL-1β and IL-18, as well as GSDM [C] can be excreted through GSDM dependent and independent pores. Pores made by the GSDM [N] can lead to a cell death called pyroptosis. Gasdermin D can also be activated by the non-canonical pathway through caspase-4 or caspase-5, leading to pyroptosis. The non-canonical activation cascade is not shown. PSTPIP1, WDR and MVK-associated autoinflammatory disease indirectly influence the pyrin inflammasome, leading to an activation of the pyrin inflammasome. AIDs, autoinflammatory diseases; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; DAMPs, damage-associated molecular patterns; GSDM, gasdermin D; GSDM [C], C-terminal fragment; GSDM [N], N-terminal fragment; IL, interleukin; PAMPs, pathogen-associated molecular patterns; ROS, reactive oxygen species.

inflammasome complex, the NLRP3 inflammasome.[3] Gain of function mutations of *NLRP3* drive the group of AIDs termed cryopyrin-associated periodic syndrome (CAPS).[12 13] CAPS is a rare, autosomal dominant genetic disorder with a prevalence of 2.8 cases per 1 million people.[14] It refers to three clinical entities with a continuous severity spectrum and considerable overlap: familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset-multisystem inflammatory disease (NOMID; also known as chronic infantile neurological cutaneous articular syndrome, CINCA) (figure 2-1).[15] Another AID involving the NLRP3 inflammasome is Majeed syndrome with an estimated prevalence of <1–2 cases per 1 million. It is

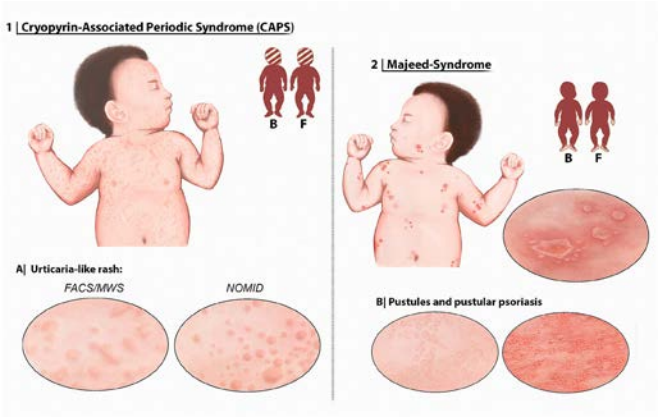

Figure 2 Cutaneous signs of NLRP3-associated inflammasomopathies CAPS (1). Three clinical entities with a continuous clinical severity spectrum and considerable overlap: FCAS (mild), MWS (intermediate), NOMID/CINCA (severe). Cold-induced, non to mildly-pruritic urticaria-like rash consisting of flat wheals in symmetrical distribution, involving the trunk and/or extremities, in children sometimes also the face (B, back view; F, front view). Majeed syndrome (2). Pallor due to anaemia and Sweet-like skin lesions (dull red, raised, painful, itchy plaques with 0.5–4 cm in diameter, central depression and yellow crusts due to serosanguineous discharge, commonly studded with vesicles and pustules) on the face, trunk and extremities, palms and soles usually spared (B, back view; F, front view). Pustules and pustular psoriasis (B). CINCA, chronic infantile neurological cutaneous articular syndrome; FCAS, familial cold autoinflammatory syndrome; MWS, Muckle-Wells syndrome; NOMID, neonatal-onset-multisystem inflammatory disease.

caused by a loss of function mutation in the gene coding for lipin-2, a protein involved in the regulation of fat metabolism inside cells and a known regulator of the NLRP3 inflammasome.[16 17]

Familial cold autoinflammatory syndrome (FCAS)

FCAS, the mildest form of CAPS, has its onset in infancy, rarely in childhood or adolescence.[14] It is characterised by recurrent episodes of urticarial rashes, fever and arthralgia triggered by cold exposure.[18] An urticarial rash 1–2 hours after cold exposure is one of the first signs of a flare-up, commonly followed by low-grade fever and arthralgia 4–6 hours later. Leucocytosis appears in laboratory evaluation after about 10 hours. Additional findings include conjunctivitis, malaise, fatigue, myalgia, headache, high levels of C reactive protein (CRP) and serum amyloid A (SAA), as well as an increase in erythrocyte sedimentation rate (ESR). Attacks are usually self-limited and resolve within 12–24 hours.[19]

Cutaneous signs

FCAS is characterised by a cold-induced, non to mildly-pruritic urticaria-like rash consisting of flat wheals (figure 2-1). In adults, the lesions tend to be symmetrically distributed involving the trunk and/or extremities but usually sparing the head. In children, the rash is commonly more widespread, sometimes even involving the face. Contrarily to classical cold urticaria, direct contact with a cold object does not trigger a flare-up, hence the ice cube test is negative.[2 20]

Muckle-Wells syndrome (MWS)

MWS is characterised by a triad of urticaria, deafness, and rarely also amyloidosis.[21] It is more severe than FCAS but milder than NOMID/CINCA. The age of onset is typically in infancy.[12 22] Flares are triggered by cold and hot temperatures, stress, physical exercise or occur without an identifiable trigger and typically resolve spontaneously.[19 23] They usually occur weekly and last 12–36 hours causing an urticaria-like rash, fever, malaise, headache, arthralgia or conjunctivitis. In a subset of patients, more serious long-term complications such as progressive sensorineural hearing loss and AA amyloidosis may develop.[22] Laboratory findings include leucocytosis, and elevated levels of inflammatory markers and SAA.

Cutaneous signs

Cutaneous manifestations include an evanescent, non to mildly-pruritic urticaria-like rash consisting of flat wheals with a similar distribution as seen in FACS (figure 2-1).[20 23 24]

Neonatal-onset-multisystem inflammatory disease (NOMID)/ chronic infantile neurological cutaneous articular syndrome (CINCA)

NOMID, also known as CINCA, is the most severe form of CAPS. It usually presents shortly after birth with an urticarial rash and a chronic state of inflammation. It shows frequent flare-ups occurring randomly or triggered by cold temperatures.[13 25] Clinical features include a maculopapular rash and/or urticarial lesions, fever, malaise, lymphadenopathy, splenomegaly, headache, aseptic meningitis, cognitive deficits, sensorineural hearing loss, papilledema, conjunctivitis, uveitis, AA amyloidosis, impaired growth and arthropathy. Affected individuals exhibit characteristic facial features, such as frontal bossing, saddle nose deformity and exophthalmos. If left untreated, NOMID is fatal in 30% of cases.[26–29] Laboratory findings include leucocytosis, eosinophilia, anaemia, coagulopathy, an increase in ESR, and elevated CRP and SAA levels.[19 30 31]

Cutaneous signs

Patients usually present with a permanent, non-pruritic maculopapular and/or urticarial rash shortly after birth which intensifies during flare-ups (figure 2-1). The distribution of the rash underlies temporal changes occurring throughout the day.[20 25 32 33]

Treatment

IL-1β plays a pivotal role in the pathogenesis CAPS, thus agents interfering with this cytokine are commonly used in clinical practice. Canakinumab, an anti-IL-1β monoclonal antibody showed rapid and sustained treatment response in a randomised controlled trial including patients with CAPS.[34 35] Other trials confirmed the efficacy of canakinumab in patients with CAPS.[36–38] Anakinra,[30 39] an IL-1 receptor antagonist and rilonacept,[40 41] an IL-1 trap, also yielded high efficacy in clinical trials.

Majeed syndrome

Lipin-2 is an enzyme which gained interest as a potential target for lipodystrophies or hypertriglyceridaemia as it plays a role in the development of adipose tissue and metabolism of triglycerides.[42 43] Lipin-2, coded by *LPIN2* on chromosome 18, is abundant in the liver, in granulocytes and macrophages, the central nervous system and the gastrointestinal tract and catalyses the conversion between phosphatidic acid (PA) and 1,2,-diacylglycerol (DAG).[44] PA and DAG are two lipids highly relevant for the generation of glycerophospholipids needed in the biogenesis of the cell membrane.[45–47] In the immune system, lipin-2 is known to control proinflammatory signalling initiated by high levels of saturated fatty acids and additionally

influences the NLRP3 inflammasome through restricting the signalling cascade initiated by TLR4 (figure 1 (signal1)) and the ATP receptor P2×7R (figure 1 (signal2)).[48] Majeed syndrome is a monogenic, autosomal recessive disease caused by the loss of function of lipin-2 which leads to a chronic autoinflammatory state.[16 17 49]

Onset of Majeed syndrome is in infancy with recurrent episodes of fever, Sweet-like skin lesions, chronic recurrent multifocal osteomyelitis (CRMO) and dyserythropoitic, hypochrome, microcytic anaemia. Hepatosplenomegaly is another possible finding.[50] Disease flares usually last for 2–4 days and occur 1–4 times per month.[51 52] The trigger for attacks is unknown. CRMO bone inflammation can lead to severe arthralgia, joint swelling, joint deformities and slow growth. Due to the anaemia, patients suffer from fatigue, weakness, shortness of breath and a pale skin. Apart from anaemia, laboratory studies show high ESR and sometimes leucocytosis. Cultures of blood, bone and pustular lesions are negative for bacteria of fungi.[50 51]

Cutaneous signs

Besides pallor due to anaemia, some patients present with dull red, raised, painful, itchy plaques with 0.5–4 cm in diameter, central depression and yellow crusts due to serosanguineous discharge resembling Sweet-like skin lesions (figure 2-2). The surface of these lesions is commonly studded with vesicles and pustules. Lesions develop on face, trunk and extremities. The palms and soles are usually spared.[50] Lesions resembling pustules and pustular psoriasis have also been observed.[16]

Treatment

The treatment of Majeed syndrome—given its low prevalence—remains empiric. Anakinra and canakinumab led to a marked clinical and laboratory improvement in a case report of two patients who failed to respond to the TNF-α inhibitor etancercept and corticosteroids.[53] Other case reports support the finding of a good response to anti-IL-1 treatment as opposed to TNF-α inhibition.[49]

THE NLRC4 INFLAMMASOME-ASSOCIATED AUTOINFLAMMATORY DISEASE

NLRC4-associated autoinflammatory disease (NLRC4-AD) encompasses a spectrum of autoinflammatory entities with the milder form, FCAS4 at one end and the life-threatening form, autoinflammation with infantile enterocolitis (AIFEC) on the other (figure 3). These monogenic entities are caused by a mutation in the *NLRC4* gene with an autosomal-dominant inheritance pattern.[54–56]

Familial cold autoinflammatory syndrome 4 (FCAS4)

FCAS4 is a rare disease with symptoms similar to the FCAS (CAPS). Only two families have been reported.[56 57] In most cases, FCAS4 first presents during infancy with recurrent episodes of fever, skin rashes, conjunctivitis, arthralgia and myalgia. Common triggers for flare-ups include cold exposure, change in weather, infection and emotional stress.[56 57] Flares occur between 2 and 60 times per year. The duration of flares has not been reported in medical literature yet. In laboratory studies, ESR and levels of CRP, IL-1β and IL-18 are increased.[56 57]

Cutaneous signs

Skin manifestations in FCAS4 are age-dependent and present in paediatric patients with a non-itching urticarial rash. Adults often show painful erythematous nodes on the lower extremity.

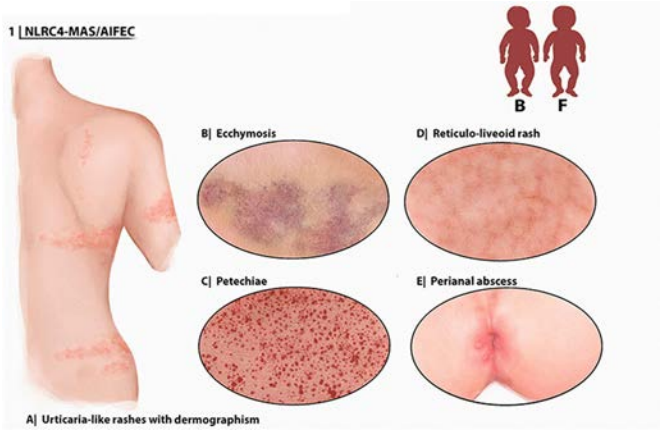

Figure 3 Cutaneous signs of NLRC4-associated inflammasomopathies. NLRC4-MAS/AIFEC. Wide range of possible lesions: evanescent, urticaria-like rashes with dermographism (A), urticaria-like rashes evolving into ecchymosis (B) and petechiae (C), and maculopapular skin rashes resolving and being replaced by a reticulo-liveoid rash (D). Lesions occur on face, trunk, arms and legs. Other possible lesions include perianal (E) and facial abscesses (not shown) and itchy nodules (not shown) (B, back view; F, front view). FCAS4 not shown. AIFEC, autoinflammation with infantile enterocolitis; FCAS, familial cold autoinflammatory syndrome; NLRC4-MAS, NLRC4-associated macrophage activation syndrome.

Those nodes can occur isolated or in combination with non-itching urticarial patches on the face, trunk, arms and legs.[56 57]

Treatment

In a case series, anakinra exhibited varying degrees of treatment response ranging from lack of treatment effect to complete remission.[57]

NLRC4-associated macrophage activation syndrome (NLRC4-MAS)/autoinflammation with infantile enterocolitis (AIFEC)

NLRC4-MAS is a rare disease with less than 15 reported patients.[54 55 58–62] The first signs and symptoms usually manifest in the neonatal period. Clinically, it presents as recurrent bouts of fever, enterocolitis with loose stools up to bloody diarrhoea and vomiting, a wide range of different cutaneous manifestations, hepatosplenomegaly and lymphadenopathy. Flares can last up to several weeks and were reported to recur up to 11 times in 6 months.[59 62] Disease episodes can be triggered by upper respiratory tract infections, minor surgery, physical stressors and emotional stress.[54 59] If left untreated, life-threatening multiorgan failure including renal failure, respiratory distress syndrome and disseminated intravascular coagulopathy can occur.[54 55] Enterocolitis was reported to subside after the first year of life.[54] Serum IL-18 remains highly elevated independent of treatment and even during interictal periods. Other laboratory findings include pancytopaenia (anaemia, thrombocytopaenia, leucopaenia), elevated levels of transaminases, hyperferritinaemia, hypertriglyceridaemia, hypofibrinaemia and elevated levels of CRP, IL-1β and IL-2R as a sign of MAS.[54 55 61] Leucocytosis was described as well.[60] Despite inflammation, ESR is only initially increased and paradoxically decreases despite systemic inflammation due to hypofibrinemia in MAS.[25 60]

Cutaneous Signs

Skin manifestations cover a wide range of lesions (figure 3). Reported lesions include evanescent, urticaria-like rashes with

dermographism, urticaria-like rashes evolving into petechiae and ecchymosis, as well as maculopapular skin rashes which may resolve and be replaced by a reticulo-liveoid rash. Common sites of involvement are the face, trunk, arms and legs.[55 58 61] Other manifestations include perianal and facial abscesses, as well as erythematous, itchy nodules.[59 60]

Treatment

Treatment with recombinant human IL-18BP (rhIL-18BP) has been used successfully in case reports.[62] Other less effective treatment options include corticosteroids, anakinra, infliximab (TNF-α inhibitor), ciclosporin, vedolizumab (anti-integrin α4β7 antibody), colchicine, and emapalumab (anti-IFNγ antibody).[55 62 63]

THE PYRIN INFLAMMASOME-ASSOCIATED AUTOINFLAMMATORY DISEASE (PAAD)

Besides NLR-associated inflammasomes, other proteins are known to form inflammasome platforms. Among those proteins is pyrin, which is abundant in neutrophils and monocytes/macrophages.[44 64] Pyrin acts as an indirect sensor for the inflammasome making use of a family of molecular switches, namely, RhoA-C GTPases. These GTPases cycle between 'Off'' (GDP-bound) and 'On' (GTP-bound) states, which changes their affinity for protein binding.[65] Through this mechanism, they regulate multiple processes, such as cytoskeleton dynamics, cell cycle, phagocytosis and immune cell signalling, and are often targeted by invading bacteria (eg, Enterohemorrhagic *Escherichia coli*, *Clostridium difficile*, Yersinia species), parasites and viruses.[66–69] Since the pyrin inflammasome is known to be influenced by various factors, there are multiple ways in which disturbances can lead to its overactivation, elevated production of IL-1β and IL-18, pyroptotic activity, and ultimately to clinically distinct autoinflammatory conditions.[70]

The most prominent disease in this AID family is familial Mediterranean fever (FMF) with thousands of known cases worldwide. FMF as well as the pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) are caused directly by the mutation of the *MEFV* gene, which encodes pyrin.[71 72] Mutations influencing the pyrin inflammasome indirectly include PAPA (pyogenic arthritis, pyoderma gangrenosum, acne)[73] and PAMI (proline–serine–threonine phosphatase-interacting protein 1 (PSTPIP1)-associated myeloid-related proteinaemia inflammatory syndrome),[74] as well as PFIT (autoinflammatory periodic fever, immunodeficiency and thrombocytopaenia)[75] and the mevalonate kinase deficiencies (MKDs) HIDS (hyperimmunoglobulinaemia D with periodic fever syndrome)[76] and MA (mevalonic aciduria).[77] Maculopapular rashes or inflammatory plaques, as well as acne, ulcers or pyoderma gangrenosum are cutaneous signs often associated with the dysregulation of the pyrin inflammasome (figures 4 and 5).

Familial Mediterranean fever (FMF)

FMF is most prevalent in the Mediterranean area (eg, Turkey, Armenia) with 1–9 cases per 1000 people. Most patients show biallelic mutations on exon 10 in the region encoding for pyrin.[78 79] FMF is usually diagnosed in adolescence with a mean age of 14 years showing recurrent attacks of fever, an erysipelas-like rash, abdominal pain (peritonitis), chest pain (pleuritis, pericarditis), joint pain (monarthritis), exertional myalgia and/or headache.[80–82] AA amyloidosis, a long-term sequelae due to chronic inflammation, is a major cause of mortality.[83] Several triggers like cold exposure, emotional stress, exhaustion, long-duration travel, exercise, infection, surgery, menstruation and

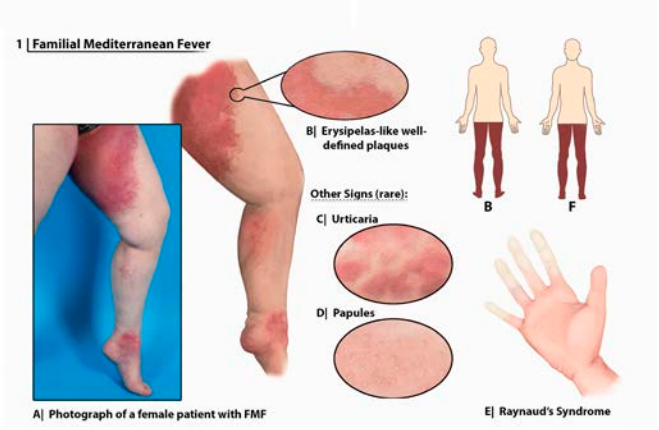

Figure 4 Cutaneous signs of FMF. Mostly well-defined erysipelas-like skin lesions (A, B) on the lower extremity (B, back view; F, front view). Rare skin manifestations: pruritic urticarial lesions (C), scattered pruritic papules (D), palmoplantar erythema and Raynaud-like lesions (E) and vasculitis (not shown). FMF, familial Mediterranean fever.

intrauterine devices have been described.[80 84] Flares usually last for 1–3 days and recur in intervals from days to several years.[2 25 81 85] Laboratory findings during flares include leucocytosis with neutrophilia and an elevation of acute phase reactants (CRP, ESR, SAA, fibrinogen). The presence of proteinuria warrants further work-up to exclude renal amyloidosis.[86 87]

Cutaneous Signs

FMF skin lesions usually present as erysipelas-like, erythematous, tender, swollen and well-defined plaques on the lower extremity (figure 4).[88] Rare skin manifestations include scattered pruritic papules, pruritic urticarial lesions, palmoplantar erythema and Raynaud-like lesions.[2 89 90] Infrequently, vasculitis (IgA-related,

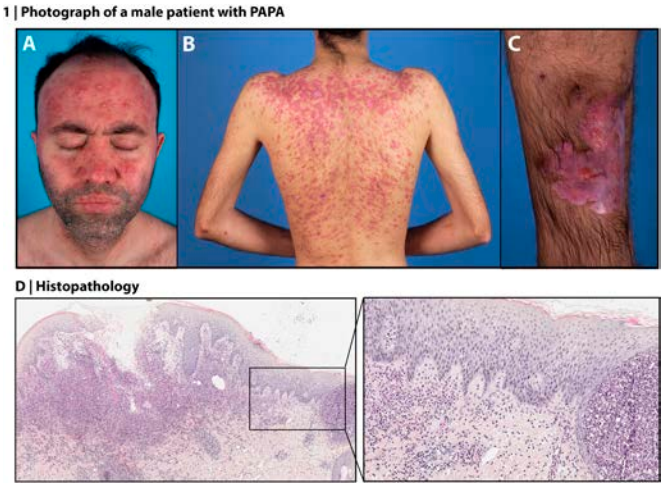

Figure 5 Cutanenaous signs of PSTPIP1-associated inflammatory diseases PAPA. Sterile abscesses (A, B), severe cystic acne (A, B) and deeply erythematous and violaceous papules (B) and ulcerated plaques (B, C) on the scalp, face, trunk and legs. A detailed case report of this patient was published by Geusau *et al*.[105] Biopsy reveals dilated follicular ostia studded with neutrophils. The surrounding dermis also shows an infiltrate mainly consisting of neutrophils (D). PAMI not shown. PAMI, PSTPIP1-associated myeloid-related proteinaemia inflammatory syndrome; PAPA, pyogenic arthritis, pyoderma gangrenosum, acne; PSTPIP1, proline–serine–threonine phosphatase-interacting protein 1.

polyarteritis nodosa, small and medium-sized vessel diseases) occurs as well.[2 91]

Treatment

Treatment goals for FMF are threefold: general symptom control, a decrease in flare frequency and the prevention of secondary AA amyloidosis. Colchicine is the first-line agent for flare control and amyloidosis prevention. In cases of colchicine resistance, other treatment options include IL-1 inhibition (canakinumab, anakinra), TNF-α inhibition (adalimumab, etanercept, infliximab), thalidomide, the IL-6 antagonist tocilizumab and the Janus kinase inhibitor tofacitinib.[92–96] Treatment options for patients who develop arthralgia despite colchicine therapy include conventional synthetic disease-modifying antirheumatic drugs (methotrexate, sulfasalazine), TNF-α antagonists and intra-articular steroid injections. Non-steroidal anti-inflammatory drugs (NSAIDs) or, if refractory, prednisone or IL-1 antagonists can be used to treat FMF-related myalgias.[92]

Pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND)

PAAND occurs in childhood with unknown prevalence.[72 97 98] It is characterised by recurrent flares of fever, a wide range of possible skin lesions, arthralgia, myalgia and lymphadenopathy. Isolated cases with associated cardiomyopathy and hepatosplenomegaly have been described. In contrast to FMF, there is no clear association with amyloidosis or serositis.[97 98] Flares take several weeks to resolve. Causal triggers and intervals remain to be determined. Laboratory abnormalities during flares include elevated levels of acute phase reactants and proinflammatory cytokines (IL-1β, IL-1Rα, IL-6, TNF-α), as well as leucocytosis with eosinophilia and anaemia.[72 97 98]

Cutaneous signs

PAAND encompasses a large variety of possible skin lesions with a predilection for the trunk, arms and face. Described lesions include severe acne, sterile skin abscesses, pyoderma gangrenosum, hidradenitis suppurativa, neutrophilic panniculitis, (pruritic) neutrophilic small-vessel vasculitis and oral ulcers.[2 97 98]

Treatment

There is no consistent treatment approach to PAAND. Some patients respond to anti-IL-1 treatment, others seem to benefit from anti-TNF-α therapy. Masters *et al* described successful treatment with the IL-1 receptor antagonist anakinra in one patient who failed to respond to corticosteroids and methotrexate. Colchicine led to partial remission in another patient.[97] The TNF-α antagonist infliximab showed a long-lasting improvement of PAAND signs and symptom in a patient nonresponsive to corticosteroids and anakinra. After a recurrence of disease symptoms, the patient was switched from infliximab to adalimumab.[72] Hong *et al* reported partial disease control with corticosteroids, cyclophosphamide, mycophenolate mofetil, methotrexate, azathioprine and TNF-α antagonists in two patients.[98]

PSTPIP1-associated inflammatory diseases (PAID)

The mutation of PSTPIP1 leads to a variety of PAID (reviewed by Boursier *et al*[99]), the most prominent are PAPA[73] and PAMI.[74 100 101]

Pyogenic arthritis, pyoderma gangrenosum and acne syndrome (PAPA)

The prevalence of PAPA is unknown with disease onset in childhood. It is characterised by recurrent pauciarticular, aseptic, pyogenic, erosive, deforming arthritis triggered by minor trauma, aseptic abscesses, cystic acne and pyoderma gangrenosum. Painful joint involvement may be the first sign of disease. By puberty, joint symptoms tend to subside while skin lesions increase.[102] Fever is rare.[2] Haemolytic anaemia, cervical lymphadenopathy, bleeding diathesis, splenomegaly and recurrent infections have been reported as well.[103 104] Laboratory studies show elevated levels of acute-phase reactants, IL-1β and leucocytosis with neutrophilia.[103 105]

Cutaneous signs

Skin lesions usually manifest or worsen during puberty and include sterile abscesses, severe cystic acne and deeply erythematous, violaceous papules as well as ulcerated plaques (figure 5). The skin pathergy test (hyper-reactivity in response to minor trauma) is commonly positive in PAPA. Skin lesions can be found on the scalp, face, trunk and legs.[73 103–108]

Treatment

IL-1 blocking agents, such as anakinra or canakinumab, yielded good treatment responses in case reports and case series.[103 105] Other effective agents include TNF-α antagonists (adalimumab, infliximab), corticosteroids, methotrexate, tacrolimus, ciclosporin and the antibiotic dapsone.[103 107 108]

PSTPIP1-associated myeloid-related proteinaemia inflammatory syndrome (PAMI)

About 35 cases of PAMI have been reported so far with the mean age of onset of 13 months.[74 106 109] It is characterised by a state of chronic inflammation including the skin, hepatosplenomegaly, failure to thrive and arthralgia or arthritis.[74] Osteomyelitis of the talus, fibula and tibia have been reported in one case.[106] Typical laboratory findings include hyperzincaemia and hypercalprotectinaemia with increased levels of MRP8 and MRP14 as well as pancytopaenia including anaemia, thrombocytopaenia and neutropaenia as a result of bone marrow dysfunction.[74 100] One report described a mild increase in ESR and CRP.[106]

Cutaneous signs

Pyoderma gangrenosum, characterised by ulcers surrounded by a purple halo with undermined borders occur in 44% of all patients, typically on the lower extremities.[74 100 106] Another common type of skin lesion is acne of the face and back.[100 106] Pustular rashes and abscesses may manifest as well. A positive skin pathergy test has been reported in one patient.[106]

Treatment

Several agents have been used with varying degrees of success in PAMI. In the initial report by Holziger *et al*, anti-IL-1 treatment (anakinra, canakinumab), ciclosporin or corticosteroids were partially beneficial. Other treatments (etanercept, adalimumab, infliximab, tacrolimus, methotrexate, intravenous immunoglobulins, colchicine) were less effective.[74] Klötgen *et al* showed that ciclosporin and a combination of corticosteroids and topical tacrolimus were efficacious, whereas treatment with infliximab, canakinumab or the IL-17A inhibitor secukinumab showed no additional benefit.[106] If disease control cannot be achieved, allogenic haematopoietic stem cell transplantation might be considered.[110]

Autoinflammatory periodic fever, immunodeficiency, and thrombocytopaenia (PFIT)

PFIT is caused by a missense mutation in the *WDR1* gene which promotes F-actin depolymerization and regulates cytoskeletal dynamics.[75] Its prevalence is unknown and disease onset is between the neonatal period and early infancy.

It is characterised by fever attacks with unknown triggers, lasting for 3–7 days, recurring every 6–12 weeks. Additional clinical features include oral inflammation and ulcers leading to scarring and microstomia as well as perianal ulcerations. Furthermore, a predisposition for recurrent infections, such as pneumonia and septic arthritis, has been described. Laboratory studies show an elevation in acute phase reactants (CRP, SAA, ferritin), elevated levels of IL-18, leucocytosis with neutrophilia, and thrombocytopaenia during flares.[75]

Cutaneous signs

PFIT presents with severe recurrent oral inflammation and ulcers leading to scarring and microstomia. Recurrent perianal ulcerations can occur as well.[75]

Treatment

In the initial report by Standing *et al*, corticosteroids, colchicine and anakinra were associated with a partial response in two patients. In another patient, allogeneic hematopoietic stem cell transplantation was beneficial. As a key pathogenic mediator in PFIT, IL-18 was suggested as a possible treatment target.[75]

Mevalonate kinase deficiencies (MKD)

MKD describes a reduction of the mevalonate kinase (MK) activity caused by autosomal recessive mutations in the *MVK* gene[111–113] and encompasses two genetically similar conditions with distinct clinical manifestations.[114] As the name suggests, the disease severity depends on the remaining activity of MK, with the milder phenotype hyperimmunoglobulinaemia D with periodic fever syndrome (HIDS) and the more severe condition mevalonic aciduria (MA).[114 115]

Hyperimmunoglobulinaemia D with periodic fever syndrome (HIDS)

In 2007, a total of 244 patients were recorded in the International HIDS Database.[115] The exact prevalence is unknown. Disease onset is at a mean age of 6 months. According to published data, 78.1% of patients experience their first flare within the first year of life.[115] HIDS is characterised by flares of fever, malaise, abdominal complaints including pain, diarrhoea, and vomiting, (cervical) lymphadenopathy, skin rash, arthralgia, arthritis and headache. Flares typically last 3–7 days and recur every 2–12 weeks with a decreasing frequency during the course of life.[76 115 116] Skin and joint symptoms resolve slowly.[117] Disease attacks can occur without any preceding event, however reported precipitating factors include vaccination, surgery, minor trauma and physical as well as emotional stress.[76 115 117] Elevated polyclonal IgD antibodies, as a hallmark of the disease, are reported in 78% of patients, accompanied by elevated IgA levels in 64% of patients.[115] Other findings during a flare-up include leucocytosis, a rise of ESR, CRP and SAA levels, as well as MA.[113 115]

Cutaneous signs

Approximately 70%–80% of patients develop cutaneous manifestations during disease flares.[115 118] The most common skin lesions are erythematous macules and papules, followed by urticarial lesions, erythematous nodules, annular erythema, palpable purpura, erythematous pustules and petechiae involving the extremities, trunk and neck.[118] Roughly every second patient develops oral or bipolar ulcers.[115] Cutaneous manifestations are usually asymptomatic or are only rarely accompanied by pain or itching.[76 118]

Treatment

The approach to treatment in patients with HIDS consists of two main components: symptomatic and preventive care. Symptomatic therapy includes NSAIDs,[119 120] if refractory, glucocorticoids[120 121] or anakinra.[120 122] Since canakinumab has a slower onset of action compared with anakinra, it is preferably used as a preventive treatment of patients with HIDS.[123] Other preventive treatment options include etanercept[120] and tocilizumab.[124] Statins are currently not recommended, despite blocking epigenetic and transcriptional changes in HIDS monocytes. In a preliminary analysis of six patients with HIDS, Simon *et al* showed no significant reduction in febrile days in patients taking simvastatin compared with placebo.[114]

Mevalonic aciduria (MA)

The exact prevalence of MA is unknown as only approximately 30 cases have been reported so far.[125] The first signs and symptoms of MA are present at birth. Patients experience recurrent flares of fever, lymphadenopathy, morbilliform skin rashes, subcutaneous oedema, diarrhoea, vomiting and arthralgias. Flares usually last 4–5 days and may occur up to 25 times a year.[126] No causative trigger has been identified so far. In addition, patients exhibit characteristic facial features (dolichocephaly with a delayed closure of the skull sutures and open fontanelles, a triangular face with down-slanting palpebral fissures and large, posteriorly rotated, low set ears), as well as ocular involvement (cataracts, blue sclera and retinitis pigmentosa), psychomotor retardation, seizures, failure to thrive and hepatosplenomegaly.[126–128] Apart from persistent MA, laboratory findings include elevated inflammatory markers (CRP and ESR), anaemia as well as increased serum IgD, IgA, IgE, creatine kinase and transaminases.[77 125–127]

Cutaneous signs

Available data on cutaneous manifestations are scarce. Hoffmann *et al* reported a morbilliform rash without specifying the site of involvement.[126]

Treatment

MA treatment remains challenging. In a small case series, treatment with anakinra induced partial remission in one of two patients.[122] Canakinumab can be used as an alternative to anakinra.[129 130] A trial of the statin lovastatin had to be discontinued due to worsening of clinical symptoms and rhabdomyolysis.[126] Stem cell transplantation might be considered as a treatment option; however, relapses can occur.[131 132]

THE NLRP1-ASSOCIATED AUTOINFLAMMATORY DISEASE (NLRP1-AD)

Keratinocytes make up most of the epidermis. They are important defenders of the body against trauma, exposure to ultraviolet radiation as well as one of the first cellular defenses in the fight against pathogens. The NLRP1 inflammasome is the main inflammasome in keratinocytes and mutations lead to an over-reactive immune response to these stressors. This leads to AID affecting the skin as well as a heightened risk of cancer.[133] Epithelial skin cancers, such as squamous cell carcinoma commonly develop after many years of sun exposure and

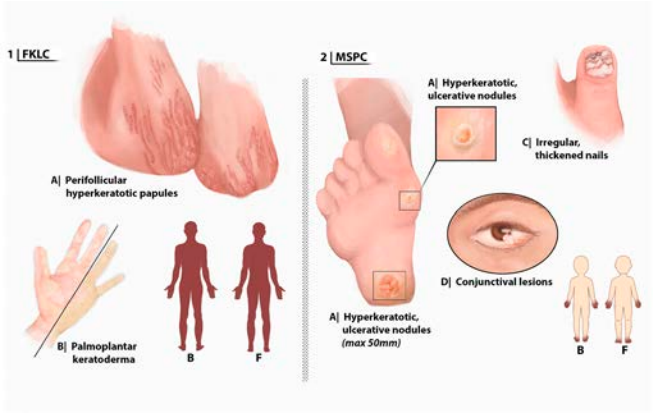

Figure 6 Cutaneous signs of NLRP1-associated inflammasomopathies: FKLC (1). Perifollicular, hyperkeratotic papules arranged in a linear or reticular pattern on the arms, legs and trunk (1A). Palmoplantar keratoderma (1B). Other manifestations (not shown): seborrheic-like dermatitis or rosacea on the face, mucosal involvement, nail changes, ocular involvement, alopecia (B, back view; F, front view). MSPC (2). Numerous, hyperkeratotic, ulcerative nodules on the palms and soles resembling keratoacanthomas, usually 8–15 nodes with a size between 5 and 50 mm in diameter (2A, 2B). Dystrophic nails with thickening of the nail plate and mottling edges (2C). Conjunctival lesions such as squamous dyskeratotic lobules (2D) (B, back view; F, front view). Hyperkeratosis pilaris not shown. NAIAD not shown. FKLC, familial keratosis lichenoides chronica; MSPC, multiple self-healing palmoplantar carcinoma; NAIAD, NLRP1- associated autoinflammation with arthritis.

tumor-promoting inflammation represents one of the hallmarks of cancer. Autoinflammatory syndromes with prominent skin involvement should be monitored carefully for skin cancer development. MSPC (multiple self-healing palmoplantar carcinoma), NAIAD (NLRP1-associated autoinflammation with arthritis) and FKLC (familial keratosis lichenoides chronica) broadly represent these NLRP1-AD (figure 6).

NLRP1-associated disease (NAIAD)

NAIAD is a rare autoinflammatory keratinisation disease of unknown prevalence caused by autosomal dominant or recessive mutations in the *NLRP1* gene.[134–136] The first signs usually develop in childhood as recurrent episodes of unprovoked fever lasting 3–4 days, dyskeratosis, oligoarticular and polyarticular arthritis, vitamin A deficiency and intermittent elevated CRP levels. Other findings include chronic infection, antinuclear antibodies and high transitional B cell levels.[134]

Cutaneous signs

NAIAD is characterised by skin dyskeratosis with phrynoderma (follicular hyperkeratosis), (filiform) hyperkeratosis, and papules with pseudo-comedones on the trunk, arms, hands, legs and feet. Ungual dyskeratosis has been described as well.[134]

Treatment

NAIAD skin manifestations have been successfully treated with the retinoid acitretin. For arthritis, etanercept and anakinra improved clinical symptoms, whereas methotrexate was not helpful. Supplementation of vitamin A led to either no disease modification or worsening of the disease.[134]

Familial keratosis lichenoides chronica (FKLC)

FKLC is a rare semidominant disease with only about 80 reported cases.[137] It shows scaly papules on the trunk and extremities appearing at a mean age of 35.6 years, ranging from 6 months to 78 years. About a quarter of all FKLC are paediatric cases.[137] Skin lesions occur without any preceding event such as sun exposure or trauma. FKLC was reported to be associated with glomerulonephritis, haematological malignancies, hypothyroidism and hepatitis. Laboratory studies are usually unremarkable. Alterations in laboratory values—if occurring—are related to the associated diseases.[137]

Cutaneous signs

FKLC is characterised by perifollicular, hyperkeratotic papules arranged in a linear or reticular pattern (figure 6-1).[137–139] The papules are mostly asymptomatic, a mild pruritus was reported to occur only in a third. The papules are usually symmetrically distributed and located on the arms, legs and trunk (88.7%, 84.5% and 59.2%, respectively).[137] Another common finding (69% of cases) is seborrhoeic-like dermatitis or rosacea on the face. Seborrhoeic-like dermatitis presents as infiltrated papules or plaques with hyperkeratotic scales typically on the convex areas of the face and usually sparing body folds. Other cutaneous manifestations include palmoplantar keratoderma (28.2%), mucosal involvement (28.2%), nail changes (26.7%; yellow discolouration, thickening, ridging), ocular involvement (19.3%) and alopecia (7%).[137]

Treatment

Phototherapy, sunlight exposure and systemic retinoids were associated with a clinical improvement. Treatment with topical corticosteroids, salicylic acid or vitamin D derivatives was not or only to a limited extent helpful.[137]

Multiple self-healing palmoplantar carcinoma (MSPC)

MSPC is a rare monogenic AID caused by an autosomal dominant mutation in the *NLRP1* gene with less than 30 reported cases as of 2021.[140 141] Mean onset of MSPC is 8.8 years ranging from 1 to 25 years of age.[140 142] It manifests as self-healing ulcerative nodules on the palms and soles as well as ocular and nail lesions. These lesions usually occur at sites of friction. There are no reports on laboratory values in patients with MSPC .[140]

Cutaneous signs

MSPC is characterised by numerous hyperkeratotic, ulcerative nodules on the palms and soles resembling keratoacanthomas (figure 6-2). Patients usually display 8–15 nodes with a size between 5 and 50 mm in diameter, which grow rapidly, evolve over weeks to years and regress spontaneously after 6 months, leaving atrophic scars.[140] In addition, 80% of patients with MSPC develop conjunctival lesions such as squamous dyskeratotic lobules in their second decade of life.[140–142] Less common manifestations include dystrophic nails with thickening of the nail plate and mottling edges, and hyperkeratosis pilaris.[142]

Treatment

Cutaneous lesions usually regress spontaneously after 6 months. Surgical removal of atrophic scars is an additional treatment option. The use of retinoids leads to a stop of lesion formation, but regression as well.[140 143]

THE EMERGING FIELD OF INFLAMMASOME-ASSOCIATED DISEASES

The growing interest of the clinical and scientific community as well as sequencing efforts have steadily expanded the field of inflammasome-associated diseases. For some of these conditions, a definitive link to genetic alterations in the inflammasome has not been established but is suspected. While beyond the scope of this review to list all of these diseases, an example is the NLRP12-associated AID FCAS2 with over 60 reported cases worldwide and a similar clinical presentation as the mildest form of CAPS.[144–146] NLRP12 is a known inhibitor of the inflammasome mainly influencing the canonical and non-canonical NFκB-pathway, as well as neutrophil recruitment and migration during infection.[147–151] To what extent FCAS2 is driven by the inflammasome is uncertain and treatment with anakinra (anti-IL-1R), while showing initial clinical improvement, was followed by a relapse in one patient.[152]

The effector cytokines of the inflammasome, namely IL-1β and IL-18, can also be induced by non-canonical activation of caspases-4 or caspases-5. The functional consequences of canonical and non-canonical inflammasome activation remain largely the same, as crosstalk between these systems regularly triggers the activation of the canonical NLRP3 inflammasome (reviewed by Downs *et al*).[153] Göös *et al* recently described a mutation in a transcription factor resulting in the constitutive activation of the caspase-5 mediated non-canonical inflammasome.[154] The disease is termed CAIN (C/EBPε-associated autoinflammation and immune impairment of neutrophils) and shows high fever and purulent paronychia, eventually progressing into ascending lymphangitis, oral ulcerations, abscesses, pyoderma gangrenosum, impaired wound healing, and abdominal pain. Treatment data targeting IL-1β or IL-18 are still outstanding.[154]

Future genetic and functional assays and treatment regimens targeting the inflammasome or its effector proteins are needed to identify the actual impact the inflammasome dysregulation has on the respective emerging diseases.

CONCLUDING REMARKS

AIDs, including the subgroup of inflammasomopathies, commonly involve the skin with the range of possible lesions being vast (online supplemental table 1). While NLRP3-associated and NLRC4-associated inflammasomopathies commonly manifest as urticarial rashes on the skin, patients suffering from pyrin-associated inflammasomopathies exhibit erysipelas-like rashes (FMF), acne, abscesses and pyoderma gangrenosum (PAAND, PAID), maculopapular rashes (MKD) or oral and perianal ulcers (PFIT). Hyperkeratosis is a frequent finding of NLRP1-associated inflammasomopathies (NAIAD, FKLC, MSPC).

A definite diagnosis of AID should be made by the interplay of clinical and cutaneous presentation, as well as histopathological, laboratory and genetic findings. It is important to be aware of the possible skin manifestations, as cutaneous lesions may direct clinicians towards the correct diagnosis, even though the pattern variability and overlap with other, non-autoinflammatory conditions represents a significant challenge.

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No efficacy of anti-IL-23 therapy for axial spondyloarthritis in randomised controlled trials but in post-hoc analyses of psoriatic arthritis-related 'physician-reported spondylitis'?

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ABSTRACT

The three monoclonal antibodies ustekinumab, guselkumab and risankizumab targeting the p 40 or the 19 subunit of interleukin -23 have now been approved for the indication psoriasis and the former two also for psoriatic arthritis (PsA). Ustekinumab and risankizumab have appeared ineffective in randomised controlled trials with patients with axial spondyloarthritis (axSpA), but post-hoc analyses of PsA trials have now suggested that they may improve back pain symptoms potentially induced by axial inflammation based on PsA. Here we argue that, based on the absence of efficacy in axSpA, this is unlikely and more probably due to generic, non-specific effects, which are not adequately covered by the tools developed for the assessment of inflammation in axSpA.

INTRODUCTION

The introduction of anti-interleukin -23 (IL-23) therapy with monoclonal antibodies against both subunits of IL-23, p 19 and p 40 such as ustekinumab, risankizumab and guselkumab has brought major achievements for patients with psoriasis, psoriatic arthritis (PsA) and chronic inflammatory bowel diseases. In contrast, there was no efficacy in several randomised controlled trials (RCTs) with patients with axial spondyloarthritis (axSpA) including ankylosing spondylitis (AS[1 2]), initiated after an early pilot study had suggested that there may be a clinically relevant response. Ustekinumab and guselkumab have been shown to clearly work in PsA. The manufacturer of ustekinumab, a drug approved for the indication of PsA a long time ago on the basis of two central studies PSUMMIT 1 and PSUMMIT 2, has published the results of two post-hoc analyses performed on those patients with PsA who had axial symptoms that now has obfuscated the picture—one already some time ago[3] and one just recently.[4] A new diagnosis has been introduced, named 'physician-reported spondylitis', with potentially far stretching consequences. Recently, a small study has reported a positive effect on low back pain in patients with PsA treated with guselkumab,[5] and this was followed by a post-hoc analysis on PsA with assumed axial involvement based on the guselkumab in PsA trials DISCOVER 1 and DISCOVER 2.[6] Similarly, in a trial that intended to treat patients with axial PsA with secukinumab[7]—the first time that this diagnosis was used as the target population in an RCT. This diagnosis was assumed to be sufficiently met, when the CASPAR criteria were fulfilled and the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) was higher than 4 (as usually done in all axSpA trials). Taking a critical look on this development, we think that it is time to stress the problems and limitations of these approaches, not only since there is a clear marketing incentive here, but also since study results are based on fallible assumptions, unlikely to hold when critically challenged.

WHAT IS BEHIND THIS SCIENTIFICALLY FALLIBLE APPROACH TO INVENT A DIAGNOSIS OF 'PHYSICIAN-REPORTED SPONDYLITIS'?

It has been discussed for a long time whether axial PsA is a separate entity of PsA or if it should just be seen as a form of axSpA. There were some arguments favouring the latter view,[8 9] in line with a recent study stressing the influence of HLA-B27 on inflammatory axial disease in PsA.[10] Differences between axial PsA and axSpA have also been highlighted[11] even though the radiographic part of the New York criteria has been found useful also for axial PsA.[12] Altogether, this means that regarding the existential question of one versus two diseases, the jury is still out.

In addition, the unexpected failure of the anti-IL-23 RCTs in axSpA[1 2] has undoubtedly been a disappointment for the pharmaceutical companies involved, and the suggestion of improvements in a few typical axSpA measures may have incentivised the post-hoc analyses of the PsA trials. There is no trial on guselkumab in axSpA though. However, the STAR study with guselkumab in axial PsA is currently recruiting patients (NCT04929210).

WHAT IS THE CONTENT OF THESE TWO POST-HOC ANALYSES ?

Taken together, we have two formal RCTs, which have clearly shown that ustekinumab works in PsA, and we have two post-hoc analyses of these RCTs, looking at patients with back pain present on request, as well as axSpA instruments suggesting increased disease activity: as if these patients with PsA were patients with axSpA. The main difference between the two studies is that the second one[4] focused on 127 anti-TNF-naive patients of PSUMMIT 1 and PSUMMIT 2, while the first one[3] had looked at all patients with 'signs of axial involvement' as explained (n=256). In this post-hoc study, a modification of a (published) modified version of the BASDAI (mBASDAI) was used, which

Box 1 Bath Ankylosing Spondylitis Disease Activity Index (BASDAI

1. Fatigue
2. Spinal pain
3. Arthralgia (joint pain) or swelling
4. Enthesitis or inflammation of tendons and ligaments (areas of localised tenderness where connective tissues insert into bone)
5. Morning stiffness duration
6. Morning stiffness severity

All scores are made on a visual analogue or a numerical rating scale (0–10).
In the original publication on the modified BASDAI[12] questions 3 and 4 were omitted.

simply excluded BASDAI question 3 (the original modification excluded questions 3 and 4[13]).

Another disease activity measure for axSpA, the disease activity score ASDAS has been shown to be superior to the BASDAI but both measures are still widely used (boxes 1 and 2). The ASDAS was used in the second post-hoc analysis.[4] Both, BASDAI and ASDAS had been evaluated in patients with AS, implying that these patients had been included on the basis of back pain, stiffness and decreased mobility. However, the performance of the BASDAI when evaluated in patients with PsA was not convincing.[14]

The baseline demographics[3] reveal a rather typical peripheral PsA population: 45 years old on average, 45% women, less than 7 years PsA duration and on average 14 swollen and 24 tender joints, half of them with dactylitis. More than 80% of patients had signs of enthesitis (mean Maastricht Ankylosing Spondylitis Enthesitis Score (MASES) around 5), and not <90% had confirmed peripheral erosions. The mean Psoriasis Area and Severity Index (PASI) was 14.5 and half of the patients was on treatment with methotrexate. In spite of this typical peripheral PsA-*Gestalt*, the BASDAI (only the back pain question: how would you describe the overall level of AS neck, back or hip pain you have had during last week?) was more than 6/10, which made the authors suggest that axial involvement was present.[3]

The second study[4] showed slightly lower values; 20% of patients were on glucocorticoids. The mean ASDAS was 3.9 and the BASDAI as well as the mBASDAI were approximately 7, while only a minority (25%) was human leukocyte antigen (HLA)-B27+. Looking at efficacy, there were no differences between the ustekinumab doses, and the mean change of BASDAI was −1.8 at week 12 and −2.1 at week 24, independent of HLA-B27 status.[4] Since international recommendations prescribe a change of at least two for an individual patient with axSpA to prove efficacy, this appears to be a rather mild improvement. However, this is only approximative since these are changes at the group and not at an individual level.

Box 2 Ankylosing Spondylitis Disease Activity Index (ASDAS)

Back pain (BASDAI question 2)
Peripheral pain/swelling (BASDAI question 3)
Duration morning stiffness (BASDAI question 6)
Patient global
CRP
(C reactive protein (CRP) value <2 mg/L (0.2 mg/dL) is not allowed. If CRP is below the limit of detection or is <2 mg/L (<0.2 mg/dL), the fixed value of 2 mg/L (0.2 mg/dL) will be entered).
All scores are made on a visual analogue or a numerical rating scale (0–10).

Of the 1120 patients in the two DISCOVER studies, 312 (28%) were included in this analysis,[6] of whom 118 were on placebo, and 194 in the guselkumab every 4 weeks or 8 weeks groups with 61% being male, mean age 45 years. Out of 190 patients with known HLA-B27 status 30% were positive. There was one major methodological difference regarding patient selection in this post-hoc analysis, since patients were included if there was 'evidence' of axial disease documented by previous imaging or pelvic radiography at screening consistent with sacroiliitis, as confirmed by the investigator[6]—which may or may not be any better than simply claiming 'physician-reported spondylitis'. In any case it is known that diagnostic decisions only based on imaging of the sacroiliac joints are problematic, and that is even more true for PsA where degenerative changes may cause problems for the differential diagnosis. This may also have influenced the results of a study which reported, based on sacroiliac radiographs, underdiagnosed axial disease especially in female patients with PsA.[15]

In this study[6] increased disease activity—as if these patients were active patients with axSpA—was assumed if the (m)BASDAI was >4 as in the ustekinumab studies described above.[3 4] At week 24, mean changes from baseline in BASDAI and ASDAS were significant: −2.7 and −1.4 in both guselkumab groups versus −1.3 and −0.7 in the placebo group, respectively. The results for mBASDAI and spinal pain were similar. Improvements with guselkumab were seen at week 24 independent of HLA-B27 status.[6] The latter is rather an argument against the claim to have successfully treated axial PsA.[10]

IS THERE AN EFFECT OF ANTI-IL-23 ON SUPPOSED BUT UNPROVEN AXIAL INFLAMMATION IN PSA ?

On the basis of a rather negative evaluation, the absence of evidence that BASDAI works in PsA[16 17] and the fact that none of the patients in PSUMMIT 1 and PSUMMIT 2 had been included because of back pain but rather because of peripheral arthritis, known to be frequently associated with fatigue, generalised entheseal pain and morning stiffness, we find it difficult to believe that the mild change in the mBASDAI observed in a post-hoc selection of patients with PsA, can be attributed to assumed but unproven inflammation in the axial skeleton of patients with the leading symptom of peripheral PsA. We rather think that the improvement of back pain is a bystander effect of general improvement in patients who had severe peripheral PsA, with involvement of many joints and skin and a high burden of disease. It is well known that pain-spreading mechanisms (central sensitisation) may cause back pain which may improve on improvement in general well-being.

In conclusion, we think that post-hoc analyses of peripheral PsA trials do not suffice to prove that ustekinumab and guselkumab (or anti-IL-23 treatment in general) are efficacious for 'real inflammation' in the axial skeleton of patients with PsA. Far better definitions for axial PsA are needed to be used for inclusion in clinical trials and we strongly support the ongoing Assessment of Spondyloarthritis International Society (ASAS)/Group for Research and Assessment of Psoriasis and Psoriatic Arthritis (GRAPPA) initiative in this regard.

Disease-specific measures, such as BASDAI and ASDAS, should be applied in the context in which they have been developed, in this case proven axial inflammation. Once axial inflammation of patients with peripheral PsA has been proven, for example, by MRI, BASDAI and ASDAS may be appropriate tools to follow these patients over time, but not before that has been achieved. We also do not apply DAS28 in patients with fibromyalgia without a diagnosis of rheumatoid arthritis.

In conclusion, we propose to abandon the term 'physician-reported spondylitis'. The likelihood that axial PsA is much different from axSpA in terms of treatment response in patients with proven axial inflammation is, in our opinion, rather low.

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CLINICAL SCIENCE

Olokizumab, a monoclonal antibody against interleukin 6, in combination with methotrexate in patients with rheumatoid arthritis inadequately controlled by methotrexate: efficacy and safety results of a randomised controlled phase III study

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ABSTRACT

Objective To evaluate the efficacy and safety of olokizumab (OKZ) in patients with active rheumatoid arthritis despite treatment with methotrexate (MTX).

Methods In this 24-week multicentre, placebo-controlled, double-blind study, patients were randomised 1:1:1 to receive subcutaneously administered OKZ 64 mg once every 2 weeks, OKZ 64 mg once every 4 weeks, or placebo plus MTX. The primary efficacy endpoint was the proportion of patients achieving an American College of Rheumatology 20% (ACR20) response at week 12. The secondary efficacy endpoints included percentage of subjects achieving Disease Activity Score 28-joint count based on C reactive protein <3.2, Health Assessment Questionnaire Disability Index at week 12, ACR50 response and Clinical Disease Activity Index ≤2.8 at week 24. Safety and immunogenicity were assessed throughout the study.

Results A total of 428 patients were randomised. ACR20 responses were more frequent with OKZ every 2 weeks (63.6%) and OKZ every 4 weeks (70.4%) than placebo (25.9%) (p<0.0001 for both comparisons). There were significant differences in all secondary efficacy endpoints between OKZ-treated arms and placebo. Treatment-emergent serious adverse events (TESAEs) were reported by more patients in the OKZ groups compared with placebo. Infections were the most common TESAEs. No subjects developed neutralising antidrug antibodies.

Conclusions Treatment with OKZ was associated with significant improvement in signs, symptoms and physical function of rheumatoid arthritis without discernible differences between the two regimens. Safety was as expected for this class of agents. Low immunogenicity was observed.

Trial registration number NCT02760368.

Key messages

What is already known about this subject?

- Olokizumab (OKZ) is a new humanised monoclonal antibody targeting interleukin 6 ligand.
- Two placebo-controlled randomised phase II trials of OKZ in rheumatoid arthritis (RA) showed that it was significantly better than placebo across a range of doses; however, these studies were conducted in patients who had previously failed antitumour necrosis factor therapy and were of 12 weeks' duration.
- Long-term extension studies of these two controlled trials were conducted, but they were open-label and uncontrolled and all patients received the same dose of OKZ, 120 mg given every 2 weeks.

What does this study add?

- This study is the first of three phase III randomised controlled trials of OKZ in RA.
- In contrast to the phase II studies that were conducted in patients who had failed anti-TNF therapy, the current study was performed in patients who had an inadequate response to methotrexate.
- This phase III study was of 6 months' duration and tested two regimens of OKZ versus placebo and met all primary and ranked secondary efficacy endpoints.
- This study provides important information on the efficacy, safety and quality of life effects of OKZ that were not previously known.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease that if left inadequately treated can lead to significant disability, morbidity and mortality.[1–3] Current guidelines recommend a treat to target strategy in order to attain acceptable level of disease control and prevent long-term disability.[1 3] A number of effective therapies with different modes of action are currently available for RA; however, many patients with active RA fail to achieve defined targets of therapy, namely low disease activity or remission.[1 3 4]

The proinflammatory cytokine interleukin 6 (IL-6) plays a significant role in the pathogenesis of RA and two anti-IL-6 receptor (IL-6R) antibodies have been shown to be relatively safe and effective

Key messages

How might this impact on clinical practice or future developments?

- The current phase III study of OKZ will be part of future registration of this agent in various countries.
- OKZ was already approved for use in the Russian Federation.
- The data provided in the study will be very important for clinicians who might want to use this agent in their practice once it is approved since it provides meaningful controlled data on the efficacy and safety of this agent in a population of patients with inadequate response to methotrexate.

and are approved for treatment of RA.[5–9] Olokizumab (OKZ) is an anti-IL-6 monoclonal antibody that binds directly to IL-6 at a specific site and neutralises its activity through blocking hexamer formation of the extracellular signalling complex inhibiting transmembrane signalling.[10] In early clinical studies it was shown that OKZ resulted in a rapid reduction in the level of IL-6 and C reactive protein (CRP) that lasted over an extended period of time due to OKZ's long half-life of approximately 31 days.[11]

OKZ in doses ranging from 60 mg to 240 mg administered every 2 weeks or every 4 weeks was relatively safe and effective in reducing signs and symptoms of RA in two phase II randomised controlled trials in patients with RA who had failed to respond to antitumour necrosis factor (anti-TNF) therapy.[12 13] Based on findings from these two studies, as well as information from earlier studies, two doses of OKZ, 64 mg every 2 weeks and 64 mg every 4 weeks, were selected for advancement to phase III.[11] The lowest two doses tested in phase II were chosen to achieve efficacy while minimising potential adverse effects. Here we report the full results of the first completed phase III study of OKZ in patients with active RA despite treatment with methotrexate (MTX).

METHODS

Study design

This phase III, randomised, double-blind, placebo-controlled, parallel-group, multicentre trial was conducted at 42 hospitals in Russia, Belarus and Bulgaria from May 2016 to April 2019. Written informed consent was obtained from each patient.

After week 24, patients had the choice of either enrolling into an ongoing open-label study or entering the safety follow-up period.

Patient inclusion and exclusion criteria

Adults were eligible for inclusion if they had active RA (swollen joint count ≥6 (66-joint count), tender joint count ≥6 (68-joint count) and CRP >6 mg/L) classified by the American College of Rheumatology/European League Against Rheumatism 2010 revised classification criteria[14] for at least 12 weeks prior to screening and had an inadequate response to treatment with MTX for at least 12 weeks at a dose of 15–25 mg/week (or ≥10 mg/week if intolerant to higher doses). The dose and route of administration of MTX must have been stable for at least 6 weeks.

Exclusion criteria were other inflammatory or rheumatic diseases and Steinbrocker class IV functional capacity. Also excluded were those who had a prior exposure to IL-6 or IL-6R inhibitors, Janus kinase inhibitors, those treated with cell-depleting agents or those concurrently on disease-modifying antirheumatic drugs (DMARDs) other than MTX. Prior use of biologic DMARDs was an exclusion criterion with the exception of subjects who discontinued anti-TNF therapy due to reasons other than lack of efficacy. Non-steroidal anti-inflammatory drugs and glucocorticoids in doses less than or equal to 10 mg/day prednisone or equivalent were allowed if their doses were stable during the 2 weeks prior to study enrolment. Patients with a history of malignancies within the last 5 years (successfully treated carcinoma of the cervix in situ, basal cell carcinoma and squamous cell carcinoma of the skin were allowed if beyond 1 year prior to screening), recurrent infections, primary or secondary immunodeficiency, hepatitis B or C, active tuberculosis (TB) or other uncontrolled medical conditions, or prespecified abnormal laboratory values were excluded. Patients with latent TB infection were allowed to participate if they had started appropriate anti-TB therapy at least 30 days prior to randomisation (see online supplemental material for additional selection criteria).

Randomisation and blinding

Patients were randomised 1:1:1 to receive subcutaneous injections of OKZ 64 mg every 2 weeks, OKZ 64 mg once every 4 weeks, or placebo (PBO) for 24 weeks with continuation of their background MTX using an automated randomisation system. Subjects who discontinued the randomised treatment earlier were required to continue the study without study treatment administration; patients could discontinue study treatment but completed the study.

All patients, investigators, clinical site staff, contract research organisation's staff and the sponsor's staff directly involved in the study were blinded. Joint assessments were performed by independent assessors, blinded to study drug assignment and all other study assessments (see online supplemental material for additional details).

Rescue medication

Starting at week 14, non-responders, defined as subjects in any treatment group who did not improve by at least 20% in both swollen and tender joint counts (66–68 joints), were prescribed rescue medication (sulfasalazine and/or hydroxychloroquine) in addition to their study treatment (see online supplemental material for details of the prior and concomitant medications).

Endpoints

The primary endpoint was the proportion of patients achieving the American College of Rheumatology 20% (ACR20) response at week 12.

Ranked secondary endpoints were percentage of subjects achieving Disease Activity Score 28 based on C reactive protein (DAS28-CRP) <3.2 at week 12, improvement in physical ability from baseline to week 12 measured by the Health Assessment Questionnaire Disability Index (HAQ-DI), ACR50 response at week 24 and percentage of subjects with Clinical Disease Activity Index (CDAI) ≤2.8 (remission) at week 24.

Quality of life was assessed using several questionnaires including Short Form-36 (SF-36) Physical Component Summary (PCS), Mental Component Summary (MCS) and total scores, and the Functional Assessment of Chronic Illness Therapy-Fatigue Scale (FACIT-F).

Standard safety monitoring, including assessment of adverse events, serious adverse events and laboratory tests via the central laboratory, was performed regularly.

Determination of antidrug antibodies (ADA) in plasma samples was done using electrochemiluminescence assay (Covance

Laboratories, Harrogate, North Yorkshire, UK). For detection of neutralising ADAs, a cell-based assay was used (Eurofins BioPharma Product Testing Munich, Planegg/Munich, Germany).

An independent external Data and Safety Monitoring Board reviewed the safety data throughout the study. Major adverse cardiovascular events (MACE) were adjudicated by a Cardiovascular Adjudicated Committee and were defined as cardiovascular death or death from undetermined cause, non-fatal myocardial infarction, non-fatal stroke, transient ischaemic attack, hospitalisation for unstable angina requiring unplanned revascularisation and coronary revascularisation procedures.

Statistical analyses

The ACR20 response at week 12 for each of the active treatment groups was compared with PBO using a 2×2 χ^2 test for equality of proportions. To control the overall type I error rate at a one-sided $\alpha=0.025$, Bonferroni adjustment was used for the tests related to each of the OKZ dose regimens versus PBO (ie, one-sided $\alpha=0.0125$ for each dose). A gate-keeping strategy with a fixed order of hypothesis was used for the primary and secondary endpoints within each OKZ dose regimen independently (figure 1).

To detect a difference between at least one OKZ dose regimen and PBO, a sample size of 420 patients randomised in a 1:1:1 ratio was estimated to ensure sufficient disjunctive power (100% for testing the primary hypothesis (ACR20 at week 12) and 98% for the secondary endpoint of DAS28-CRP <3.2 rate at week 12).

The secondary endpoints that were binary in nature were analysed as per the primary endpoint. For analyses of binary

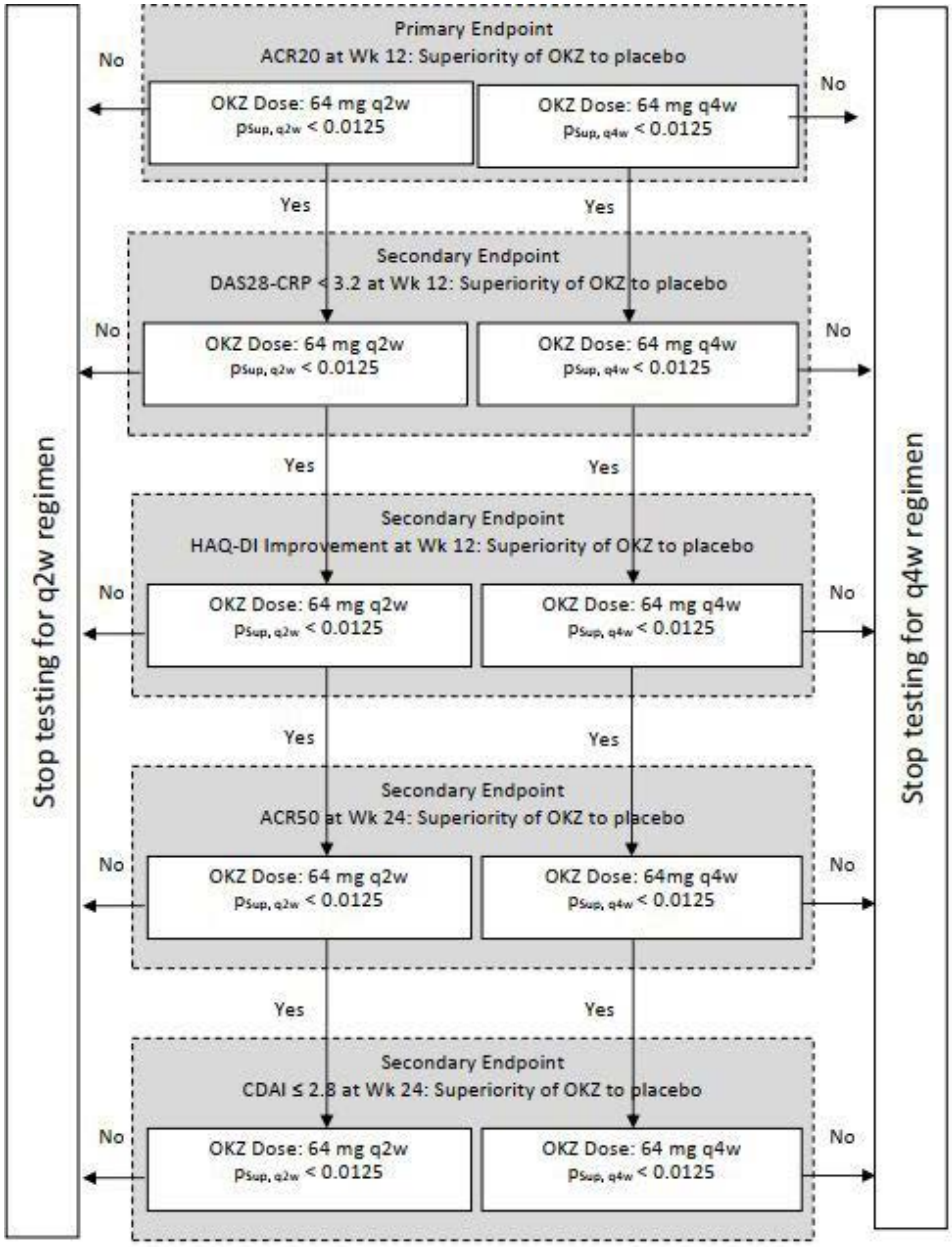

Figure 1 Gate-keeping strategy. $p_{Sup,\ q2w}$ and $p_{Sup,\ q4w}$ represent p values from a one-sided test of superiority versus placebo for OKZ dose regimens 64 mg q2w and q4w. ACR, American College of Rheumatology response; CDAI, Clinical Disease Activity Index; DAS28-CRP, Disease Activity Score 28 based on C reactive protein; HAQ-DI, Health Assessment Questionnaire Disability Index; OKZ, olokizumab; q2w, every 2 weeks; q4w, every 4 weeks; Wk, week.

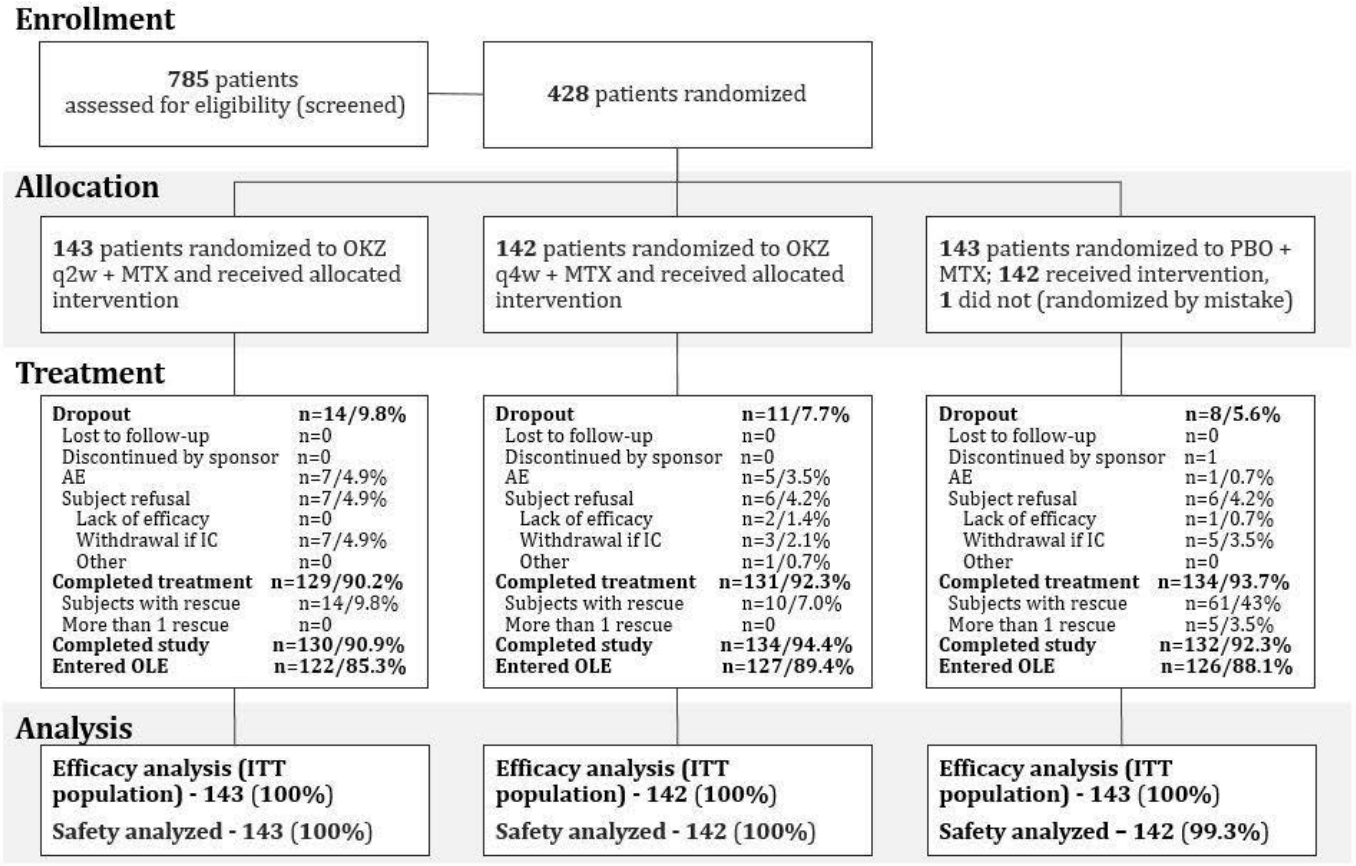

Figure 2 Patient disposition. AE, adverse event; IC, informed consent; ITT, intention-to-treat; MTX,methotrexate; OKZ, olokizumab; OLE, open-label extension; PBO, placebo; q2w, every 2 weeks; q4w, every 4 weeks.

variables, inability to remain on randomised treatment through the time point of interest was defined as non-response with respect to the corresponding endpoint. For analyses of binary variables, in case of missing visits or assessments not performed for reasons other than treatment or study discontinuation intermediate missing data were imputed using surrounding visits.

Efficacy endpoints that were continuous in nature were analysed using an analysis of covariance model adjusted for the baseline value of the corresponding parameter. For analyses of continuous endpoints, subjects who discontinued randomised treatment prematurely but remained in the study through the time point of interest were included using all collected measurements, including those from assessments post treatment discontinuation. In case of missing values, return to baseline values was assumed and was implemented using multiple imputation accounting for the uncertainty of missing data according to the methodology of Rubin.[15]

The primary analysis was performed for intention-to-treat population, defined as all randomised patients. The safety population included all subjects who received at least one dose of the study treatment.

Protocol-specified statistical analyses were performed using Statistical Analysis System V.9.4 or higher.

RESULTS

Disposition

A total of 428 patients were randomised to OKZ 64 mg every 2 weeks (n=143), OKZ 64 mg every 4 weeks (n=142) or PBO (n=143). One patient failed screening, was randomised in error to the PBO group and was withdrawn once the error was discovered, before receiving study treatment; the safety population consisted of 427 subjects (figure 2). The three treatment groups were well balanced for baseline demographic and disease characteristics (table 1).

A total of 92.1% (n=394) of subjects completed the treatment period: 92.3% (n=131) in OKZ every 4 weeks, 90.2% (n=129) in OKZ every 2 weeks and 93.7% (n=134) in the PBO group. The most common reasons for treatment discontinuation were withdrawal of informed consent and adverse events (figure 2).

A higher proportion of patients in the PBO group (43%) received rescue medication(s) compared with patients on OKZ every 4 weeks (7%) or OKZ every 2 weeks (9.8%).

At week 24 of the study, 122 (85.3%) patients on OKZ every 2 weeks, 127 (89.4%) on OKZ every 4 weeks and 126 (88.1%) on PBO were enrolled in the open-label extension study .

Efficacy

The primary efficacy endpoint, ACR20 response rate at week 12, was 70.4% in OKZ every 4 weeks and 63.6% in OKZ every 2 weeks, both significantly greater than 25.9% in the PBO group ($p<0.0001$ for both comparisons) (table 2). Separation of the ACR20 response in the OKZ treatment groups from PBO was seen starting around week 2 and plateauing at week 12 (figure 3).

The secondary endpoint of DAS28-CRP <3.2 at week 12 was achieved by 33.6% and 38.7% of patients on OKZ every 2 weeks and every 4 weeks, respectively, significantly higher than those in the PBO group (3.5%, $p<0.0001$ for both comparisons) (table 2, figure 3).

Significant improvements in physical function as assessed with HAQ-DI were observed at week 12 for subjects in both OKZ

Table 1 Demographic and other baseline characteristics (ITT population)*

Characteristics, mean (SD) unless otherwise specified	OKZ every 2 weeks N=143	OKZ every 4 weeks N=142	PBO N=143
Age (years)	52.0 (11.8)	49.1 (12.1)	52.7 (11.3)
Female (%)	81.1	83.1	83.9
Duration of RA (years)	8.7 (8.0)	7.3 (7.0)	8.4 (7.8)
MTX dose (mg)†	16.1 (3.4)	16.3 (3.4)	16.1 (3.7)
Duration of prior MTX use (weeks)	201.5 (232.1)	157.4 (165.6)	210.1 (208.2)
Glucocorticoid use, n (%)	52 (36.4)‡	50 (35.2)‡	41 (28.7)‡
Prednisone dose or equivalent (mg)	7.6 (6.0)	6.1 (2.3)	6.6 (2.4)
Prior exposure to TNF inhibitors, n (%)	0	0	4 (2.8)
BMI (kg/m^2)	26.6 (5.1)	26.4 (5.5)	26.9 (5.0)
RF+ (≥15 IU/mL), n (%)	115 (80.4)	122 (85.9)	127 (88.8)
Anti-CCP+ (>10 IU/mL), n (%)	110 (76.9)	115 (81.0)	117 (81.8)
CRP (mg/L)§	23.5 (23.1)	22.7 (22.7)	25.8 (28.7)
TJC¶	24.4 (11.4)	22.2 (10.3)	24.0 (11.3)
SJC¶	14.8 (6.5)	14.5 (6.7)	14.6 (6.9)
DAS28-CRP	6.0 (0.7)	5.9 (0.7)	6.0 (0.8)
CDAI score (0–76)	40.5 (9.8)	38.7 (9.4)	40.4 (10.5)
HAQ-DI score	1.74 (0.47)	1.64 (0.50)	1.78 (0.49)
PtGA (VAS) (mm)	70.4 (16.0)	68.5 (14.5)	69.6 (15.9)
Pain (VAS) (mm)	70.2 (16.3)	67.4 (18.5)	68.3 (17.6)
PGA (VAS) (mm)	70.5 (13.9)	66.4 (14.2)	68.0 (14.3)

Pain: patient assessment of pain.
*All patients with exception of one were Caucasian.
†100% patients were on MTX.
‡P=0.33 (χ^2 test).
§Upper limit of normal: >6 mg/L.
¶Joint counts were assessed based on 66–68 joint counts.
anti-CCP+, anticyclic citrullinated peptide positivity; BMI, body mass index; CDAI, Clinical Disease Activity Index; CRP, C reactive protein; DAS28-CRP, Disease Activity Score 28 based on C reactive protein; HAQ-DI, Health Assessment Questionnaire Disability Index; ITT, intention-to-treat; MTX, methotrexate; N, number of subjects; OKZ, olokizumab; PBO, placebo; PGA, Physician Global Assessment of Disease Activity; PtGA, Patient Global Assessment of Disease Activity; RA, rheumatoid arthritis; RF+, rheumatoid factor positivity; SJC, swollen joint count; TJC, tender joint count; TNF, tumour necrosis factor; VAS, Visual Analogue Scale.

Table 2 Efficacy results in the intent-to-treat population (NRI)

	OKZ every 2 weeks N=143	OKZ every 4 weeks N=142	PBO N=143
ACR20 response, n (%), week 12 (primary endpoint)	91 (63.6)*	100 (70.4)*	37 (25.9)
ACR50 response, n (%), week 24	61 (42.7)*	69 (48.6)*	11 (7.7)
ACR70 response†, n (%), week 24	28 (19.6)	32 (22.5)	3 (2.1)
DAS28-CRP <3.2, n (%), week 12	48 (33.6)*	55 (38.7)*	5 (3.5)
HAQ-DI week 12			
LSM (SE)	−0.54 (0.04)	−0.56 (0.04)	−0.20 (0.04)
Treatment comparison vs PBO LSM difference (SE)	−0.34* (0.06)	−0.36* (0.06)	
97.5% CI for LSM difference	−0.47 to −0.21	−0.49 to −0.23	
CDAI ≤2.8, n (%), week 24	12 (8.4)‡	11 (7.7)‡	0
DAS28-CRP <2.6†, n (%), week 24	31 (21.7)	40 (28.2)	5 (3.5)
DAS28-CRP, change from baseline, week 24 LSM (SE)	−2.5 (0.1)	−2.8 (0.1)	−1.2 (0.1)
Treatment comparison vs PBO LSM difference (SE)	−1.4 (0.1)	−1.7 (0.2)	
97.5% CI for LSM difference	−1.7 to −1.0	−2.0 to −1.4	
CDAI <10†, n (%), week 12	37 (25.9)	40 (28.2)	7 (4.9)

*P value difference from PBO <0.0001.
†Results for other than primary and secondary endpoints were not tested for significance.
‡P value difference from PBO <0.001.
ACR, American College of Rheumatology response; CDAI, Clinical Disease Activity Index; DAS28-CRP, Disease Activity Score 28 based on C reactive protein; HAQ-DI, Health Assessment Questionnaire Disability Index; LSM, least squares mean; N, number of subjects; n, number of responders; NRI, non-responder imputation; OKZ, olokizumab; PBO, placebo.

dosage groups compared with PBO. HAQ-DI improvements from baseline (least squares mean change) were 0.56, 0.54 and 0.20 for every 4 weeks, every 2 weeks and PBO groups, respectively (p<0.0001 for both comparisons) (table 2, figure 3).

The ACR50 response at week 24 was achieved by 48.6% of patients on OKZ every 4 weeks, 42.7% on OKZ every 2 weeks and 7.7% on PBO (p<0.0001 for comparisons of OKZ groups vs PBO) (table 2, figure 3).

Disease remission, defined as CDAI ≤2.8, was achieved at week 24 by 7.7% of patients on OKZ every 4 weeks and by 8.4% on OKZ every 2 weeks. No subjects achieved this endpoint in the PBO group (p=0.0003 for OKZ every 4 weeks vs PBO and p=0.0002 for OKZ every 2 weeks vs PBO comparisons) (table 2, figure 3). The percent mean changes in ACR response criteria parameters and CDAI score parameters are presented in online supplemental figure 1. The number of missing observations for key efficacy outcomes is presented in online supplemental table 1. The results of the primary and ranked secondary endpoints were confirmed by predefined sensitivity analyses and a post-hoc linear mixed model analysis (data available on request).

Subgroup analyses of the ACR20 response did not show influence of country, gender, age, weight, body mass index, baseline disease severity, time since diagnosis, duration of prior MTX use, or anticyclic citrullinated peptide and rheumatoid factor status on the efficacy of OKZ (data available on request).

In parallel with the main efficacy endpoints, there were marked increases (improvement) in SF-36 mental component scores from baseline to week 24 of approximately 8.9, 6.2 and 2.5 in patients on OKZ every 4 weeks, OKZ every 2 weeks and PBO, respectively. Corresponding values for SF-36 physical component scores were 8.7, 7.8 and 3.5. Likewise, FACIT-F improvements were 10.6, 8.5 and 3.7 (table 3). Other quality of life measures showed similar trends in improvement (table 3, online supplemental table 2).

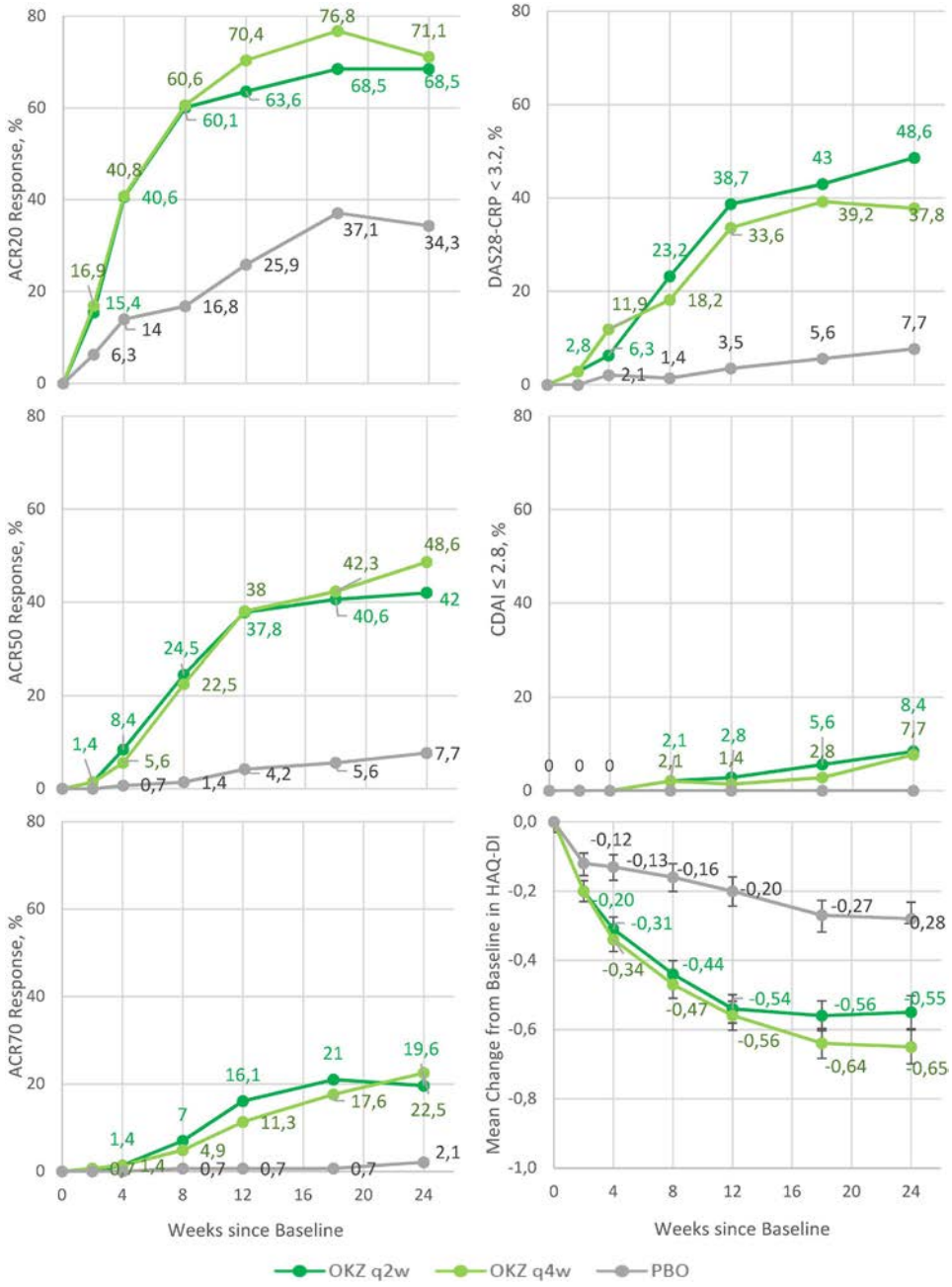

Figure 3 Efficacy results during the double-blind treatment period (ITT population). ACR, American College of Rheumatology response; CDAI, Clinical Disease Activity Index; DAS28-CRP, Disease Activity Score 28 based on C reactive protein; HAQ-DI, Health Assessment Questionnaire Disability Index; ITT, intention-to-treat; OKZ, olokizumab; PBO, placebo; q2w, every 2 weeks; q4w, every 4 weeks.

Safety

Two hundred and twenty-six patients (52.9%) reported treatment-emergent adverse events (TEAE) with similar incidences across the treatment groups (table 4).

Most TEAEs were mild to moderate in severity and non-serious, leading to study treatment discontinuation in 3.5%, 4.9% and 0.7% of patients on OKZ every 4 weeks, OKZ every 2 weeks and PBO, respectively. The most common TEAEs were investigations reported for 35.9% of patients on OKZ every 4 weeks, 35.0% on OKZ every 2 weeks and 18.3% on PBO, and infections reported for 14.1% on OKZ every 4 weeks, 15.4% on OKZ every 2 weeks and 16.2% on PBO. Injection site reactions were reported by two subjects (1.4%) in each OKZ group. A total of 20 treatment-emergent serious adverse events (TESAEs) were reported.

Incidences of TESAEs were numerically higher in patients on OKZ every 4 weeks and OKZ every 2 weeks, compared with PBO: 5.6%, 5.6% and 2.8%, respectively. The most frequently reported serious events were serious infections: 2.8% in patients on OKZ every 2 weeks and 1.4% on PBO (no serious infections were reported for OKZ every 4 weeks). One TEAE leading to death was reported in the study, septicaemia due to *Staphylococcus aureus* and toxic shock syndrome in the OKZ group every 2 weeks. There were no reports of gastrointestinal perforations or anaphylaxis.

As reported with other anti-IL-6 therapies, there were early rises in mean serum lipids noted from week 4, with a plateau that reached around week 8 (figure 4); however, no MACE was observed. Likewise, early decreases in mean blood platelets and neutrophils were seen, with a plateau reached at week 4. No

Table 3 Patient-reported outcome measures at months 3 (12 weeks) and 6 (24 weeks)*

	Week 12			Week 24		
	OKZ every 2 weeks N=143	OKZ every 4 weeks N=142	PBO N=142	OKZ every 2 weeks N=143	OKZ every 4 weeks N=142	PBO N=142
PtGA	−30.6 (1.7) 17.5 (2.5) −23.0 to −12.0	−31.0 (1.7) −17.9 (2.5) −23.4 to −12.4	−13.1 (1.8)	−32.1 (1.9) −12.7 (2.7) −18.8 to −6.6	−36.3 (2.0) −16.8 (2.8) −23.0 to −10.6	−19.4 (1.9)
Pain	−31.6 (1.8) −18.7 (2.6) −24.6 to −12.9	−31.8 (1.8) −19.0 (2.6) −24.8 to −13.1	−12.8 (1.9)	−34.5 (2.1) −13.0 (2.9) −19.5 to −6.5	−37.1 (2.1) −15.7 (2.9) −22.3 to −9.1	−21.4 (2.1)
Pain, patients with >30% improvement, n (%)	94 (65.7)	86 (60.6)	37 (25.9)	96 (67.1)	95 (66.9)	57 (39.9)
Pain, patients with >50% improvement, n (%)	69 (48.3)	60 (42.3)	18 (12.6)	69 (48.3)	74 (52.1)	25 (17.5)
Pain, patients with level of <10 mm, n (%)	12 (8.4)	13 (9.2)	0 (0.0)	23 (16.1)	24 (16.9)	6 (4.2)
Pain, patients with level of <20 mm, n (%)	38 (26.6)	27 (19.0)	8 (5.6)	41 (28.7)	37 (26.1)	16 (11.2)
Pain, patients with level of <40 mm, n (%)	78 (54.5)	80 (56.3)	29 (20.3)	80 (55.9)	85 (59.9)	41 (28.7)
HAQ-DI†				−0.55 (0.05) −0.27 (0.07) −0.43 to −0.12	−0.65 (0.05) −0.37 (0.07) −0.53 to −0.22	−0.28 (0.05)
HAQ-DI <0.5, n (%)	13 (9.1)	13 (9.2)	2 (1.4)	17 (11.9)	21 (14.8)	5 (3.5)
SF-36 PCS	6.7 (0.6) 4.5 (0.8) 2.7 to 6.3	6.0 (0.6) 3.8 (0.8) 2.0 to 5.6	2.2 (0.6)	7.8 (0.7) 4.3 (0.9) 2.2 to 6.4	8.7 (0.7) 5.2 (1.0) 3.1 to 7.4	3.5 (0.7)
SF-36 MCS	6.5 (0.7) 3.0 (1.0) 0.7 to 5.3	7.0 (0.7) 3.6 (1.1) 1.2 to 5.9	3.5 (0.8)	6.2 (0.8) 3.7 (1.1) 1.2 to 6.2	8.9 (0.8) 6.4 (1.1) 3.8 to 8.9	2.5 (0.8)
EQ-5D score	19.7 (1.7) 12.2 (2.4) 6.8 to 17.6	18.7 (1.7) 11.2 (2.4) 5.8 to 16.7	7.4 (1.7)	20.9 (2.0) 12.6 (2.7) 6.5 to 18.7	23.6 (2.0) 15.3 (2.8) 8.9 to 21.7	8.3 (2.0)
FACIT-F	8.2 (0.7) 4.6 (1.0) 2.4 to 6.8	8.7 (0.7) 5.1 (1.0) 2.9 to 7.3	3.6 (0.7)	8.5 (0.8) 4.8 (1.1) 2.3 to 7.3	10.6 (0.8) 6.9 (1.1) 4.3 to 9.5	3.7 (0.8)

Pain: patient's assessment of arthritis pain.
*With the exception of pain, n (%) LSM change from baseline (SE), treatment comparison vs placebo LSM difference (SE), and 97.5% CI for LSM difference are presented.
†Secondary endpoint (refer to table 2).
EQ-5D, European Quality of Life-5 Dimensions; FACIT-F, Functional Assessment of Chronic Illness Therapy-Fatigue Scale (MCID ≥4 units); HAQ-DI, Health Assessment Questionnaire Disability Index; LSM, least squares mean; MCID, minimal clinically important difference; MCS, Mental Component Score (MCID ≥2.5 units); N, number of subjects; OKZ, olokizumab; PBO, placebo; PCS, Physical Component Score (MCID ≥2.5 units); PtGA, Patient Global Assessment of Disease Activity; SF-36, Short Form-36.

patients had grade 3 or higher neutropaenia in accordance with the Common Terminology Criteria for Adverse Events version 4.0. Elevations in serum alanine aminotransferase values above 3× ULN at any time during the study were seen in 11.4%, 9.2% and 5.0% of patients on OKZ every 4 weeks, OKZ every 2 weeks and PBO, respectively, with no concomitant elevations in serum bilirubin above 2× ULN. Selected abnormal haematology and chemistry assessments are presented in online supplemental tables 3 and 4.

Immunogenicity

Positive confirmed ADA tests at any time post baseline were reported in six subjects (4.4%) on OKZ every 2 weeks and in nine subjects (6.6%) on OKZ every 4 weeks. No subjects had a positive result for neutralising antibodies.

DISCUSSION

CREDO 1 trial, a phase III study of OKZ in patients with active RA despite MTX, achieved the primary and all ranked secondary efficacy endpoints. This study evaluated two effective doses with a frequency of injection of once per 2 weeks and once per month, and both regimens of OKZ were superior to PBO in reducing signs and symptoms and improving disability and quality of life over a period of 24 weeks. The onset of efficacy of OKZ was rapid as detected by differences in ACR20 response rates between OKZ and PBO that were apparent within 2 weeks from the start of treatment. The study was specifically designed and sized to detect differences between OKZ and PBO, so small differences seen between OKZ doses in one variable could be by chance, especially since they were not consistently detected across efficacy endpoints. ACR20 was used as the primary endpoint due to its widely accepted and validated value in assessing the efficacy of drugs in RA over many years. While higher levels of response such as ACR50 or ACR70 responses could have been chosen as the primary outcome, use of ACR20 allows for easier comparisons with other compounds evaluated in the past that used ACR20. While ACR20 was the primary endpoint, the study included ACR50 as a ranked secondary endpoint, as well as DAS28-CRP <3.2 and CDAI ≤2.8 (remission), all of which confirmed the results of the ACR20 analysis. In this study patients had relatively high disease activity at baseline, making it more difficult to achieve DAS28-CRP <3.2 status by week 12, as compared with becoming ACR20 responders. Despite this, the data regarding DAS28-CRP <3.2 are consistent with what has previously been reported for anti-IL-6R antibodies, in the same population.[8 9 16]

Disability is an important aspect of RA that originates from joint pain and joint damage and should be directly assessed in RA clinical trials.[17] One of the secondary endpoints in the study was assessment of disability using the HAQ-DI questionnaire.[18 19] The study showed that both regimens of OKZ resulted in significantly more improvement in disability than PBO. Additionally,

Table 4 TEAE by system organ class and preferred term and key serious treatment-emergent adverse events (safety population)

System organ class (preferred term)	OKZ every 2 weeks N=143, n (%)	OKZ every 4 weeks N=142, n (%)	PBO N=142, n (%)
Number of subjects with at least one TEAE reported for 4% of subjects in any treatment group	83 (58.0)	81 (57.0)	62 (43.7)
Investigations	50 (35.0)	51 (35.9)	26 (18.3)
ALT increased	25 (17.5)	33 (23.2)	11 (7.7)
AST increased	16 (11.2)	22 (15.5)	10 (7.0)
White cell count decreased	7 (4.9)	6 (4.2)	4 (2.8)
Neutrophil count decreased	6 (4.2)	7 (4.9)	3 (2.1)
Blood cholesterol increased	6 (4.2)	4 (2.8)	3 (2.1)
Gamma-glutamyltransferase increased	3 (2.1)	6 (4.2)	4 (2.8)
Infections and infestations	22 (15.4)	20 (14.1)	23 (16.2)
Nasopharyngitis	4 (2.8)	3 (2.1)	6 (4.2)
Upper respiratory tract infection	2 (1.4)	6 (4.2)	4 (2.8)
Blood and lymphatic system disorders	17 (11.9)	18 (12.7)	15 (10.6)
Leucopenia	8 (5.6)	7 (4.9)	4 (2.8)
Neutropaenia	5 (3.5)	9 (6.3)	2 (1.4)
Anaemia	4 (2.8)	3 (2.1)	6 (4.2)
Metabolism and nutrition disorders	9 (6.3)	7 (4.9)	3 (2.1)
Musculoskeletal and connective tissue disorders	6 (4.2)	7 (4.9)	6 (4.2)
Skin and subcutaneous tissue disorders	8 (5.6)	3 (2.1)	2 (1.4)
Number and percentage with at least one key TESAE	8 (5.6)	8 (5.6)	4 (2.8)
Investigations	2 (1.4)	4 (2.8)	1 (0.7)
ALT increased	2 (1.4)	4 (2.8)	1 (0.7)
AST increased	0	3 (2.1)	0
Infections and infestations	4 (2.8)	0	2 (1.4)
Subcutaneous abscess	2 (1.4)	0	0
Gastroenteritis	0	0	1 (0.7)
Pneumonia	0	0	1 (0.7)
Pulmonary tuberculosis	1 (0.7)	0	0
Staphylococcal sepsis	1 (0.7)	0	0
Toxic shock syndrome	1 (0.7)	0	0
Herpes zoster	0	0	0
Hepatobiliary disorders	0	1 (0.7)	0
Drug-induced liver injury	0	1 (0.7)	0
Neoplasms benign, malignant and unspecified (including cysts and polyps)	0	1 (0.7)	0
Cervix carcinoma stage II	0	1 (0.7)	0
Gastrointestinal disorders	0	1 (0.7)	0
Obstructive pancreatitis	0	1 (0.7)	0
Gastrointestinal perforation	0	0	0
Vascular disorders	0	1 (0.7)	0
Diabetic vascular disorder	0	1 (0.7)	0
Venous thromboembolism	0	0	0
Death	1 (0.7)	0	0

All AEs were collected from the signature of the informed consent form until the last visit of the subject in the study (up to 22 weeks after the final dose of study treatment) regardless of relationship to study treatment, thus up to approximately 44 weeks.
A TEAE is defined as an AE that first occurred or worsened in severity after the first dose of the study treatment.
%, percentage of subjects calculated relative to the total number of subjects in the population.
MedDRA (Medical Dictionary for Regulatory Activities, V.21.1) was used to code AEs.
AE, adverse event; ALT, alanine aminotransferase; AST, aspartate aminotransferase; n, number of subjects with events; N, number of subjects; OKZ, olokizumab; PBO, placebo; TEAE, treatment-emergent adverse event; TESAE, treatment-emergent serious adverse event.

in this patient population and investigational setting, 89 (62.2%) and 94 (66.2%) patients treated with OKZ had improvement in their HAQ-DI score with more than minimally detectable difference of 0.22, compared with 63 (47.6%) in the PBO group.

Chronic arthritis can have a profound effect on patients' quality of life.[20] In this study it was shown that the improvements seen in signs and symptoms and disability of RA were mirrored by positive effects on quality of life measures including SF-36 and FACIT-F. SF-36 is a multidomain questionnaire that assesses different aspects of a person's life, summarised into PCS and MCS. Treatment with OKZ resulted in improvements across all of these domains (table 3). Certain mental ailments such as sleep disorders and fatigue in RA may be linked to high levels of circulating IL-6.[21 22] OKZ treatment resulted in marked improvements in fatigue, consistent with its mechanism of action as an inhibitor of IL-6.

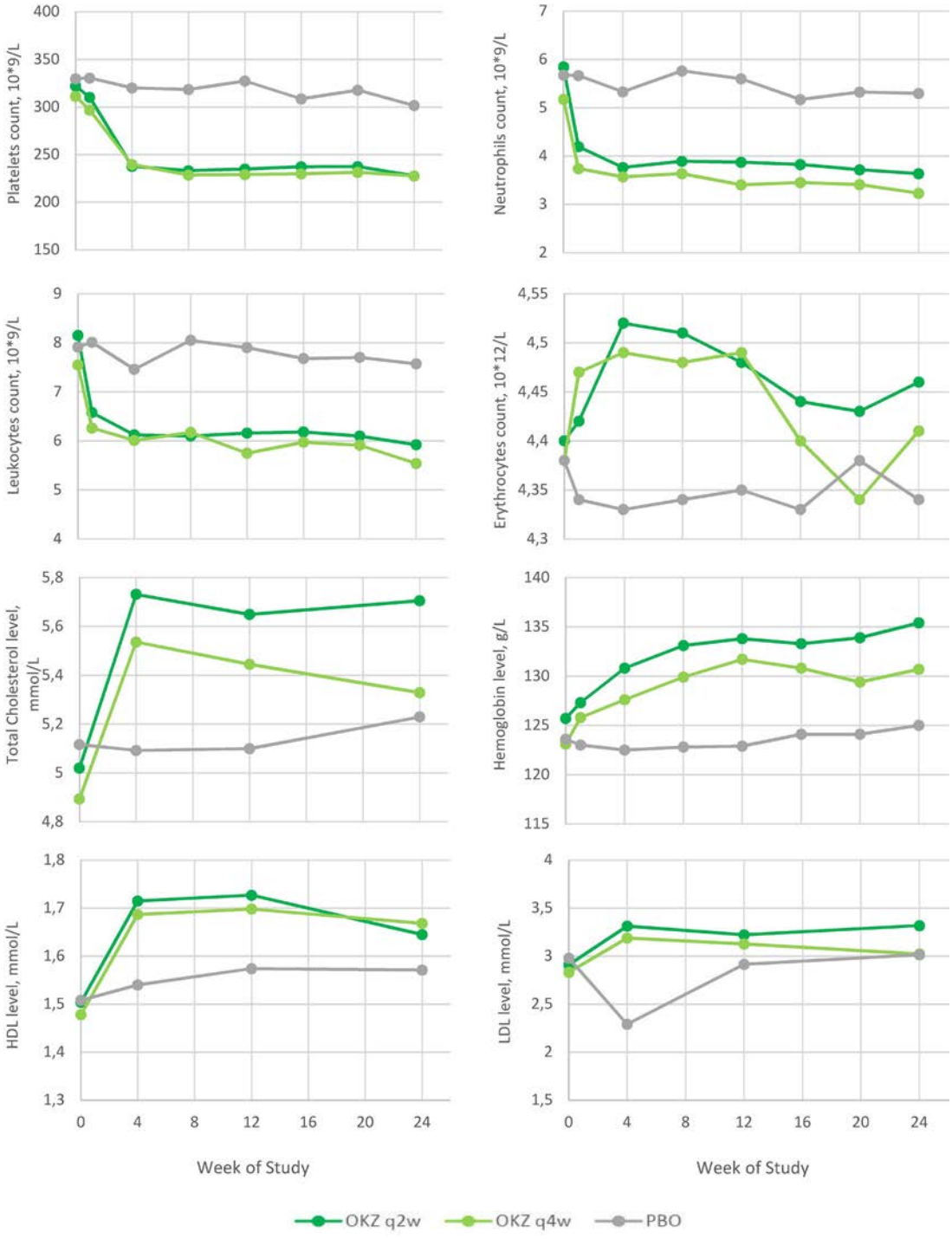

Figure 4 Mean changes in laboratory values during the double-blind treatment period (safety population). HDL, high-density lipoproteins; LDL, low-density lipoproteins; OKZ, olokizumab; PBO, placebo; q2w, every 2 weeks; q4w, every 4 weeks.

CREDO 1 trial also evaluated the safety of OKZ over 24 weeks and confirmed that OKZ has a safety profile similar to approved anti-IL-6R antagonists and no unexpected safety findings.[23 24]

As expected, there were more adverse events observed in the OKZ-treated patients, but they were mostly mild to moderate with few serious adverse events and no unexpected safety findings and relatively low number of dropouts due to an adverse event. In this relatively small study few serious infections, including opportunistic infection (pulmonary TB) and one fatal event, were reported for OKZ every 2 weeks and none for OKZ every 4 weeks.

There are several limitations to the study. First, there was no active comparator in this study, limiting the ability to compare with other agents. Second, the study did not include radiographic assessments. An analysis of RA trials of anti-TNF biologics showed a trend towards decreasing rate of radiographic progression, possibly due to more effective patient management, and to reliably show a positive radiographic effect one must include large numbers of patients on PBO, a possible ethical issue.[25] Third, this study was conducted in a limited geographical location with limited racial diversity and its findings should be confirmed in other phase III controlled trials that include a more diverse patient population.

CONCLUSION

In this first phase III trial of OKZ in patients with active RA despite treatment with an adequate dose of MTX, OKZ demonstrated significant improvements in signs and symptoms of RA, including in disability and quality of life measures, compared with PBO. OKZ was reasonably well tolerated over a period of 24 weeks with no unexpected safety findings.

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TRANSLATIONAL SCIENCE

Key interactions in the trimolecular complex consisting of the rheumatoid arthritis-associated DRB1*04:01 molecule, the major glycosylated collagen II peptide and the T-cell receptor

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ABSTRACT

Objectives Rheumatoid arthritis (RA) is an autoimmune disease strongly associated with the major histocompatibility complex (MHC) class II allele DRB1*04:01, which encodes a protein that binds self-peptides for presentation to T cells. This study characterises the autoantigen-presenting function of DRB1*04:01 (HLA-DRA*01:01/HLA-DRB1*04:01) at a molecular level for prototypic T-cell determinants, focusing on a post-translationally modified collagen type II (Col2)-derived peptide.

Methods The crystal structures of DRB1*04:01 molecules in complex with the peptides $HSP70_{289-306}$, citrullinated $CILP_{982-996}$ and galactosylated $Col2_{259-273}$ were determined on cocrystallisation. T cells specific for $Col2_{259-273}$ were investigated in peripheral blood mononuclear cells from patients with DRB1*04:01-positive RA by cytofluorometric detection of the activation marker CD154 on peptide stimulation and binding of fluorescent DRB1*0401/$Col2_{259-273}$ tetramer complexes. The cDNAs encoding the T-cell receptor (TCR) α-chains and β-chains were cloned from single-cell sorted tetramer-positive T cells and transferred via a lentiviral vector into TCR-deficient Jurkat 76 cells.

Results The crystal structures identified peptide binding to DRB1*04:01 and potential side chain exposure to T cells. The main TCR recognition sites in $Col2_{259-273}$ were lysine residues that can be galactosylated. RA T-cell responses to DRB1*04:01-presented $Col2_{259-273}$ were dependent on peptide galactosylation at lysine 264. Dynamic molecular modelling of a functionally characterised $Col2_{259-273}$-specific TCR complexed with DRB1*04:01/$Col2_{259-273}$ provided evidence for differential allosteric T-cell recognition of glycosylated lysine 264.

Conclusions The MHC-peptide-TCR interactions elucidated in our study provide new molecular insights into recognition of a post-translationally modified RA T-cell determinant with a known dominant role in arthritogenic and tolerogenic responses in murine Col2-induced arthritis.

Key messages

What is already known about this subject?

- Rheumatoid arthritis (RA) is closely associated with HLA-DRB1*04:01-encoded major histocompatibility complex II molecules.
- DRB1*04:01-restricted $CD4^+$ T-cell responses to citrullinated autoantigens and post-translationally modified collagen II (Col2) have been described.

What does this study add?

- This study contributes new crystal structure information that reveals the DRB1*04:01 function in presenting post-translationally modified antigenic determinants to T cells in patients with RA.
- Through comparative analysis of RA T cells, T cell receptor (TCR) cloning and molecular modelling of a prototypic trimolecular complex, we gained insights into TCR recognition of unmodified and glycosylated Col2 in a DRB1*04:01 context.

How might this impact on clinical practice or future developments?

- Our findings contribute to a better understanding of the role of posttranslational Col2 modification for TCR recognition in $CD4^+$ T cells and has potential implications for induction of tolerance and onset of pathogenic autoimmunity.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease targeting diarthrodial cartilaginous joints. The disease is believed to be initiated by the development of autoantibodies to various altered self-antigens, predominantly modified by citrullination for yet unknown reasons,[1–4] followed years later by onset of joint inflammation and spreading of autoimmune responses to new structures, including cartilage proteins.[5–7] The strong association of RA with major histocompatibility complex class II (MHCII) suggests activation of T cells and a maturation of an autoreactive B cell response, capable of orchestrating the immune attack on the joints.[8] The origin of the T-cell activation is unknown but is likely to be dependent on presentation of antigenic peptides bound to the MHCII molecules. These peptides

could have been derived from non-self-proteins, for example, from infectious agents giving help to autoreactive B cells, or could be modified self-peptides that have escaped tolerance selection.

The MHCII association with RA has been mapped to the DRB1 locus,[9 10] which encodes a DR β-chain (DRB) and forms a peptide-binding receptor together with an invariant α-chain (DRA).[11] According to a popular hypothesis, alleles of the highly polymorphic DRB1 locus associated with RA encode a shared peptide binding pocket for a selected set of self-peptides, thereby predisposing individuals to pathogenic T-cell activation.[12 13] Most of the RA-associated DRB1 molecules have positively charged amino acids at position 71, favouring interactions with peptides that contain a negatively charged amino acid at the P4 position. Although a favoured binding of peptides with citrulline at this position (in contrast to a positively charged arginine) has been proposed,[13 14] this hypothesis could not be confirmed in studies on larger sets of peptides.[15 16]

A potentially relevant self-antigen in arthritis pathogenesis is type II collagen (Col2) due to its abundance in cartilage and proven role as an arthritogenic immunogen in experimental arthritis.[17] Moreover, antibodies to native and citrullinated Col2[18–22] and Col2-specific T cells[18] are detectable in patients with RA. Interestingly, RA T-cell responses directed to the dominant $Col2_{259\text{-}273}$ peptide were restricted by the RA-associated DR alleles 0401 and 0101, which were demonstrated to confer susceptibility to collagen-induced arthritis on transgenic expression in mice.[23–25] However, it is crucial to consider that the major $Col2_{259\text{-}273}$ peptide can be both hydroxylated and galactosylated at lysine residues and that RA T cells predominantly recognise the galactosylated form.[18] Initial insight into the positioning of the galactosylated (gal) $Col2_{259\text{-}273}$ peptide ($galCol2_{259\text{-}273}$) in the binding pocket of the DRB1*04:01 molecule was provided by molecular modelling.[26 27] The same target peptide and its post-translational modification (PTM) is also recognised by arthritogenic T cells in mice, provided that they express the natural murine antigen MHCII allele or transgenic human DRB1*04:01 or DRB1*01:01 molecules.[18 23–25] Critical differences in central tolerance induction of T cells based on their specificity for unmodified ($nCol2_{259\text{-}273}$) or galactosylated $Col2_{259\text{-}273}$ ($galCol2_{259\text{-}273}$)[28] and in the tolerogenic potential of Col2 peptide vaccination against experimental arthritis have been observed in mice.[29]

To further elucidate the role of PTM in Col2 recognition by human T-cell receptors (TCR), we performed a comparative study of $Col2_{259\text{-}273}$ recognition in either galactosylated or unmodified form using binding of tetramerised recombinant DRB1*04:01/Col2-peptide complexes and analysed Col2 peptide-induced T-cell activation in peripheral blood mononuclear cells (PBMCs) from patients with DRB1*04:01-positive RA. Moreover, we solved the X-ray crystallographic structure of DRB1*04:01(HLA-DRA1*01:01/HLA-DRB1*04:01) complexed with $galCol2_{259\text{-}273}$ to gain the first molecular insights into the structural basis of PTM-dependent differential Col2 recognition. Together with sequence information obtained from a cloned human galCol2-specific TCR, these crystal structures allowed us to perform molecular modelling on the trimolecular MHCII/peptide/TCR complex.

MATERIALS AND METHODS

See online supplemental material.

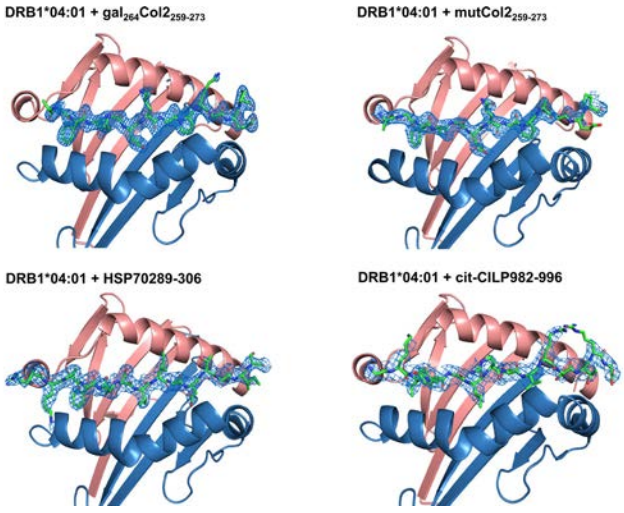

Figure 1 Top view of the DRAxDRB1*04:01 molecule binding peptides. The DRA (alpha chain) is shown in pink and the DRB (beta-chain) shown in blue. The $2F_o$-F_c electron density map of each peptide is shown in blue mesh contoured at 1 σ.

RESULTS

The molecular complex of DRB1*04:01 with bound peptides

The investigated peptides $nCol2_{259\text{-}273}$(GIAGFKGEQGPKGET),[18] heat shock protein (HSP) $HSP70_{289\text{-}306}$(TRKPFQSVIADTGISV) and citrullinated (cit) cartilage-intermediate protein (CILP) $citCILP_{982\text{-}996}$ (GKLYGI[Cit]DV[Cit]STRDR) represent autoantigenic determinants recognised by T cells in RA.[30 31] To establish the structural basis of their autoimmune recognition, we solved the crystal structures of DRB1*04:01 complexed with $Col2_{259\text{-}273}$, mutated $Col2_{259\text{-}273}$ containing alanine replacements at 264 (K264A, at P2) and 270 (K270A, at P8), $HSP70_{289\text{-}306}$ and the $CILP_{982\text{-}996}$ peptide citrullinated at positions 988 and 991 (figures 1 and 2, and online supplemental figure S1). All investigated T cell determinants, either unmodified or altered by citrullination or galactosylation, were bound in a conserved linear and extended conformation located in the classic binding groove of DRB1*04:01, thereby closely resembling previously solved DRB1*04:01 structures.[32]

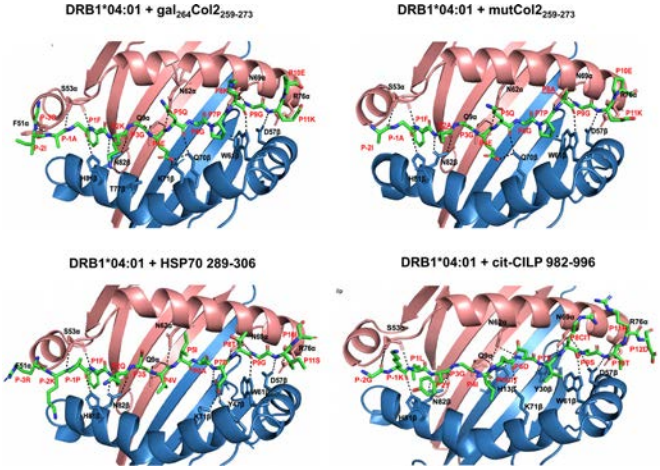

Figure 2 Interaction of peptides with the DRAxDRB1*04:01 molecule. Hydrogen bonds are indicated by dashed black lines. Peptide residues are numbered in accordance with the numbering of the binding pockets. The residues from both α and β chains important for contacts with the peptide are represented as sticks.

The crystal structure of $Col2_{259-273}$-bound DRB1*04:01 was determined at 1.9 Å resolution (online supplemental table S1). A weakly bound mutated human CLIP peptide (PVSKARMATGALAQA) occupying the DRB1*04:01 binding groove was exchanged with a synthetic glycosylated $Col2_{259-273}$ (mono-[β-D-galactopyranosyl]-moiety at a lysine [K264] side chain) prior to protein crystallisation. However, we did not observe electron density for the galactose moiety electron density in the structure, most likely because it is mobile at its solvent-exposed position on the protein surface. As expected, the peptide occupied the P1, P4, P6, P7 and P9 pockets with P1-Phe, P4-Glu, P6-Gly, P7-Pro and P9-Gly, respectively, whereas the potential TCR contact residues are P2-Lys, P5-Gln and P8-Lys. We also determined DRB1*04:01 in complex with covalently attached mutated $Col2_{259-273}$ (online supplemental table S1, figures 1 and 2, online supplemental figure S1 and figure S2). To investigate a possible influence on peptide binding by the two potential TCR contact residues P2-Lys and P8-Lys, we mutated them to Ala. We have previously found that the mutation of P2 from Lys to Ala slightly decreased the affinity of the peptide with DRB1*04:01, but also abolished T cell reactivity.[25] As can be seen from the structure, the mutated peptide mimics the conformation and location of the wildtype peptide (online supplemental figure S2), but its P4-Glu did not engage with Lys71β, thus explaining the reduced affinity.

The $HSP70_{289-306}$ peptide binds in a linear, extended manner with P1-Phe, P4-Val, P6-Ala and P9-Gly occupying the P1, P4, P6 and P9 pockets of DRB1*04:01, respectively, whereas P2-Gln, P5-Ile, P7-Asp, P8-Thr and P10-Ile represented potential TCR contact sites. The Gln70β within the shared epitope motif does not contact P6-Ala as seen in DRB1*04:01 where Gln70β hydrogen bonds to both P4E and P6G of $Col2_{259-273}$. In contrast, P7-D is bound by both Lys71β and Tyr47β.

As expected, the $citCILP_{982-996}$ peptide also binds in a linear, extended manner with P1-L, P4-I, P6-D and P9-S interacting with the DR molecules. Thus, the citrulline does not bind to the P4 pocket but instead is likely to face the TCR.

An overall comparison of the binding sites confirmed that the strongest DR binding site had a hydrophobic amino acid in the P1 position, whereas a considerable degree of flexibility was allowed at other positions. An important DR binding site is P4, in which an acidic side chain (glutamic acid) is favoured as it interacts with basic amino acids at position 71 in the beta chain, in line with the known association with RA. In contrast, the citrulline side chains of the CILP peptide did not bind within the P4 pocket. The TCR recognition sites for the Col2 peptide were the lysines at P2 and P10 as well as the glutamine at P5.

Detection of T lymphocytes specific for $Col2_{259-273}$

DRB1*04:01 allele carriers (patients with RA and healthy donors) were investigated for the presence of $Col2_{259-273}$ peptide (nCol2 or $gal_{264}Col2$)-specific $CD4^+$ T cells in peripheral blood by flow cytometric analysis of tetramer-stained PBMCs. A representative result in figure 3A shows the detection of Col2 epitope-specific cells that stain double-positive for two identical but differently fluorescence-labelled DRB1*04:01/Col2 tetramer complexes. The frequency of double-positive cells was 0.030%

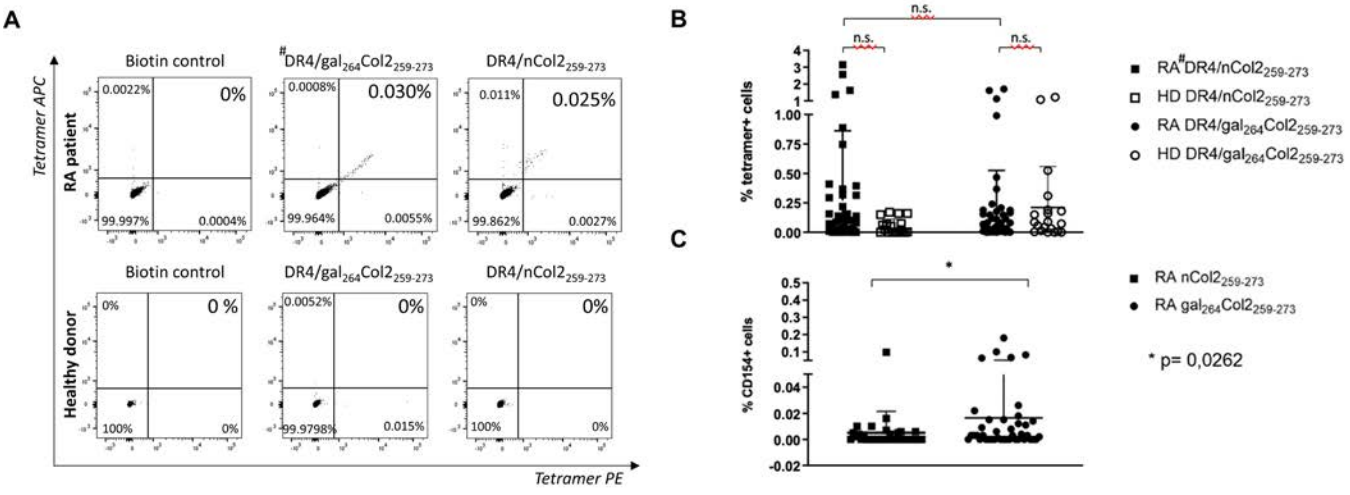

Figure 3 Detection of human antigen-specific T cells in the peripheral blood of HLA-DRB1*04:01 carriers by flow cytometry using #DR4 (HLADRB1*04:01)-tetramers with specificity for the $gal_{264}Col2_{259-273}$ or $nCol2_{259-273}$ peptide. (A) Representative dot blots: the $CD4^+$ enriched T cells from PBMCs were stained with a dead/live marker and DRB1*04:01/Col2 peptide tetramers conjugated with two different fluorophores (PE and APC). Subsequent flowcytometric analysis reveals the Col2-specific cells in the live double positive stained subpopulation. Representative dot blots of specific DR4/gal_{264}Col2 and DR4/nCol2 tetramer binding to T helper cells in samples from a patient with RA and a HD are shown. Biotin-streptavidin complexes without a specific peptide conjugate served as a negative control. (B) Specific tetramer binding of T helper cells from patients with RA (n=55) and HD (n=20) using #DR4 (DRB1*04:01)-tetramers with different peptide specificity (gal_{264}Col2 vs nCol2). In PBMCs of patients with RA, the frequencies of DR4/nCol2 and DR4/gal_{264}Col2 staining CD4+T cells do not differ significantly (n.s.). Depicted values represent processed data in which for each tetramer staining datapoint the respective biotin background has already been subtracted from the raw value. (C) Detection of antigen-specific T cells in PBMCs of patients with RA (HLA-DRB1*04:01) on in vitro stimulation by synthetic Col2 peptides using flow cytometry. PBMCs from patients with RA were stimulated with a Col2 peptide (gal_{264}Col2, nCol2) and anti-CD40 for 7 hours. Subsequently, peptide-induced upregulation of the activation marker CD154 on the surface of the live CD4+T cell population was detected by flow cytometry. The frequency of CD154-positive CD4-positive T helper cells in PBMCs was significantly elevated in response to vitro challenge by the galCol2 peptide (n=41) compared with unmodified nCol2 (n=35, p=0.0262). Statistical significance was determined using the Mann-Whitney test. APC, allophycocyanin; HD, healthy donors; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; RA, rheumatoid arthritis; TCR, T-cell receptor.

for the $gal_{264}Col2_{259-273}$-peptide and 0.025% for the $nCol2_{259-273}$-peptide containing tetramers in the total $CD4^+$ T-lymphocyte population. In a concomitantly analysed blood sample from a healthy donor, no DRB1*04:01/Col2-specific T cells could be identified.

The studies on our entire sample size using DRB1*04:01 tetramers containing either unmodified nCol2 or $gal_{264}Col2_{259-273}$ peptide confirmed detectability of antigen-specific T cells in PBMCs from patients with RA at the expected low precursor frequencies. The frequencies of DRB1*04:01/nCol2-staining and DRB1*04:01/gal_{264}Col2-staining $CD4^+$ T cells staining $CD4^+$ T cells were identical: 27.27%. Staining positivity was defined by a value exceeding the threshold set by the mean of negative biotin control +3 SD (see Methods section). Interestingly, $CD4^+$ T cells staining with the DRB1*04:01/nCol2-tetramers as well the DRB1*04:01/gal_{264}Col2-tetramers were also detectable at a percentage of 5% and 30%, respectively, in the small cohort of healthy DRB1*04:01 carriers (figure 3B). No significant differences were detectable between the RA and healthy donor groups (figure 3B). In this respect, the small sample size constitutes a certain constraint of our study mainly due to limited access to biomaterial from HLA-typed healthy blood donors.

Interestingly, the DRB1*04:01/Col2-tetramer-positive T-cell population detectable in the peripheral blood of patients with RA exhibited a difference in responsiveness to in vitro stimulation of PBMCs with synthetic Col2 peptides. Stimulation with the gal_{264}Col2 peptide resulted in an elevated frequency of antigen-activated $CD4^+$ T lymphocytes compared with nCol2 as determined by peptide-induced upregulation of the activation marker CD154 detected by flow cytometry (figure 3C). Taken together, the results demonstrate a functional impact of the Col2 peptide structure presented in the context of a DRB1*04:01-encoded MHCII molecule on autoimmune recognition by $CD4^+$ T cells in the peripheral blood of patients with RA.

Analysis of a cloned TCR derived from a single sorted Col2-reactive T lymphocyte

We next aimed to characterise TCRs with DRB1*04:01-restricted recognition of $Col2_{259-273}$. Single cells of in vitro expanded $gal_{264}Col2_{259-273}$ peptide-reactive $CD4^+$ T lymphocytes from patients with DRB1*04:01-positive RA were sorted according to staining with DRB1*04:01/$gal_{264}Col2_{259-273}$ tetramers and used for Vα-TCR and Vβ-TCR gene amplification by PCR. A prototypic TCR (TCR#16), for which we obtained the complete cDNA sequence of the paired α-chain and β-chain (see online supplemental figures S3 and S4), was further characterised by recombinant expression in the TCR-deficient Jurkat 76 cell line on lentiviral gene transfer (see online supplemental figure S5 for studies on two additionally transduced human TCRs). As shown in figure 4A, TCR-transduced Jurkat cells exhibited a specific positive staining with the DRB1*04:01/gal_{264} $Col2_{259-273}$ tetramers and at a clearly reduced level with DRB1*04:01/$nCol2_{259-273}$ tetramers, whereas control constructs consisting either of MHCII complexes in which DRB1*04:01 is replaced by the murine analogue A^q (A^q/$gal_{264}Col2_{259-273}$) or DRB1*04:01 complexed with the influenza hemagglutinin (HA) peptide ($HA_{306-318}$: PKYVKQNTLKLAT) (DRB1*0401/HA-peptide) remained negative. In addition, Jurkat cells transduced with a human HA-specific TCR (HA1.7)[32] stained positive with DRB1*04:01/HA306-318 while remaining negative when stained with the DRB1*04:01/$gal_{264}Col2_{259-273}$ tetramer (data not shown).

Subsequent functional studies using lentiviral gene transfer from a single sorted $gal_{264}Col2_{259-273}$-specific T-cell of a patient with HLA-DRB1*04:01-positive RA demonstrated the selective capability of recombinant monomeric DRB1*04:01/Col2-peptide complexes to induce IL-2 production in TCR-reconstituted Jurkat 76 cells (figure 4B). The challenge with monomeric DRB1*04:01/$gal_{264}Col2_{259-273}$ induced the strongest IL-2 response. Stimulation with DRB1*04:01 complexes containing the nCol2 peptide resulted in a considerably reduced IL-2 release that nevertheless clearly exceeded the levels induced by DRB1*04:01/HA306-318 or A^q/$gal_{264}Col2_{259-273}$ control complexes (figure 4B). To confirm this result, we tested antigen-presenting cells (APCs) homozygously expressing DR*0401 obtained from DRB1*04:01 knock-in mice as well as APCs from the peripheral blood of DR*0401individuals (online supplemental figure S6). The presentation of $gal_{264}Col2_{259-273}$ in a DRB1*04:01 context on the surface of either murine or human fixed APC after preloading with peptides was specifically recognised by TCR#16 mRNA transfected nuclear factor of activated T cells (NFAT) luciferase Jurkat reporter cells and associated with stronger NFAT activation compared with the stimulatory effect of the nCOL2 peptide under identical conditions. The CLIP control peptide did not lead to any activation of the TCR mRNA transfected Jurkat reporter cells. Accordingly, these results are in agreement with the studies on the specificity of tetramer-induced IL-2 responses via the recombinantly expressed TCR #16 in lentivirally transduced Jurkat 76 cells lacking an endogenous TCR.

Modelling of molecular interactions in the trimolecular complex of the DRB1*04:01, $Col2_{259-273}$ peptide and TCR

Based on the identified sequence of the human Col2-specific TCR#16 and the solved crystal structure of the DRB1*04:01/$Col2_{259-273}$ complex, molecular modelling was performed using the template of a published TCR cocrystallised with an influenza peptide-containing DRB1*04:01 molecule (HA1.7).[32] The overview of the entire modelled 3D structure of the multicomponent system consisting of the DRB1*04:01/Col2 peptide/TCR complex is shown in online supplemental figure S7. More detailed insights into critical amino acid residues involved in interactions between the unmodified or gal_{264}Col2 peptides and TCR #16 variable regions in the DRB1*04:01 complex are provided in figure 5, which depicts the superimposition of the minimised starting geometries for the two trimolecular complexes. Molecular interaction of the Col2 peptide with the TCR occurs via three side chain bonds irrespective of $Col2_{259-273}$ galactosylation. Two interactions involve the CDR3 region of the TCRα-chain (Asp94—Lys264 [$Col2_{259-273}$] and Asn97—Glu266 [$Col2_{259-273}$]) and an additional salt bridge involves the CDR1 region of the TCRβ-chain (Asp29—Lys270 [$Col2_{259-273}$]). The galactose residue at Lys264 is in close contact with the TCRα-CDR3 backbone but is not involved in side chain interactions.

Additional insight was provided by comparative molecular dynamic simulations of both trimolecular complexes (unmodified or galactosylated Col2 peptide). Snapshots at 950 ns of dynamic modelling revealed critical differences imposed by the galactosyl residue at lysine 264 in the Col2 peptide (figure 6). TCR#16 interaction with the complex containing nCol2 at the initial salt bridge Lys264—Asp94 (TCRα-CDR3) caused the complex to open up to allow neoformation of a bond to Asn97 (TCRα-CDR3). Consequently, Glu266 of $Col2_{259-273}$ formed a new salt bridge with Lys71 in the β-chain of DRB1*04:01 at expense of the initial bonding to Asn97 (TCRα-CDR3)

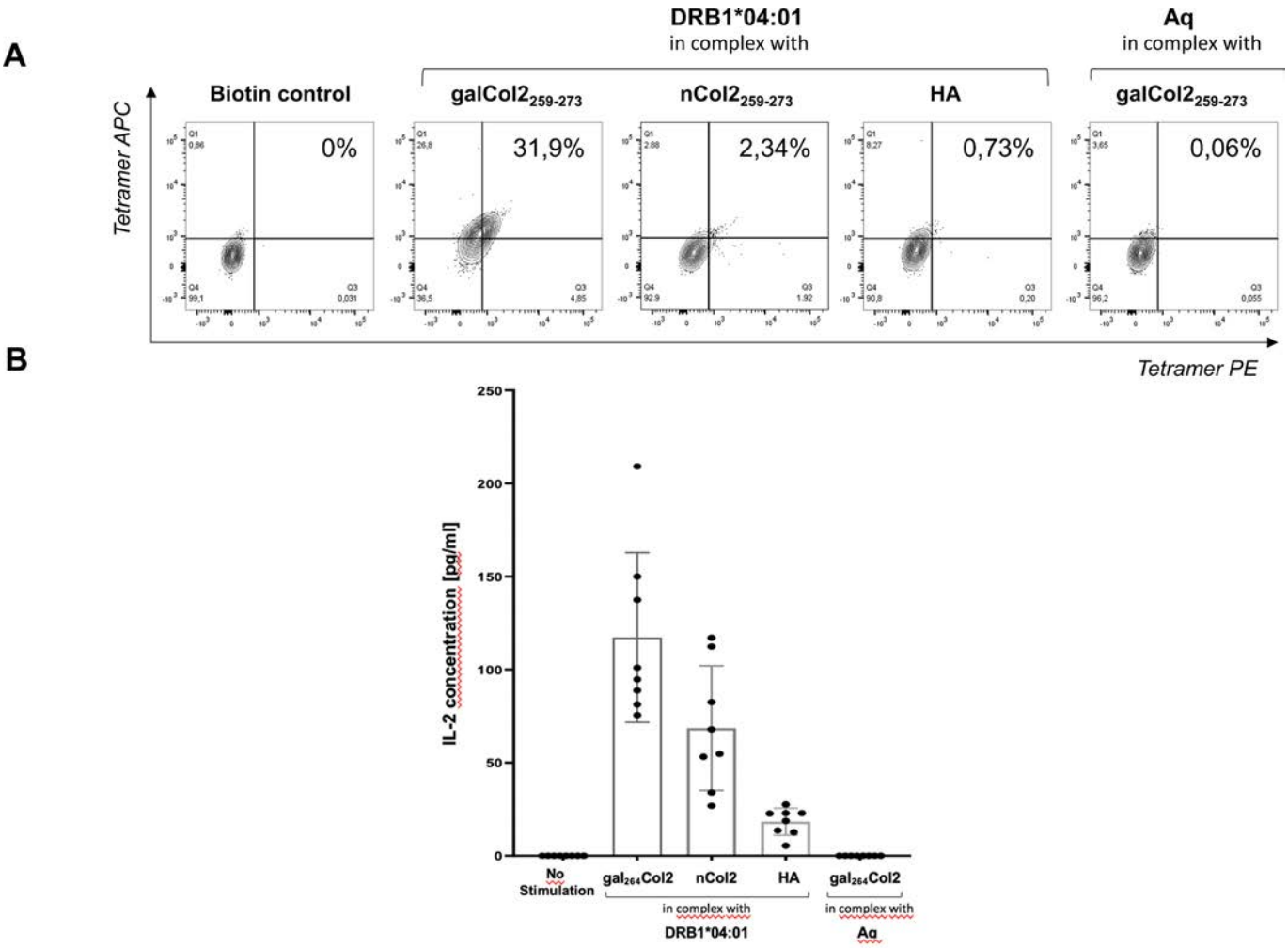

Figure 4 Binding of DRB1*04:01 tetramers to TCR-deficient Jurkat cells and induction of IL-2. (A) Flow cytometric analysis of binding of DRB1*04:01/gal$_{264}$Col2$_{259-273}$ tetramers to TCR-deficient Jurkat 76 cells after gene transfer of the cloned α and β chains of TCR #16. Transduction of a TCR-deficient Jurkat 76 cell line was performed with a lentiviral vector encoding the α and β chains of TCR #16 cloned from a single-cell sorted CD4$^+$ gal$_{264}$Col2-specific T cell. Transduced Jurkat cells were stained with a dead/live marker and DRB1*04:01/peptide tetramers conjugated with two different fluorophores (PE and APC). Flow cytometric analysis revealed tetramer-specific cells in the live double-positive stained subpopulation. A biotin-streptavidin complex without a specific peptide conjugate served as negative control. (B) Induction of specific IL-2 responses in transduced Jurkat 76 cells expressing the human TCR #16 receptor by stimulation with DRB1*04:01/Col2$_{259-273}$ peptide complexes. Transduced Jurkat cells were incubated with soluble DRB1*04:01/peptide complexes for 24 hours. Specific activation of cells via the TCR was measured by induced IL-2 release specific capture ELISA. Unstimulated cells served as a negative control. The MHCII restriction of TCR was performed by stimulation with the murine Aq/gal$_{264}$Col2 peptide complex. Bars indicate mean values, lines indicate SD and dots represent separate experiments. APC, allophycocyanin; Aq, murine MHCII allele; HA, influenza hemagglutinin 306–318; IL, interleukin; PE, phycoerythrin; TCR, T-cell receptor.

(figure 6A). By contrast, the presence of a galactose residue at position 264 allowed formation of a hydrogen bond to the TCRα-CDR3 backbone, also resulting in the stabilisation of both side chain bonds likely due to limitation in lysine 264 mobility (figure 6B). The stabilising allosteric effect is depicted in figure 7, which shows a comparative overview of all intermolecular bonds formed in the trimolecular complexes consisting of TCR#16 and DRB1*04:01 associated with either nCol2 or gal$_{264}$Col2. The graphic illustrates that the galactose is the only moiety bonded to all three molecules (the Col2 peptide, the DRB1*04:01 β-chain and the TCR). The molecular dynamics simulations for a 1000 ns period exhibited a rather low degree of molecular fluctuation in the TCR V-regions contacting the DRB1*04:01/Col2 peptide complex (figure 8). An exception was a peak of molecular mobility detectable in a solvent-exposed loop with reduced protein contacts carboxyterminal of TCRβ-CDR2 in the complex with nCol2-bound DRB1*04:01 (figure 8). Even more remarkable was the increase in molecular fluctuations in the constant region of TCR#16 affecting a Cβ domain just proximal to the so-called FG-loop and a Cα region that included the AB-loop.[33] The molecular flexibility of these functional domains, which localise near ectodomains of the signal-transducing CD3 membrane complex,[33] was clearly more pronounced in the trimolecular complex containing gal$_{264}$Col2 (figure 8).

DISCUSSION

Our studies characterised molecular details of antigen presentation by the DRB1*04:01 molecule, an allelic variant strongly associated with RA in Caucasian patients, by structure determinations following co-crystallisation with several peptides

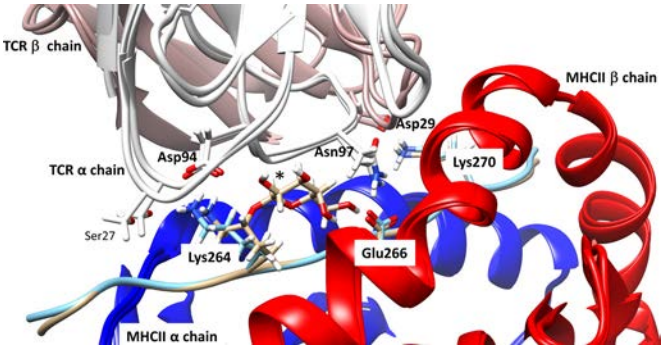

Figure 5 Molecular model of the three-dimensional structure of the multicomponent DRB1*04:01/Col2 peptide/TCR complex. Superimposition of minimised starting geometries for the trimolecular complexes of TCR#16 with DRB1*04:01 containing either the unmodified (K264) or galactosylated (gal264) version of the $Col2_{259\text{-}273}$ peptide. The image depicts three side chain bonds irrespective of $Col2_{259\text{-}273}$ galactosylation: two involving the CDR3 region of the TCRα-chain, Asp94 - Lys264 ($Col2_{259\text{-}273}$) and Asn97—Glu266 ($Col2_{259\text{-}273}$) and one in the CDR1 region of the TCRβ-chain, Asp29—Lys270 ($Col2_{259\text{-}273}$). The galactose (*) residue at Lys264 is not involved in side chain interactions with the TCR. TCR, T-cell receptor.

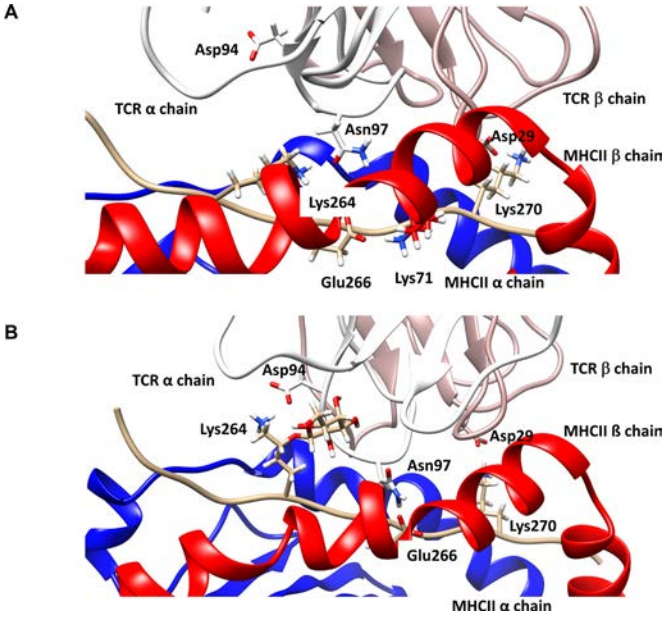

Figure 6 Geometry of the trimolecular complexes after 950 ns of molecular dynamics simulation. (A) Trimolecular complex consisting of TCR#16 and DRB1*04:01 complexed with the unmodified $Col2_{259\text{-}273}$ peptide. The initial salt bridge Lys264 - Asp94 (TCRα-CDR3) breaks to allow for neoformation of a bond between Lys264 of $Col2_{259\text{-}273}$ and Asn97 (TCRα-CDR3). Glu266 of $Col2_{259\text{-}273}$, which initially interacted with Asn97 in the TCRα (figure 5), now forms a salt bridge with Lys71 in the β-chain of DRB1*04:01, whereas the initial bond between Lys270 ($Col2_{259\text{-}273}$)-Asp29 (TCRβ-CDR1) remains preserved. (B) Trimolecular complex consisting of TCR#16 and DRB1*04:01 complexed with the $gal_{264}Col2_{259\text{-}273}$ peptide. The initial salt bridge Lys264 - Asp94 (TCRα-CDR3) remains intact, likely due to the stabilising impact of the sugar ring by decreasing the mobility of Lys264. The galactose ring in close contact to the CDR3 backbone of TCRα forms a hydrogen bond to the backbone. The two other side chain bonds of the starting geometry (figure 5), Glu266 ($gal_{264}Col2_{259\text{-}273}$)-Asn97 (TCRα-CDR3) and Lys270 ($gal_{264}Col2_{259\text{-}273}$)-Asp29 (TCRβ-CDR1), remain detectable. CDR, complementarity determining region; MHC, major histocompatibility complex; TCR, T-cell receptor.

known to trigger autoreactive RA T cells. A crucial residue for DR molecules associated with RA is position 71 in the β-chain. This residue, which is a lysine in DRB1*04:01, critically interacts with glutamic acid in position 266 of the Col2 peptide. However, there is some freedom in this interaction as other peptides derived from HSP and CILP contain different amino acids in position 266. The residues of valine 11 (V11b) and histidine 13 (H13b) of the β chain in HLA-DRB1*04:01 have been shown previously to be associated with susceptibility of seropositive RA in genome-wide association studies.[11] Our crystal structure analysis reveals that both residues likely contribute to the stability of the MHCII molecule as they are located where α and β chains pair in the beta-plated sheet. Moreover, a likely contribution to antigen presentation of the $CILP_{982\text{-}996}$ peptide is provided by the residue H13β that forms a hydrogen bond with the carboxyl group of the aspartic acid residue in the P6 pocket (P6D) of the CILP peptide. This hydrogen bond is missing in the other three complexed MHCII/peptide crystals, as the P6A ($HSP70_{289\text{-}306}$) and P6G ($Col_{259\text{-}273}$) lack the corresponding carboxyl group. In addition, the side chains of A74β orientate toward the P4 pocket, likely influencing the binding specificity of the P4 residue.

Similar to its interaction with murine A^q,[34] the DRB1*04:01 bound $Col2_{259\text{-}273}$ peptide exposes two lysine residues that are physiologically modified by hydroxylation and glycosylation, and these modified variants can be recognised by T cells.[18 35] Whereas in the mouse Col2 immunisation activates $Col2_{259\text{-}273}$ -specific T cells, thereby inducing a severe form of erosive arthritis, it is not clear to what extent MHCII-restricted Col2-specific T cells play a regulatory role in humans. In this context, there has been a long-standing question concerning why these T cells are not deleted from the repertoire by central tolerance. A possible explanation could be that TCR affinity for PTM variants of Col2 peptides promotes escape from thymic selection; this hypothesis is supported by our previous finding that non-modified Col2, but not glycosylated Col2-determinants, could be expressed by mouse or human thymic epithelium.[29] Notably, earlier studies have detected glycopeptide-reactive T cells in patients with DRB1*04:01-positive and DRB1*01:01-positive RA,[18 36] and the present investigation provides new evidence that peripheral T cells of healthy individuals also express TCRs with binding affinity for DRB1*01:01/ $gal_{264}Col2$ complexes in respective MHCII allele carriers.

The present investigation of human PBMCs from RA and healthy DRB1*04:01 carriers, which used tetramer staining as well as parallel peptide-induced T cell activation assays, provides new insight into the impact of PTM on Col2 T-cell recognition. In contrast to a comparable prevalence of $CD4^+$ T cells that recognise nCol2 or $gal_{264}Col2$ in DRB1*04:01 tetramer complexes, T-cell responsiveness was increased on peptide challenge with the galactosylated variant. The mechanism for this glycosylation-dependent impact on T-cell activation is not entirely clear and might be multifactorial, but one attractive hypothesis is that an altered TCR interaction with the DRB1*04:01-bound galactosylated Col2-peptide affects TCR signal transmission. Accordingly, our functional and structural characterisation of a prototypic human TCR and its interaction with nCol2 or $gal_{264}Col2$ in the context of DRB1*04:01 presentation provide first experimental support for this hypothesis. The comparative dynamic modelling of the respective trimolecular complexes containing either the galactosylated or unmodified

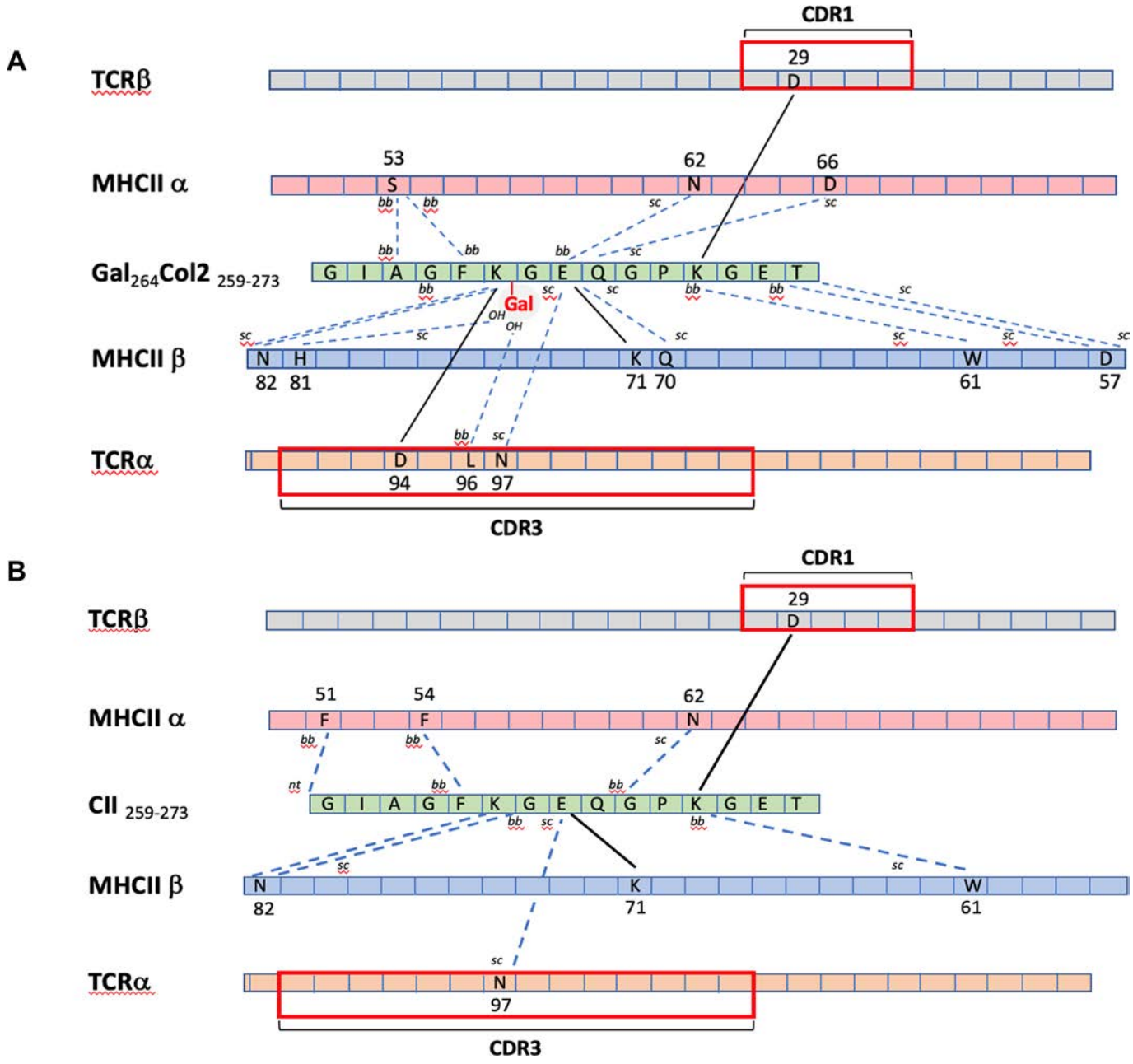

Figure 7 Comparative overview of intermolecular bonds formed in the trimolecular complexes of TCR#16 and DRB1*04:01 (MHCII) associated with either (A) gal$_{264}$Col2 or (B) nCol2. Amino acids are shown in one letter code and their positions in the respective protein sequences indicated by number. Bold lines indicate salt bridges and dashed lines indicate hydrogen bonds. ai, aromatic interaction; bb, backbone; CDR, complementarity determining region; MHC, major histocompatibility complex; sc, side chain, TCR, T-cell receptor.

Col2 variant revealed a major difference in the propagation of TCR dynamics from the V-regions to key allosteric sites in the Cα and Cβ region pertaining particularly to the so-called CαAB loop.[33] This domain was previously described for its role in the allosteric regulation of TCR signalling as evidenced by fluorescence-based conformational changes on MHCII/peptide perception and signalling impairment by mutational analysis.[37] Another region exhibiting reinforced molecular fluctuations on TCR recognition of the DRB1*04:01/gal$_{264}$Col2 in our studies is in immediate proximity to the CβFG loop that has been critically incriminated in T-cell activation and thymic selection.[38 39]

Thus, our dynamic modelling studies provide evidence for allosteric changes initiated by CDR3α-region interaction with a single galactose residue in the DRB1*04:01-bound gal$_{264}$Col2 peptide, which propagates to result in increased conformational flexibility at distant sites in the constant TCR regions contacting the CD3 signalling complex. Whereas it remains enigmatic how these allosteric changes are transmitted across the cell membrane, our complementary functional T-cell studies provide experimental support for translation into a reinforced TCR signal in response to challenge by gal$_{264}$Col2-bound versus nCol2-bound DRB1*04:01complexes. Moreover, the data obtained by analysis of RA T cells and the prototypic TCR #16 might reflect mechanisms of central tolerance by thymic medullary epithelial cells that do not express galactosylated Col2 and accordingly execute clonal deletion exclusively via presentation of unmodified Col2 determinants.[29] Thus, T cells rescued from thymic selection due to weak TCR recognition of DRB1*04:01/

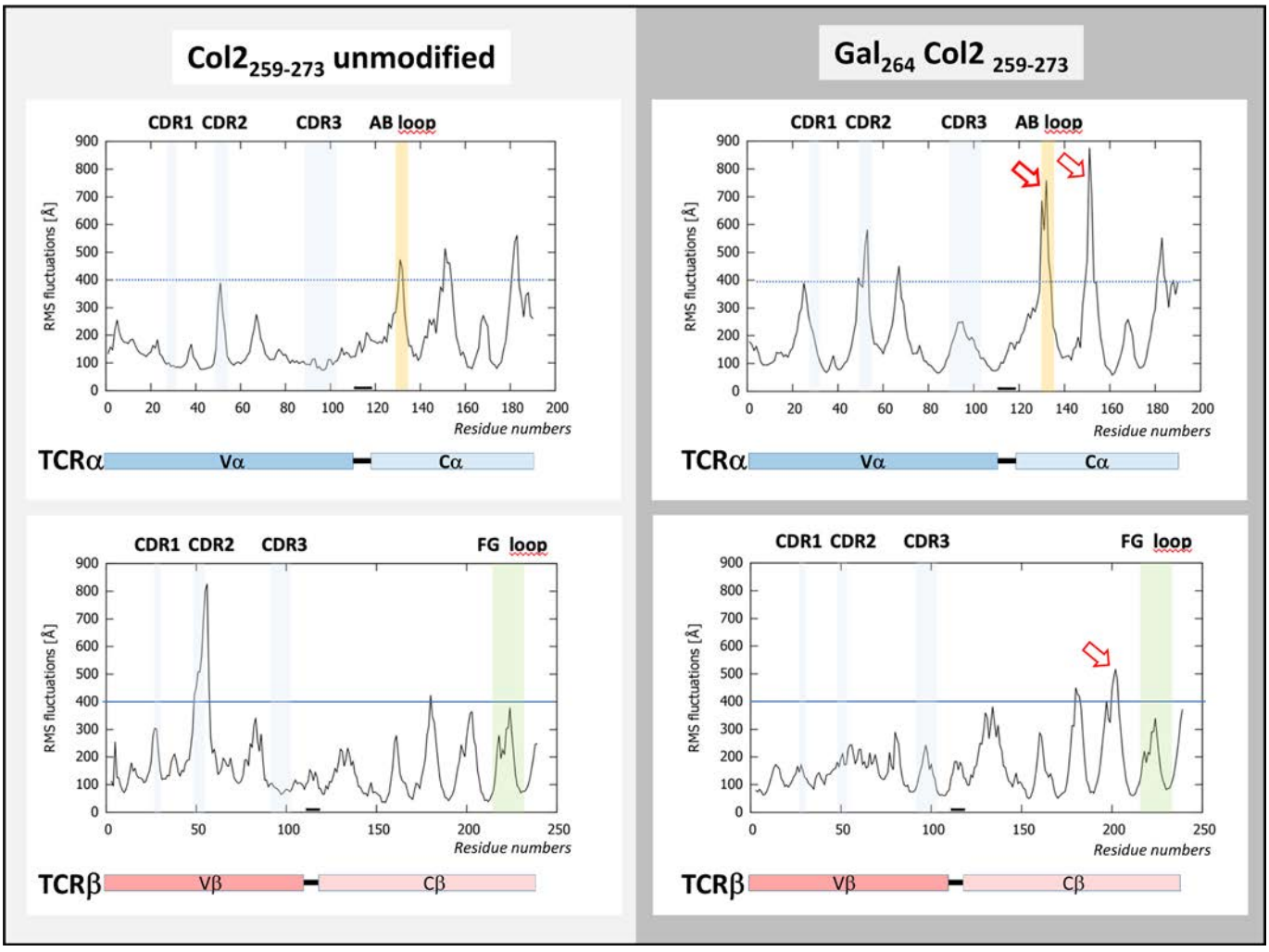

Figure 8 Molecular dynamics simulations of trimolecular complexes consisting of TCR#16 and DRB1*04:01 associated with either the galactosylated (gal264) (left) or unmodified $Col2_{259-273}$ peptide (right). Depicted are the root mean squares of fluctuation values for the variable and constant region residues of TCRα and TCRβ within the trimolecular complex over a simulation period of 1000 ns. In complex with the unmodified Col2 peptide, a peak of molecular mobility is detectable carboxyterminal of the TCRβ-CDR2 domain and to a minor degree in TCRα-CDR2 in complex with the gal264Col2 peptide. Most notable is the increase of molecular fluctuations in the constant region of TCR#16 (arrows), especially in the α-chain, also affecting the CαAB loop and clearly much more pronounced in complex with gal264 compared with the unmodified Col2 peptide. CDR, complementarity-determining region; RMS, root mean square; TCR, T-cell receptor.

nCol2 complexes and the resulting low TCR signal intensities could subsequently become activated on MHCII presentation of physiologically galactosylated Col2 in peripheral tissues[39] via reinforced signalling dynamics initiated by the recognition of posttranslational Col2 peptide modifications. However, the outcome of the activation, which can result in either arthritogenic or tolerogenic T cell responses, would remain context dependent. An obvious objection to our findings on the lack of a contribution of citrulline residues to MHCII binding in the two crystallised control complexes containing citrullinated epitopes of RA T cell responses and our focus on TCR recognition of Col2 peptides is how our proposed concept could be linked to citrulline-specific immunity and its strong association with DRB1*04:01 in RA. However, it has been shown that Col2 can be citrullinated in vivo, both in mice and in humans with RA.[22] Moreover, the autoantibody response to citrullinated Col2 is prominent in B cell recognition of Col2. Accordingly, it is easily conceivable that B cells specific for citrullinated Col2 can present the non-citrullinated 259–273 peptide to T cells and vice versa.[40] Such T cells could help to activate B cells specific for citrullinated epitopes, potentially breaking tolerance and allowing pathogenic epitope spreading of the anticitrullinated protein antibody response target, joint cartilage.[40]

Although we have provided indirect evidence for the proposed scenario, further studies are clearly needed to provide additional experimental support and to answer the question of whether this hypothesis might also apply to citrullinated antigens, such as the CILP-derived peptide cocrystallised with DRB1*04:01 in this study.

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Ethics approval All blood sample donors gave prior written consent for study inclusion. The current study has been approved by the ethical approval committee of the University Hospital Frankfurt, Germany.

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Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as supplementary information. The crystallographic coordinates and structure factors elucidated in this study have been deposited in the Protein Data Bank with the accession codes listed in online supplemental table S1 (7NZE, 7NZF, 7NZH, 7O00).[41–44]

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CLINICAL SCIENCE

Dactylitis is an indicator of a more severe phenotype independently associated with greater SJC, CRP, ultrasound synovitis and erosive damage in DMARD-naive early psoriatic arthritis

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ABSTRACT

Objective To characterise the impact of dactylitis in disease-modifying antirheumatic drug (DMARD)-naive early psoriatic arthritis (PsA).

Methods Patients with early PsA meeting the classification criteria for PsA (CASPAR) were recruited. Clinical outcomes were recorded, and ultrasonography was conducted to assess grey scale (GS) and power Doppler (PD) synovitis, periarticular cortical bone erosions and enthesitis. The cohort was dichotomised by the presence or absence of dactylitis.

Results Of 177 patients with PsA, those with dactylitis (dactylitic PsA (81/177, 46%)) had higher tender joint count (p<0.01), swollen joint count (SJC) (p<0.001) and C reactive protein (CRP) (p<0.01) than non-dactylitic PsA. Dactylitis was more prevalent in toes (146/214 (68.2%)) than fingers (68/214 (31.8%)); 'hot' dactylitis was more prevalent than 'cold' (83.6% vs 16.4%). Ultrasound (US) synovitis and erosions were significantly more prevalent in dactylitic PsA (p<0.001 and p<0.001, respectively). Exclusion of dactylitis in dactylitic PsA confirmed significantly greater SJC (3 vs 1, p=0.002), US synovitis (GS ≥2: 20.6% vs 16.1%, p<0.001, or PD ≥1: 5.1% vs 3.3%, p<0.001) and erosions (1.1% vs 0.5% joints, p=0.008; 26.1% vs 12.8% patients, p=0.035%) than non-dactylitic PsA. Synovitis (GS ≥2 and/or PD ≥1) occurred in 53.7% of dactylitis. No substantial differences were observed for US enthesitis.

Conclusion Dactylitis signifies a more severe disease phenotype independently associated with an increased disease burden with greater SJC, CRP, US-detected synovitis and bone erosions in DMARD-naive early PsA and may be a useful discriminator for early risk stratification.

Key messages

What is already known about this subject?

⇒ Dactylitis is a typical lesion in psoriatic arthritis (PsA) and is associated with radiographic progression in chronic disease.

⇒ In early PsA, dactylitis is a common finding, but the associated impact of this lesion on the disease burden is unknown.

What does this study add?

⇒ This study demonstrates that disease-modifying antirheumatic drug (DMARD)-naive patients with early PsA with dactylitis (dactylitic PsA) have a greater burden of disease than patients with PsA without dactylitis (non-dactylitic PsA), which was confirmed independently (ie, when dactylitis was excluded) demonstrating greater swollen joint count, C reactive protein, ultrasound (US) synovitis and US erosions.

⇒ Our data confirm that dactylitis is a clinical marker of a more severe phenotype in DMARD-naive early PsA.

How might this impact on clinical practice or future developments?

⇒ In patients with early PsA, the presence of dactylitis identifies a more severe disease phenotype and may be an important discriminator for risk stratification in early arthritis clinics and clinical research trials.

INTRODUCTION

Dactylitis is defined as diffuse swelling of a finger or toe and represents a specific lesion typically associated with psoriatic arthritis (PsA). The prevalence of dactylitis in PsA has been estimated at 33%–55%, with approximately 70% occurring at presentation.[1] Dactylitis is the epitome of PsA pathophysiology, encompassing multiple underlying pathologies including inflammation to joints (synovitis) and tendons/ligaments (enthesitis). Flexor tenosynovitis, surrounding diffuse peritendinous inflammation and soft tissue oedema, are typically responsible for the 'sausage digit' appearance.[2] Importantly, synovitis and bone erosion can develop, adding to further structural and functional impairments.[3] Bone marrow oedema and ligamentous enthesitis have also been demonstrated using high-resolution MRI.[4] At the bedside, the accuracy of ultrasound (US) for detecting inflammatory arthritis in PsA is regarded as comparable to MRI, with studies suggesting US may be superior for the assessment of synovitis.[5]

The presence or history of dactylitis adds high sensitivity and specificity towards classifying PsA (CASPAR criteria).[6] Further, dactylitis is associated with greater radiographic damage in chronic established PsA.[7] However, to our knowledge, direct evaluation of the impact of dactylitis on overall disease phenotype and severity in early, untreated PsA has not been characterised. The objective of this study was to determine the impact of dactylitis on clinical phenotype, US synovitis and erosion in disease-modifying antirheumatic drug (DMARD)-naive early PsA.

METHODS

Patients, clinical details and examination

In total, 177 DMARD-naive patients with early PsA meeting CASPAR criteria were recruited into the Leeds Spondyloarthropathy Register for Research and Observation for baseline cross-sectional analysis.[6] Clinical examination included tender joint count (TJC) (78) and swollen joint count (SJC) (76). The early PsA cohort was dichotomised by the presence or absence of dactylitis at baseline (PsA with dactylitis (dactylitic PsA) or PsA without dactylitis (non-dactylitic PsA)). Dactylitis was recorded per digit via the dichotomous (Clegg *et al*) method including tender ('hot') or non-tender ('cold') status.[8 9] Clinical enthesitis was measured by the Maastricht Ankylosing Spondylitis Enthesitis Score (MASES) to include peripheral and axial entheses.

US examination

Experienced ultrasonographers blinded to clinical details (four operators with over 5 years' experience) scanned 50 joints per patient using the GE Logiq E9 machine with matrix linear (ML) 15–6 MHz or small-footprint linear array 18–8 MHz transducer and had regular training and calibration on the US examination protocol and quality of sonographic assessment throughout the study period, conducted every 6 months to maintain high consistency for US assessment, image interpretation and scoring.

Synovitis

Synovitis was graded by semiquantitative scores (0–3) and defined as grey scale (GS) ≥2) or abnormal power Doppler (PD) signal (PD ≥1), and GS of ≤1 was determined as non-significant as it occurs frequently in healthy individuals.[10]

Wrists (radiocarpal, intercarpal and ulnar carpal recesses), metacarpophalangeal (MCP) joints 1–5, proximal interphalangeal (PIP) joints 1–5, distal interphalangeal joints 2–5, elbows, knees (suprapatellar, medial parapatellar and lateral parapatellar recesses), ankles (tibiotalar joint), subtalar joints, talonavicular joints and metatarsophalangeal (MTP) joints 1–5 were scanned in longitudinal and transverse planes (online supplemental table S1A).

Erosions

Erosions were determined by periarticular cortical bone discontinuity present in two perpendicular planes (longitudinal and transverse), with MTP1 excluded as it is a frequent site of osteoarthritis.

Enthesitis

Enthesitis was determined by the Outcome MEasures in Rheumatology (OMERACT)-defined elementary lesions and modified Glasgow Ultrasound Enthesitis Severity Score (GUESS), calculated per patient based on all the enthesitis sites and domains (except bursitis at the quadriceps tendon insertion—not recorded in the study protocol).[11 12] The US data recorded for entheses are shown in online supplemental table S1 (B).

Statistical analysis

Statistical tests were two-tailed, statistical significance prespecified at 5% ($p<0.05$) with 95% CIs. Differences between mean, medians and proportions were calculated using Student's t-test, quantile regression (continuous variables), χ^2 test (binary variables) and Kruskal-Wallis (categorical variables) via Stata V.16.1.

RESULTS

Clinical characteristics

Dactylitic PsA versus non-dactylitic PsA

Dactylitic PsA occurred in 81/177 (46%) patients vs non-dactylitic PsA in 96/177 (54%) patients. Mean ages were similar; 43.7 and 44.4 years, respectively. More patients in the dactylitic group had a symptom duration of <24 months (68/81 (84%) vs 64/96 (66.7%), $p=0.008$). The median TJCs and SJC were significantly greater in patients with dactylitic PsA compared with patients without dactylitic PsA (TJC: 9 vs 4, $p<0.01$; SJC 7 vs 1, $p<0.001$), with polyarthritis being the predominant phenotype in dactylitic PsA (65.4%, $p<0.01$), while oligoarthritis was dominant in non-dactylitic PsA (86.5%, $p<0.001$). Excluding dactylitis affected digits, dactylitic PsA remained predominantly polyarticular (51/81 patients, 62.9%), and the SJC (but not the TJC) still significantly greater (total SJC: 326 joints (81 patients) vs 209 joints (96 patients), median 3 vs 1, $p=0.002$).

Clinical enthesitis was more prevalent in patients with dactylitic PsA (42/81 (51.9%) vs 34/96 (35.4%); $p=0.027$), with greater median MASES (1.0 (0.0–2.0) vs 0.0 (0.0–2.0); $p<0.01$). The prevalence of nail dystrophy did not differ between groups, however the median modified nail psoriasis severity index (mNAPSI) was greater in non-dactylitic PsA (2.0 (0.0–7.5) vs 0.0 (0.0–8.0); $p<0.05$).

Elevated C reactive protein (CRP >10 mg/L) occurred more frequently in dactylitic versus non-dactylic patients (44% vs 25% ($p=0.006$)) including with a greater median CRP and erythrocyte sedimentation rate (ESR; mm/hr) (CRP: 8.1 vs 5.0 ($p<0.01$), ESR: 16.5 vs 11 ($p<0.05$)). Disease Activity in Psoriatic Arthritis (DAPSA) scores were greater in dactylitic PsA but not significant (median 24.4 vs 20.8, $p=0.07$). No significant differences were observed in PsAQoL, HAQ, and DLQI. Comparison of patient characteristics between groups are shown in table 1.

Characteristics of dactylitis

Of 81/177 (45.8%) patients with dactylitic PsA, dactylitis affected 214 digits, predominantly with multiple digit involvement (>1) in 51/81 (63%) patients (median digits: 2 (IQR 1–3)) and was distributed asymmetrically (52/81 (64%) patients). Hands were affected in 23/81 (28.4%) patients, feet in 40/81 (49.4%), and both in 18/81 (22.2%). Dactylitis was more prevalent in toes (146/214, 68.2%) than fingers (68/214, 31.8%) with the majority of digits classified as hot dactylitis (179/214 digits, 83.6%) (cold dactylitis (35/214, 16.4%)). The second finger (23/179, 12.8%) and fourth toe (40/179; 22.3%) were most frequently affected by hot dactylitis, and the third finger (2/35; 5.7%) and fourth toe (10/35; 28.6%) by cold dactylitis (online supplemental figure S1A).

US synovitis

In total, 155/177 (87.5%) patients with PsA underwent ultrasonography (6143 joints): 69/155 (44.5%) patients with dactylitic PsA and 86/155 (55.5%) without dactylitic PsA

Table 1 Characteristics of the early PsA cohort dichotomised by the presence or absence of dactylitis

Characteristics and outcomes	Non-dactylitic PsA (96/177 (54.2%))	Dactylitic PsA (81/177 (45.8%))	Difference/p value
Clinical			
Age (years), mean (SD)	44.4 (12.8)	43.7 (13.3)	0.7 (−3.2 to 4.5)
Male	38 (39.6%)	42 (51.9%)	p=0.10
Symptom duration (months), median (IQR)	18.0 (10.5–36.0)	12.0 (6.0–24.0)	−6.0 (−13.1 to 1.1)
Duration from diagnosis (months), median (IQR)	1.1 (0–2.7)	1.2 (0.3–4.6)	0.03 (−0.9 to 1.0)
Symptoms <24 months, patients (%)	64/96 (66.7)	68/81 (84.0)	p=0.008
Early morning stiffness (min), median (IQR)	50.0 (15.0–90.0)	60.0 (15.0–180.0)	0 (−24.1 to 24.1)
TJC (78), median (IQR)	4.0 (1.0–10)	9.0 (5.0–19.0)	5.0 (2.0 to 8.0)**
SJC (76), median (IQR)	1.0 (0.0–3.0)	7.0 (4.0–13.0)	6.0 (4.3 to 7.6)***
TJC (78) median (IQR) (excluding dactylitis)	4.0 (1.0–10.0)	5.0 (2.0–11.0)	1.0 (−1.4 to 3.4)
SJC (76) median (IQR) (excluding dactylitis)	1.0 (0.0–3.0)	3.0 (1.0–6.0)	2.0 (0.8 to 3.3)**
Current psoriasis	96/96 (100.0%)	74/81 (91.4%)	p=0.003**
Family history of psoriasis	52/94 (55.3%)	49/78 (62.8%)	p=0.32
PASI, median (IQR)	2.9 (0.8–4.9)	1.9 (0.4–4.2)	−1.2 (−2.4 to 0.0)
Psoriatic nail dystrophy	49/96 (51.0%)	44/81 (54.3%)	p=0.66
mNAPSI, median (IQR)	2.0 (0.0–7.5)	0.0 (0.0–8.0)	−2.0 (−3.7 to −27.9)*
Clinical Enthesitis	34/96 (35.4%)	42/81 (51.9%)	p=0.027*
MASES score, median (IQR)	0.0 (0.0–2.0)	1.0 (0.0–2.0)	1.0 (0.4 to 1.6)**
BMI, median (IQR)	28.2 (24.0–32.1)	28.6 (25.0–31.5)	0.3 (−1.7 to 2.4)
Smoking (current)	19 (19.8%)	9 (11.1%)	p=0.11
Disease phenotype			
Oligoarthritis (defined by SJC <5)	83/96 (86.5%)	28/81 (34.6%)	p<0.001***
Oligoarthritis (defined by TJC and/or SJC <5)	48/96 (50%)	29/81 (35.8%)	p=0.058
DIP joint disease	7/93 (7.5%)	13/77 (16.9%)	p=0.058
Axial disease	17/94 (18.1%)	9/78 (11.5%)	p=0.23
Arthritis mutilans	0	0	0
Laboratory markers			
CRP (mg/L), median (IQR)	5.0 (5.0–9.3)	8.1 (5.0–18.4)	3.1 (0.9–5.3)**
Elevated CRP (>10 mg/L)	24/96 (25.0%)	36/81 (44.4%)	p=0.006**
ESR, median (IQR)	11.0 (5.0–25.0)	16.5 (7.0–27.0)	7.0 (0.4–13.6)*
Composite outcomes			
DAPSA score, median (IQR)	20.8 (12.6, 30.5)	24.4 (14.9, 36.5)	p=0.07
Patient-reported outcomes (PROs)			
PsAQoL, median (IQR)	6.0 (0.0–13.0)	6.0 (2.0–12.0)	0.0 (−4.1 to 4.1)
DLQI, median (IQR)	3.0 (1.0–9.0)	2.0 (1.0–6.0)	−1.0 (−3.3 to 1.3)
HAQ, median (IQR)	0.75 (0.25–1.50)	0.75 (0.38–1.38)	0.125 (−0.23 to 0.48)

*p<0.05, **p<0.01, ***p<0.001.
BMI, body mass index; CRP, C reactive protein; DAPSA, Disease Activity in Psoriatic Arthritis; DIP, distal interphalangeal; DLQI, Dermatology Quality of Life Index; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire Disability Index; MASES, Maastricht Ankylosing Spondylitis Enthesitis Score; mNAPSI, Modified Nail Psoriasis Severity Index; PASI, Psoriasis Area Severity Index; ; PsA, psoriatic arthritis; PsAQoL, PsA quality of life; SJC, swollen joinr count; TJC, tender joint count.

(online supplemental figure S1B). US synovitis was significantly more prevalent in dactylitic PsA (GS ≥2: 23.6% vs 16.1% joints (p<0.001); PD ≥1: 7.3% vs 3.3% joints (p<0.001); GS ≥2+PD ≥1: 6.3% vs 2.6% joints (p<0.001)) as outlined in table 2A. GS ≥2 synovitis was most frequently observed at MCP 2–5, PIP1–3, MTP2–5, and PD ≥1 synovitis at MCP 2 and MTP 4–5. On exclusion of digits affected by dactylitis, US synovitis remained significantly more prevalent in patients with dactylitic PsA (GS ≥2: 21.3% vs 16.1% joints (p<0.001); PD ≥1: 5% vs 3.3% joints (p<0.001); GS ≥2+PD ≥1: 4.1% vs 2.6% joints (p<0.003); table 2B). Further subgroup analyses stratified for phenotype (defined by SJC ≥5 as polyarticular) confirmed greater GS ≥2 and PD ≥1 synovitis, respectively, for polyarticular subsets in dactylitic PsA (p<0.001), including greater GS ≥2 synovitis when dactylitis was excluded (p=0.01) (online supplemental table S2). Application of the second definition inclusive of tender joints (tender and/or swollen joints<5 as oligoarticular) indicated significantly greater GS of ≥2 and PD of ≥1 synovitis, respectively, independent of phenotype in dactylitic PsA. Exclusion of dactylitis from dactylitic PsA also confirmed greater GS of ≥2 synovitis in oligoarticular and polyarticular subsets independent of dactylitis affected joints (online supplemental table S3).

US erosions

Periarticular cortical bone erosions were identified in a significantly greater proportion of patients with dactylitic PsA, compared with those without dactylitis (20/69 (29.0%) vs 11/86 (12.8%), p=0.012). There was also a significant difference in the total number of erosions detected in dactylitic versus patients without dactylitic PsA (33/2557 joints vs 15/3206 joints, p<0.001; table 2A). The anatomical sites for joints most prone to erosive damage were MCP2 (9/33 (27.3%)) and MTP5 (11/33 (33.3%)).

Table 2 Ultrasound synovitis and bone erosions in non-dactylitic versus dactylitic PsA: (A) including dactylitis affected digits and (B) excluding dactylitis affected digits

(A) US synovitis and erosions	Non-dactylitic PsA (86/155 (55.5%) patients)	Dactylitic PsA (69/155 (44.5%) patients)	Difference
Total GS ≥2 (joints)	551/3422 (16.1%)	642/2721 (23.6%)	p<0.001
Total PD ≥1 (joints)	114/3422 (3.3%)	198/2721 (7.3%)	p<0.001
Total GS ≥2+PD >1 (joints)	89/3422 (2.6%)	171/2721 (6.3%)	p<0.001
Total US erosions (joints)	15/3206 (0.5%)	33/2557 (1.3%)	p<0.001
Total erosion score (patient level)	Mean 0.28 (SD 0.87), median 0 (0–0)	Mean 0.72 (SD 1.63), median 0 (0–1)	p=0.016
Total US erosive patients	11/86 (12.8%)	20/69 (29.0%)	p=0.012
(B) US synovitis and erosions	**Non-dactylitic PsA (86/155 (55.5%)) (same as A)**	**Dactylitic PsA (69/155 (44.5%)) (dactylitis excluded)**	**Difference**
Total GS ≥2 (joints)	551/3422 (16.1%)	507/2466 (20.6%)	p<0.001
Total PD ≥1 (joints)	114/3422 (3.3%)	126/2466 (5.1%)	p<0.001
Total GS ≥2+PD >1 (joints)	89/3422 (2.6%)	101/2466 (4.1%)	p=0.003
Total US erosions (joints)	15/3206 (0.5%)	24/2315 (1.1%)	p=0.008
Total erosion score (patient level)	Mean 0.28 (SD 0.87), median 0 (0–0)	Mean 0.58 (SD 1.52), median 0 (0–1)	p=0.048
Total US erosive patients	11/86 (12.8%)	18/69 (26.1%)	p=0.035

GS, grey scale; PD, power Doppler; PsA, psoriatic arthritis.

On exclusion of dactylitic digits, US erosions were more frequent in dactylitic PsA than non-dactylitic PsA (24/2315 (1.1%) vs 15/3206 (0.5%) joints, p=0.008). The proportion of patients with US erosions (US erosion-positive patients) was greater for dactylitic PsA (18/69 (26.1%) vs 11/86 (12.8%) patients, p=0.035). Total erosion scores at the patient level were also higher in dactylitic PsA (p=0.016), including when dactylitis was excluded (p=0.048), as shown in table 2B. Subgroup analyses confirmed greater total US-detected erosions in polyarticular stratified subsets regardless of the phenotype definition applied and on excluding dactylitis affected joints (online supplemental table S2 and S3). The US appearances of erosions detected in the dactylitic PsA group are illustrated in figure 1B,D.

US in dactylitis

In digits affected by dactylitis, US synovitis (GS ≥2) was prevalent in 137/255 (53.7%) joints. A higher prevalence of US synovitis was observed in joints affected by hot dactylitis versus those with the cold type (129/227 (56.8%) vs 8/28 (28.6%) joints, p=0.0047). US PD synovitis (PD ≥1 regardless of GS grade) was present in 72/255 (28.2%) of the total joints clinically affected by dactylitis and was more prevalent in hot type (hot: 69/227 (30.4%) and cold: 3/28 (10.7%) joints, p=0.0289). In hot dactylitis, erosions occurred in 9/227 (2.6%) of affected joints (4/69 (6%) patients) and none in cold dactylitis (0/28) (p=0.388). Figure 1 illustrates synovitis at MTP5 (figure 1A) and shows soft tissue oedema and flexor tenosynovitis (figure 1E).

US enthesitis

Of 1534 entheses examined by US, modified GUESS scores indicated no significant differences between patients with dactylitic PsA and patients without dactylitic PsA (median 3 (IQR 2–6) vs median 4 (IQR 1–6), p=0.91). There were no relevant differences between groups on analysis of OMERACT elementary lesions.

DISCUSSION

This is the first study, to our knowledge, to evaluate the overall clinical and US disease burden in a DMARD-naive early PsA cohort based on the presence/absence of dactylitis. These study results confirmed a greater burden of disease in PsA with dactylitis. Moreover, patients with dactylitic PsA had greater SJC, CRP, prevalence of US synovitis and erosive damage independently (ie, on exclusion of digits affected by dactylitis) compared with non-dactylitic PsA. These results therefore provide an insight into the significance of dactylitis in early PsA, demonstrating that it is an indicator of a more severe phenotype. Indeed, more aggressive disease accounted for an earlier diagnosis of patients with dactylitic PsA who presented with shorter disease duration. Further analyses to include history of dactylitis and exclude patients with a symptom duration of >24 months, respectively, did not change results confirming the increased burden of disease in the dactylitic group (online supplemental table S4 and S5).

Previous studies in established PsA have shown that digits affected by dactylitis are associated with significant pathological findings. Brockbank *et al* first reported that radiographic damage occurred frequently in hot dactylitis with an average PsA cohort disease duration of 8 years.[7] Healy *et al* reported a high prevalence of synovitis in hot dactylitis present on MRI in 69%, closely matching the prevalence of US detected synovitis in our study (56.8%), confirming that synovitis is present in the majority of dactylitis in early PsA.[13] Moreover, in our early PsA study, the greater prevalence of US synovitis and erosions in patients with dactylitic PsA (vs non-dactylitic PsA), even when excluding the dactylitis digits, has to our knowledge not previously been shown and reflects an increased burden of disease. Further, this was reflected by CRP, also a marker of disease activity which was elevated (>10 mg/L) more often in patients with dactylitic PsA (44.4% vs 25%, p=0.006) and with higher median values. This is a relevant observation, since elevated baseline CRP is associated with poor radiographic outcomes.[14] Additionally, radiographic joint destruction is reportedly predicted by the development of dactylitis in men from longitudinal PsA cohort data.[15] Despite the differences found for clinical enthesitis, a possible limitation of our study was the inability to show meaningful differences in US enthesopathy possibly related to the outcomes used or the known clinical and US mismatch shown in other stuides.[16] However, our results do provide new data, including for US synovitis and erosion, showing that there is a difference in the burden of disease between patients with early PsA with and without dactylitis.

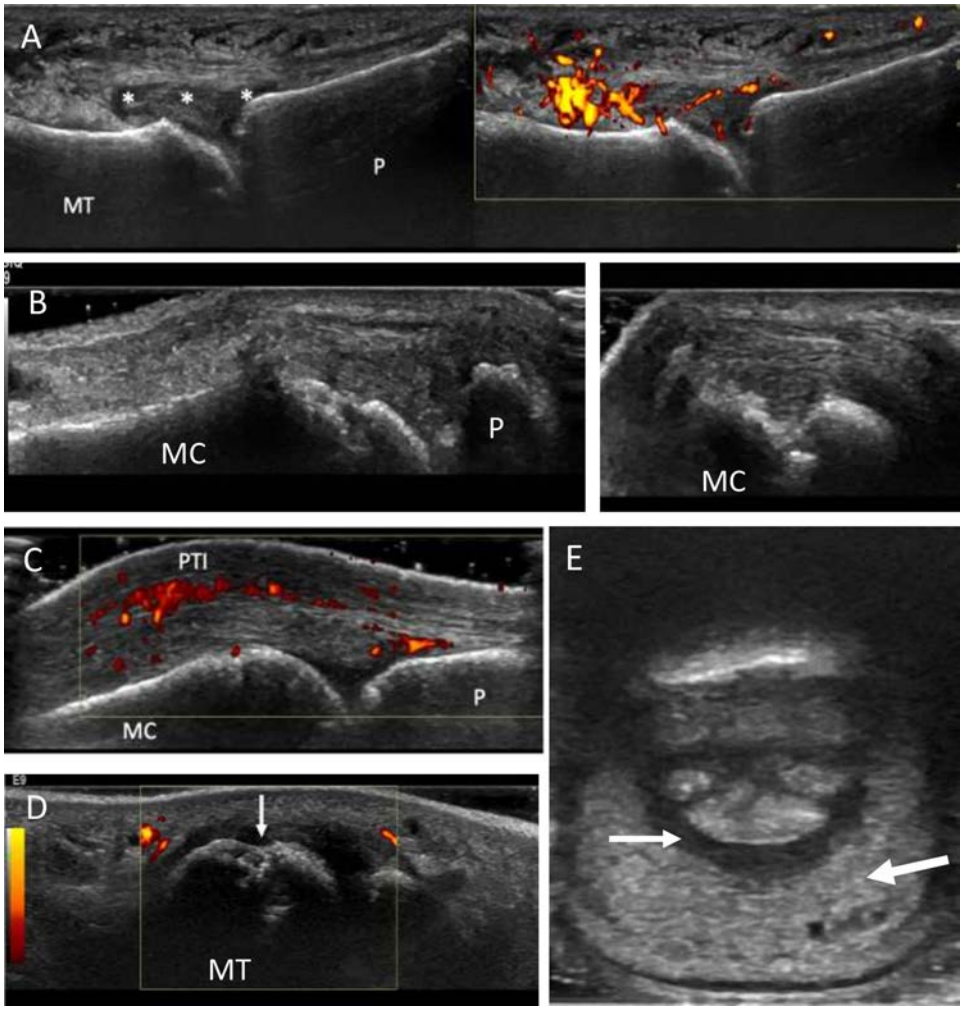

Figure 1 Characteristic ultrasound pathologies in early dactylitic patients with PsA. (A) Longitudinal view through the fifth metatarsophalangeal joint illustrating synovitis within a dactylitic toe. There is grey scale synovitis (grade 3) with effusion (*) and abnormal power Doppler signal (grade 2, right image) consistent with 'active' synovitis. (B) Periarticular cortical bone irregularity at the second MCP joint confirmed in the longitudinal (left) and transverse planes, respectively (right), confirming erosion. A common site of erosion in PsA and in dactylitis. (C) Longitudinal view at the MCP joint displaying power Doppler signal above the extensor tendon (PTI). (D) Image in the transverse plane showing the fifth metatarsal head, the most frequent site of erosion in feet, demonstrating periarticular bone irregularity (arrow). Bone irregularity was confirmed further in the longitudinal plane to signify erosion. There is also surrounding grey scale synovial hypertrophy (grade 2). (E) Transverse view of volar aspect of dactylitic third toe showing diffuse soft tissue oedema (large arrow) and flexor tenosynovitis (small arrow). MCP, metacarpophalangeal; MC, metacarpal head; MT, metatarsal head; P, phalanx; PsA, psoriatic arthritis; PTI, peritendon inflammation.

Tailoring therapy specific to PsA phenotypes is increasingly pertinent to avoid biologic disease-modifying antirheumatic drug (bDMARD) failure, especially given the diverse mode of action therapies available. Moreover, superior treatment responses have been shown for dactylitis with bDMARDs over conventional synthetic DMARDs, including first-line combination therapy.[17 18] Our study findings can facilitate early risk stratification to optimise treatment outcomes, coherent with the European Alliance of Associations for Rheumatology (EULAR) recommendations that regard dactylitis as a poor prognostic factor in early PsA and advocate rapid initiation of DMARDs.[19] Overall, these data may further inform management strategies, including clinical trials for targeted therapy to understand differential responses within PsA phenotypes.

CONCLUSIONS

The presence of dactylitis is independently associated with an increased burden of disease with greater SJC, CRP, US-detected synovitis and erosive bone damage in DMARD-naive early PsA. Dactylitis should therefore be considered a clinical marker for a more severe phenotype in early PsA and may be an important discriminator for risk stratification in early intervention strategies.

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Contributors SD conducted the study, analysis and the writing of the manuscript. OA and XM conducted statistical methods and tests. XM and LG-M contributed to the clinical assessment of patients. RJW contributed to ultrasound scan procedure and protocol. PE and ALT conducted clinical supervision of patients and writing of the manuscript. HM-O, DGM and PH contributed to writing of the manuscript. HM-O designed the study protocol and oversaw its conduct as principal investigator and guarantor.

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CLINICAL SCIENCE

Phase II randomised trial of type I interferon inhibitor anifrolumab in patients with active lupus nephritis

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ABSTRACT

Objective To assess the efficacy and safety of the type I interferon receptor antibody, anifrolumab, in patients with active, biopsy-proven, Class III/IV lupus nephritis.

Methods This phase II double-blinded study randomised 147 patients (1:1:1) to receive monthly intravenous anifrolumab basic regimen (BR, 300 mg), intensified regimen (IR, 900 mg ×3, 300 mg thereafter) or placebo, alongside standard therapy (oral glucocorticoids, mycophenolate mofetil). The primary endpoint was change in baseline 24-hour urine protein–creatinine ratio (UPCR) at week (W) 52 for combined anifrolumab versus placebo groups. The secondary endpoint was complete renal response (CRR) at W52. Exploratory endpoints included more stringent CRR definitions and sustained glucocorticoid reductions (≤7.5 mg/day, W24–52). Safety was analysed descriptively.

Results Patients received anifrolumab BR (n=45), IR (n=51), or placebo (n=49). At W52, 24-hour UPCR improved by 69% and 70% for combined anifrolumab and placebo groups, respectively (geometric mean ratio=1.03; 95% CI 0.62 to 1.71; p=0.905). Serum concentrations were higher with anifrolumab IR versus anifrolumab BR, which provided suboptimal exposure. Numerically more patients treated with anifrolumab IR vs placebo attained CRR (45.5% vs 31.1%), CRR with UPCR ≤0.5 mg/mg (40.9% vs 26.7%), CRR with inactive urinary sediment (40.9% vs 13.3%) and sustained glucocorticoid reductions (55.6% vs 33.3%). Incidence of herpes zoster was higher with combined anifrolumab vs placebo (16.7% vs 8.2%). Incidence of serious adverse events was similar across groups.

Conclusion Although the primary endpoint was not met, anifrolumab IR was associated with numerical improvements over placebo across endpoints, including CRR, in patients with active lupus nephritis.

Trial registration number NCT02547922.

Key messages

What is already known about this subject?
- Anifrolumab is generally well tolerated and efficacious across a range of clinically meaningful endpoints in patients with systemic lupus erythematosus (SLE).
- Anifrolumab targets the type I interferon signalling pathway, which plays a role in the pathogenesis of lupus nephritis (LN).

What does this study add?
- This phase II, randomised, placebo-controlled trial is the first investigation of an interferon-targeted therapy in patients with active LN.
- This study suggests that patients with LN require an intensified regimen (IR) of anifrolumab relative to non-renal SLE to obtain adequate exposure and clinical efficacy.

How might this impact on clinical practice or future developments?
- The findings of TULIP-LN merit further investigation of anifrolumab IR in larger numbers of patients with active LN.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune condition that can cause multiorgan inflammation and organ damage.[1] Lupus nephritis (LN) is one of the most prevalent severe disease manifestations of SLE, occurring in ~40% of patients.[2] Patients with Class III or IV LN[3] have poor prognoses, with up to 45% of patients progressing to end-stage kidney disease within 15 years of diagnosis.[4–6]

High type I interferon gene signatures (IFNGS) are present in >80% of patients with LN,[7] become even more pronounced in active LN,[8] and are associated with active kidney disease and treatment failure.[8 9] Therefore, there is scientific rationale to support anifrolumab, a human monoclonal antibody that binds to the type I interferon receptor subunit 1,[10] as a potential LN treatment option.

Anifrolumab has been investigated in patients with moderate to severe SLE despite standard therapy in two phase III randomised placebo-controlled trials, TULIP-1 and TULIP-2.[11 12] Anifrolumab 300 mg was generally well tolerated and provided therapeutic benefit across several clinical endpoints despite TULIP-1 not meeting its primary endpoint.[11 12] As the TULIP trials excluded patients with severe, active LN, further studies were required to evaluate anifrolumab in this patient population.[11 12] Here, we report 52-week primary analysis results of the 2-year, phase II, randomised, placebo-controlled Treatment of Uncontrolled Lupus via the Interferon Pathway - Lupus Nephritis (TULIP-LN) trial, which evaluated the safety and efficacy of two anifrolumab dosages added to standard therapy in patients with active LN.

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METHODS

Study design

This phase II trial was conducted at 66 sites in 16 countries (online supplemental table S1) in accordance with the Declaration of Helsinki and the International Conference on Harmonisation Good Clinical Practice Guideline. All patients provided written informed consent. The trial consisted of a 52-week randomised, placebo-controlled, double-blind treatment period, after which the primary endpoint was assessed. Patients then either entered an 8-week safety follow-up period or, if eligible, the ongoing second-year treatment period (online supplemental figure S1). Only the first-year data are reported here.

Patients

Eligible patients were 18–70 years old with a biopsy-proven diagnosis within 3 months of screening of Class III or IV (+/− coexistent Class V) LN, according to the WHO or International Society of Nephrology and the Renal Pathology Society (ISN/RPS) 2003 criteria.[3] Eligible patients had 24-hour urine−protein creatinine ratios (UPCR) >1 mg/mg (113.17 mg/mmol), estimated glomerular filtration rates (eGFR) ≥35 mL/min/1.73 m^2, and fulfilled ≥4 of the 11 American College of Rheumatology SLE 1997 classification criteria, including seropositivity for ≥1 of antinuclear, anti-double-stranded DNA (anti-dsDNA), and/or anti-Smith antibodies at screening.[13] For full inclusion/exclusion criteria, see online supplemental material.

Treatments

Patients were block randomised (1:1:1) to receive anifrolumab basic regimen (BR; 300 mg, corresponding to SLE dosing[10–12]), anifrolumab intensified regimen (IR; 900 mg for the first three doses, 300 mg thereafter), or placebo intravenously every 4 weeks for 48 weeks. Randomisation was stratified according to 24-hour UPCR at screening (≤3.0 vs >3.0 mg/mg) and type I IFNGS status (high vs low, determined as previously described[14]).

Investigational agents were administered alongside standard therapy of oral glucocorticoids and mycophenolate mofetil (MMF). All patients received an intravenous methylprednisolone pulse (500 mg) within 10 days of randomisation. There was a mandatory oral glucocorticoid taper to a dosage goal of ≤10 mg/day by week 12 and ≤7.5 mg/day by week 24 (prednisone or equivalent). MMF was titrated to a target dosage of 2 g/day by week 8. MMF dosage adjustments were permitted for suboptimal responses, toxicity or intolerability. Stable oral glucocorticoid and MMF dosages were required during weeks 40–52. Standard therapy requirements are detailed further in online supplemental material.

Prespecified discontinuation criteria

During the 52-week treatment period, patients were required to discontinue investigational product treatment if they had predefined worsening of LN, which was defined as an LN-related, confirmed eGFR decrease >30% from baseline to <60 mL/min/1.73 m^2 at any time, eGFR decrease <75% from baseline to <60 mL/min/1.73 m^2 at week 12 or week 24, or nephrotic range UPCR at week 12 or week 24 (>3.5 mg/mg or <60% improvement in patients >3 mg/mg at baseline).

Investigational product was discontinued in the case of failure to adhere to protocol-specified standard therapy requirements, including a mandatory oral glucocorticoid taper to a dosage of ≤15 mg/day by week 12 or <15 mg/day by week 24. Patients were also required to discontinue investigational product treatment if they received rescue treatments (eg, cyclophosphamide, high-dose glucocorticoids and/or rituximab) owing to worsening LN or SLE at any time, or if they received protocol-specified forbidden medications at any time. Standard therapy requirements and forbidden medication rules are detailed further in online supplemental material.

Outcomes

Primary endpoint

The primary endpoint was the relative difference in the mean change from baseline to week 52 in 24-hour UPCR in the combined anifrolumab (IR plus BR) versus placebo group, measured with a geometric mean (GM) ratio (GMR; <1 favours anifrolumab) using the equation:

$$\mathrm{GMR} = \frac{\mathrm{GM}\left(\frac{\text{24-hour UPCR at week 52}}{\text{24-hour UPCR at baseline}}\right)_{\text{combined anifrolumab}}}{\mathrm{GM}\left(\frac{\text{24-hour UPCR at week 52}}{\text{24-hour UPCR at baseline}}\right)_{\text{placebo}}}$$

Secondary endpoint

The secondary endpoint was the difference in the combined anifrolumab vs placebo groups in the proportion of patients with a complete renal response (CRR) at week 52, defined as 24-hour UPCR ≤0.7 mg/mg, eGFR ≥60 mL/min/1.73 m^2 or no decrease ≥20% from baseline, no investigational product discontinuation and no use of restricted medications. Restricted medications are listed in online supplemental material.

Exploratory endpoints

Exploratory endpoints included mean UPCR over time; the proportion of patients with sustained oral glucocorticoid tapers (≤7.5 mg/day prednisone equivalent from weeks 24–52, among those receiving ≥20 mg/day at baseline); the proportion of patients with an alternative CRR (aCRR), defined as a CRR that required inactive urine sediment (<10 red blood cells per high-power field); the proportion of patients with a CRR and sustained oral glucocorticoid taper; mean change from baseline in non-renal SLE Disease Activity Index 2000 (SLEDAI-2K),[15] Physician's Global Assessment (PGA),[16] Patient's Global Assessment (PtGA),[17] lupus serologies (anti-dsDNA antibodies, C3/C4); and the immunogenicity, pharmacokinetic (PK) and pharmacodynamic (PD) profile of anifrolumab. PD neutralisation was measured as the median percentage change of baseline 21-gene type I IFNGS (21-IFNGS), as described previously.[10 14 18]

Post hoc analyses included cumulative proteinuria (area under the curve in UPCR standardised by expected follow-up time), the proportion of patients with a CRR with UPCR ≤0.5 mg/mg ($CRR_{0.5}$), and probability of $CRR_{0.5}$ response sustained through week 52.

Safety assessments included adverse events (AEs), laboratory assessments and vital signs. AEs of special interest (AESI) were non-opportunistic serious infections, opportunistic infections, herpes zoster (HZ), influenza, malignancy, tuberculosis, hypersensitivity and major adverse cardiovascular events.

Sample size estimation

A 1:1:1 randomised sample size of 50 patients per treatment arm was planned to provide ~87% power at the two-sided alpha level of 0.0499 to detect a relative difference of 0.76 or less in 24-hour UPCR GMR from baseline to week 52 for combined anifrolumab versus placebo.

Statistical analysis

The primary endpoint was analysed using a mixed model for repeated measures fitted to log-transformed 24-hour UPCR values, controlling for stratification factors and based on observed data up to investigational product discontinuation. Binary endpoints, responder rates and 95% CIs were calculated using a stratified Cochran-Mantel-Haenszel approach, controlling for stratification factors. Safety was analysed descriptively.

Efficacy and safety analyses were conducted using the modified intention-to-treat (mITT) population. Patients enrolled at sites in Italy and France were excluded from the analyses of secondary and exploratory binary CRR efficacy endpoints. This exclusion was because the Italian Medicines Agency and the France Ethics Committee did not agree to a protocol amendment that included changes to the cut-off values for the renal function and proteinuria components of the CRR definition.

All analyses were performed with Statistical Analysis System (SAS; SAS Institute Inc., Cary, NC), V.9.3 or higher. Individual anifrolumab regimens versus placebo analyses were conducted using a hierarchical testing strategy to control the familywise error. Further details on statistical analyses are provided in online supplemental material.

Patient and public involvement

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

RESULTS

Trial population

Between November 2015 and November 2018, 338 patients were screened, and 147 patients were randomised (figure 1). Of the 145 patients in the mITT population, 45 received anifrolumab BR, 51 received anifrolumab IR and 49 received placebo.

Demographics and baseline disease characteristics are shown in table 1. At screening, 26.9% of patients had Class III LN and 73.1% of patients had Class IV LN (41.0% and 21.7% of whom had coexistent Class V disease, respectively). Most patients (94.5%) were IFNGS high. At baseline, 77.2% of patients had eGFR ≥60 mL/min/1.73 m^2. Demographics and baseline disease characteristics were generally balanced between groups; however, the placebo group had higher mean baseline 24-hour UPCR, lower mean baseline eGFR, longer median time from initial LN diagnosis and more patients with low C3 or C4 than both anifrolumab groups. Most patients were receiving standard therapy for LN at baseline (mean dosage 22.3 mg/day prednisone equivalent oral glucocorticoids and 1.8 g/day MMF); treatments were balanced between groups.

Overall, 126/145 patients (86.9%) completed the 52-week period (BR: 77.8%, IR: 98.0%, placebo: 83.7%), and 101/145 patients (69.7%) completed investigational product treatment at week 52 (figure 1). More patients discontinued

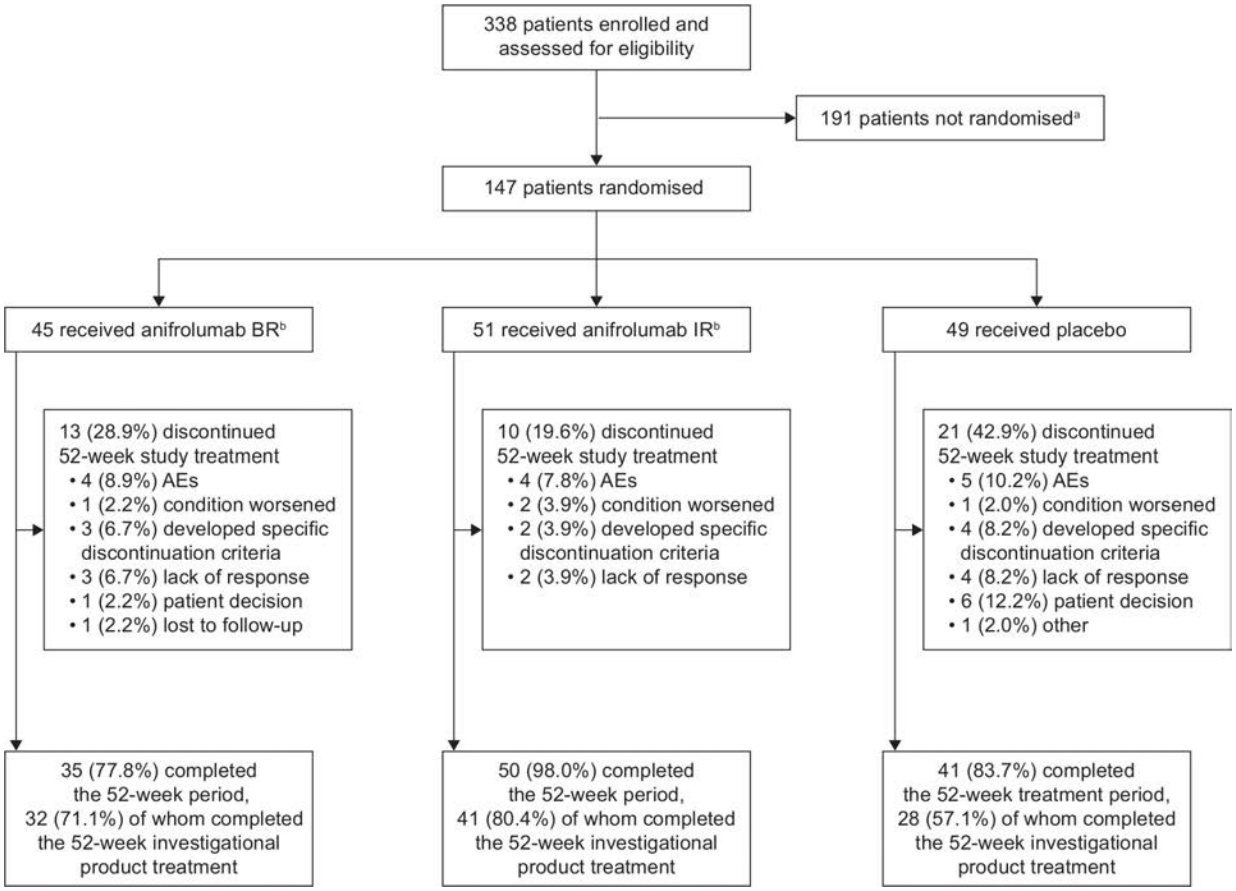

Figure 1 Patient disposition for the completed 52-week double-blind treatment period. All percentages are based on the 145 patients in the full analysis set (modified intention-to-treat population), who were included in the primary endpoint analysis. [a]Of patients not randomised, 179 did not meet the screening criteria, 7 withdrew consent, 2 experienced AEs, 1 was lost to follow-up, 1 patient was not included because of the physician's decision, and 1 patient was not included for unspecified reason ('other'). [b]One patient was assigned to but did not receive ≥1 dose of each of the anifrolumab regimens and therefore was not included in the analysis. AE, adverse event; BR, basic regimen; IR, intensified regimen.

Table 1 Patient demographics and disease characteristics

		Anifrolumab combined (n=96)	Anifrolumab BR (n=45)	Anifrolumab IR (n=51)	Placebo (n=49)
Patient demographics					
Age, years	Median (range)	34.5 (18, 67)	34.0 (19, 67)	35.0 (18, 65)	32.0 (18, 58)
Sex	Female, n (%)	82 (85.4)	37 (82.2)	45 (88.2)	38 (77.6)
Weight	Mean (SD), kg	65.4 (15.0)	62.7 (12.3)	67.7 (16.8)	65.6 (13.3)
BMI	Mean (SD)	25.1 (5.06)	24.0 (3.77)	26.0 (5.85)	24.5 (3.93)
	>28 kg/m^2, n (%)	23 (24.0)	7 (15.6)	16 (31.4)	9 (18.4)
Race, n (%)	White	42 (43.8)	17 (37.8)	25 (49.0)	24 (49.0)
	Black/African American	6 (6.3)	2 (4.4)	4 (7.8)	1 (2.0)
	Asian	18 (18.8)	11 (24.4)	7 (13.7)	10 (20.4)
	Native Hawaiian/ Pacific Islander	1 (1.0)	1 (2.2)	0	0
	American Indian/ Alaska Native	4 (4.2)	3 (6.7)	1 (2.0)	0
	Other	25 (26.0)	11 (24.4)	14 (27.5)	14 (28.6)
Hispanic or Latino ethnicity, n (%)		45 (46.9)	22 (48.9)	23 (45.1)	20 (40.8)
Geographic region, n (%)	Asia Pacific	18 (18.8)	10 (22.2)	8 (15.7)	9 (18.4)
	Europe	26 (27.1)	10 (22.2)	16 (31.4)	15 (30.6)
	Latin America	34 (35.4)	14 (31.1)	20 (39.2)	16 (32.7)
	North America	18 (18.8)	11 (24.4)	7 (13.7)	9 (18.4)
Baseline disease characteristics					
Time from initial LN diagnosis to randomisation, mean (range), months		6.8 (0.4, 306.9)	3.4 (1.1, 212.7)	15.7 (0.4, 306.9)	37.0 (0.7, 328.3)
Renal biopsy result at screening, n (%)	Class III	17 (17.7)	7 (15.6)	10 (19.6)	6 (12.2)
	Class III+V	11 (11.5)	7 (15.6)	4 (7.8)	5 (10.2)
	Class IV	53 (55.2)	26 (57.8)	27 (52.9)	30 (61.2)
	Class IV+V	15 (15.6)	5 (11.1)	10 (19.6)	8 (16.3)
24-hour UPCR, mg/mg	Mean (SD)	3.10 (2.18)	3.36 (2.50)	2.86 (1.85)	3.71 (3.20)
	>3.0, n (%)	36 (37.5)	19 (42.2)	17 (33.3)	23 (46.9)
eGFR* mL/min/1.73 m^2	Mean (SD)	97.1 (44.77)	100.2 (46.77)	94.4 (43.22)	87.3 (35.43)
	≥60, n (%)	73 (76.0)	35 (77.8)	38 (74.5)	39 (79.6)
SLEDAI-2K† score	Mean (SD)	10.7 (4.83)	10.4 (4.63)	11.0 (5.04)	11.3 (4.38)
	≥10, n (%)	51 (53.1)	23 (51.1)	28 (54.9)	29 (59.2)
Non-renal SLEDAI-2K† score	Mean (SD)	4.7 (3.12)	5.2 (3.44)	4.2 (2.74)	4.7 (2.30)
IFNGS status	High, n (%)	91 (94.8)	44 (97.8)	47 (92.2)	46 (93.9)
Serology, n (%)	ANA positive‡	90 (93.8)	44 (97.8)	46 (90.2)	49 (100)
	Anti-dsDNA positive§	76 (79.2)	37 (82.2)	39 (76.5)	39 (79.6)
	Low C3¶	57 (59.4)	30 (66.7)	27 (52.9)	42 (85.7)
	Low C4¶	24 (25.0)	10 (22.2)	14 (27.5)	20 (40.8)
Baseline treatments					
Oral glucocorticoids**	Yes, n (%)	94 (97.9)	43 (95.6)	51 (100)	48 (98.0)
	Dosage, mean (SD), mg/day	22.6 (10.63)	21.9 (10.4)	23.2 (10.88)	21.9 (11.20)
	≥20 mg/day, n (%)	67 (69.8)	31 (68.9)	36 (70.6)	33 (67.3)
MMF before randomisation	Yes, n (%)	72 (75.0)	36 (80.0)	36 (70.6)	33 (67.3)
	Dosage, mean (SD), g/day	1.81 (0.502)	1.82 (0.551)	1.79 (0.460)	1.77 (0.469)
Concomitant ACEI/ARB treatment, n (%)		63 (65.6)	27 (60.0)	36 (70.6)	33 (67.3)
Antimalarials, n (%)		57 (59.4)	31 (68.9)	26 (51.0)	35 (71.4)

Baseline is defined as the last measurement prior to randomisation and dose administration on day 1.
*eGFR is calculated using the MDRD formula.
†The SLEDAI-2K is a 24-item weighted score of lupus activity that ranges from 0 to 105, with higher scores indicating greater disease activity.
‡ANA positive was defined as a titre ≥1:40.
§Anti-dsDNA positive was defined as an anti-dsDNA level above the assay cut-off for positive.
¶Low complement level at baseline was defined as a complement level below lower limit of normal.
**Baseline oral glucocorticoid dosage is defined as the maximum daily dose of prednisone or equivalent taken between day 1 and day 7, inclusive.
ACEI, ACE inhibitors; ANA, antinuclear antibodies; anti-dsDNA, anti-double-stranded DNA; ARB, angiotensin receptor blockers; BMI, body mass index; BR, basic regimen; C3, complement 3; C4, complement 4; eGFR, estimated glomerular filtration rate; IFNGS, interferon gene signature; IR, intensified regimen; LN, lupus nephritis; MDRD, modification of diet in renal disease; MMF, mycophenolate mofetil; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; UPCR, urine protein–creatinine ratio.

investigational product early in the placebo (42.9%) than in both anifrolumab groups (BR: 28.9%, IR: 19.6%; online supplemental figure S2). There were 75 patients who entered the second-year extension period; only the first-year results are reported here.

Efficacy

Primary endpoint

The primary endpoint was not met; at week 52, the mean 24-hour UPCR improved from baseline by 69% and 70% to 0.92 mg/mg and 1.05 mg/mg in the combined anifrolumab and placebo groups, respectively, resulting in a GMR of 1.03 (95% CI 0.62 to 1.71, p=0.905; GMR <1 favours anifrolumab; figure 2A; online supplemental table S2). The outcome of the preplanned efficacy analysis for the combined anifrolumab versus placebo group was made less clinically meaningful by the suboptimal PK exposure and PD neutralisation in the anifrolumab BR group, owing to higher drug clearance associated with proteinuria in patients with active LN versus patients with non-renal SLE[19 20] (see later sections). As the primary endpoint was not met, the secondary endpoint was not formally tested per the statistical analysis plan. For all remaining endpoints, reported p values are nominal and should not be used to conclude statistical significance.

Mean 24-hour UPCR improved over time across treatment groups (online supplemental figure S3). The GM improvements in 24-hour UPCR were numerically larger in both anifrolumab groups vs placebo at weeks 12 and 24, but not at weeks 36 or 52 (figure 2A). In the anifrolumab IR group, the 24-hour UPCR improved by 71% from baseline to 0.88 mg/mg at week 52, which was similar to the improvement with placebo (GMR=0.96; 95% CI 0.55 to 1.69) (figure 2A). Both anifrolumab groups had numerically lower cumulative UPCRs than placebo throughout the treatment duration (online supplemental figure S4). There were no major differences in 24-hour UPCR changes from baseline to week 52 across predefined subgroups (online supplemental figure S5). Post hoc sensitivity analysis controlling for time from LN diagnosis did not reveal any major impact on primary results (data not shown).

Secondary endpoint

At week 52, the percentages of patients with a CRR were similar in the combined anifrolumab and placebo groups (31.0% vs 31.1%, difference −0.1% (95% CI −16.9 to 16.8)) (table 2). The proportion of patients with a CRR was greater in the anifrolumab IR group than in the placebo group (45.5% vs 31.1%, difference 14.3% (95% CI −5.8 to 34.5)) and was lower in the anifrolumab BR group than in the placebo group (16.3% vs 31.1%, difference −14.8% (95% CI −32.9 to 3.2)).

The proportions of patients in each treatment group who attained the individual components of the CRR at week 52 are displayed in online supplemental table S3. A greater proportion of patients in the anifrolumab IR group had 24-hour UPCR ≤0.7 mg/mg at week 52 compared with the anifrolumab BR or placebo groups (anifrolumab IR: 50.0%; anifrolumab BR: 32.6%; placebo: 35.6%). At week 52, 81.8% of the anifrolumab IR group, 79.1% of the anifrolumab BR group, and 73.3% of the placebo group had eGFR ≥60 mL/min/1.73 m^2 or no decrease ≥20% from baseline, with mean (SD) eGFR values of 94.5 (36.2), 95.8 (24.9) and 84.7 (30.1) mL/min/1.73 m^2, respectively.

Exploratory endpoints

The proportion of patients who had an aCRR at week 52 (which required inactive urinary sediment) was greater in the anifrolumab IR group than the placebo group (40.9% vs 13.3%, difference 27.6% (95% CI 9.4 to 45.7)), and was lower in the anifrolumab BR group than in the placebo group (7.0% vs 13.3%, difference −6.4% (95% CI −20.6 to 7.8)). The proportion of patients with inactive urinary sediment (<10 red blood cells per high-power field) was also greater in the anifrolumab IR group (77.3%) than in the anifrolumab BR group (55.8%) or placebo group (42.2%) (online supplemental table S3).

A similar trend was observed with $CRR_{0.5}$; the proportion of patients who had a $CRR_{0.5}$ at week 52 was greater in the anifrolumab IR group than the placebo group (40.9% vs 26.7%, difference 14.2% (95% CI −5.4 to 33.9)) and was lower in the anifrolumab BR group than in the placebo group (16.3% vs 26.7%, difference −10.4 (95% CI −28.1 to 7.3) (table 2).

Response rates were higher with anifrolumab IR vs placebo as early as week 12 and remained higher over time across all CRR definitions (figure 2B; online supplemental figure S6). Compared with placebo, patients in the anifrolumab IR group were more likely to have a $CRR_{0.5}$ response sustained through week 52 (sustained $CRR_{0.5}$ HR 1.46; 95% CI 0.71 to 3.14) (figure 2C). Anifrolumab BR responses for all CRR definitions were generally similar to or lower than placebo at all timepoints apart from week 12 (figure 2B, online supplemental figure S6).

The proportion of patients who had a sustained oral glucocorticoid dosage taper ≤7.5 mg/day was greater in the anifrolumab IR group than in the placebo group (55.6% vs 33.3%, difference 22.2% (95% CI −0.8 to 45.2)) and was similar in the anifrolumab BR and placebo groups (35.5% vs 33.3%, difference 2.2% (95% CI −21.4 to 25.7)). The proportion of patients who had a CRR with sustained glucocorticoid taper was also greater in the anifrolumab IR group than in the placebo group (34.1% vs 24.4%, difference 9.7% (95% CI −9.5 to 28.8)) but was lower in the anifrolumab BR group than in the placebo group (14.0% vs 24.4%, difference −10.5 (95% CI −27.6 to 6.6)) (table 2).

Compared with placebo, anifrolumab IR was associated with greater improvements from baseline in measures of disease activity (SLEDAI-2K, PGA and PtGA), whereas the anifrolumab BR was associated with greater improvements in SLEDAI-2K but not for PGA or PtGA (figure 3A–C). Improvements from baseline in lupus serologies (anti-dsDNA antibodies, C3 and C4) were variable; however, there was a trend towards greater improvements in anti-dsDNA antibodies and C3 levels with anifrolumab IR and anifrolumab BR than with placebo by week 52 (online supplemental figure S7).

Pharmacokinetics

The PK analysis included 95 patients who received anifrolumab and had ≥1 quantifiable serum PK observation after the first dose. Anifrolumab exhibited non-linear PK between the anifrolumab BR and IR groups (online supplemental figure S8). In IFNGS-high patients (94.5%), the median week 12 anifrolumab steady-state concentration was 63.4 µg/mL with anifrolumab IR and 8.2 µg/mL with anifrolumab BR (~50% lower than in non-renal SLE[21]) (online supplemental figure S9). After anifrolumab IR was tapered to 300 mg at week 12, the median trough concentrations at week 24 and week 36 were lower than in patients with non-renal SLE. Anifrolumab clearance was higher among patients with UPCR >3 mg/mg vs ≤3 mg/mg at baseline (online supplemental figure S10). Anifrolumab clearance decreased over time. Larger decreases in baseline clearance (≥33% decrease at week 52) were associated with greater reductions in baseline 24-hour UPCR after week 12 compared with patients who had smaller decreases in baseline clearance (<20% decrease at

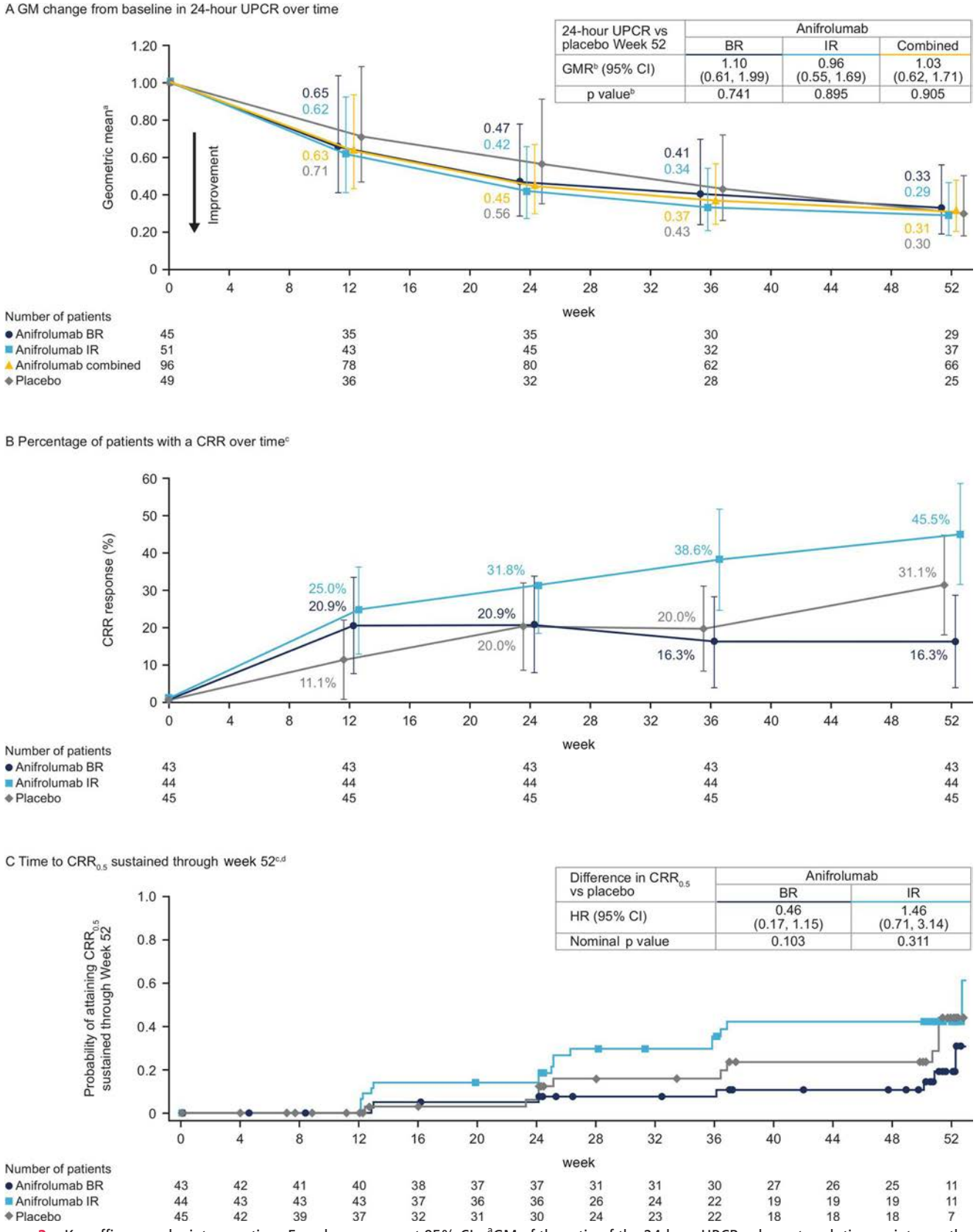

Figure 2 Key efficacy endpoints over time. Error bars represent 95% CIs. [a]GM of the ratio of the 24-hour UPCR values at each time point over the baseline value for each treatment group (values <1 indicate an improvement). [b]GMR of the relative improvement in 24-hour UPCR for anifrolumab groups vs placebo groups, where GMR <1 favours anifrolumab. A p≤0.05 for the combined anifrolumab vs placebo group was deemed significant. All other p values presented are nominal. [c]Patients from France and Italy (n=13) were excluded from the analysis (see online supplemental material). [d]Probability of obtaining a sustained $CRR_{0.5}$ was analysed post hoc using a Cox regression model controlling for stratification factors. BR, basic regimen; CRR, complete renal response; $CRR_{0.5}$, CRR with UPCR ≤0.5 mg/mg; GM, geometric mean; GMR, geometric mean ratio; IR, intensified regimen; UPCR, urine protein–creatinine ratio.

Table 2 Summary of secondary and exploratory endpoints

Endpoints		Responders, n/N (%)*	Difference (95% CI)*	Nominal p value†
CRR at week 52‡	Combined	27/87 (31.0)	−0.1 (−16.9, 16.8)	0.993
	Basic	7/43 (16.3)	−14.8 (−32.9, 3.2)	0.107
	Intensified	20/44 (45.5)	14.3 (−5.8, 34.5)	0.162
	Placebo	14/45 (31.1)	–	–
aCRR at week 52‡	Combined	21/87 (24.1)	10.8 (−3.3, 25.0)	0.134
	Basic	3/43 (7.0)	−6.4 (−20.6, 7.8)	0.380
	Intensified	18/44 (40.9)	27.6 (9.4, 45.7)	0.003
	Placebo	6/45 (13.3)	–	–
$CRR_{0.5}$ at week 52‡§	Combined	25/87 (28.7)	2.1 (−14.3, 18.4)	–
	Basic	7/43 (16.3)	−10.4 (−28.1, 7.3)	–
	Intensified	18/44 (40.9)	14.2 (−5.4, 33.9)	–
	Placebo	12/45 (26.7)	–	–
Sustained oral glucocorticoid dosage reduction (≤7.5 mg/day, week 24 to week 52¶)	Combined	31/67 (46.3)	12.9 (−7.3, 33.1)	0.209
	Basic	11/31 (35.5)	2.2 (−21.4, 25.7)	0.858
	Intensified	20/36 (55.6)	22.2 (−0.8, 45.2)	0.058
	Placebo	11/33 (33.3)	–	–
CRR with sustained oral glucocorticoid dosage reduction to ≤7.5 mg/day‡	Combined	21/87 (24.1)	−0.3 (−16.1, 15.5)	0.970
	Basic	6/43 (14.0)	−10.5 (−27.6, 6.6)	0.229
	Intensified	15/44 (34.1)	9.7 (−9.5, 28.8)	0.323
	Placebo	11/45 (24.4)	–	–

A CRR required 24-hour UPCR ≤0.7 mg/mg, eGFR ≥60 mL/min/1.73 m^2 or no decrease ≥20% from baseline, no investigational product discontinuation and no use of restricted medications. An aCRR required all of the above CRR criteria, but with inactive urinary sediment, defined as <10 red blood cells per high-power field. A $CRR_{0.5}$ required all of the above CRR criteria, but with 24-hour UPCR ≤0.5 mg/mg.

*The response rates, differences between the groups and associated 95% CIs were calculated with a weighted Cochran-Mantel-Haenszel method. Differences between anifrolumab and placebo groups were calculated in percentage points (the percentage in the anifrolumab group minus the percentage in the placebo group).

†Nominal p values are unadjusted as the primary outcome was not significant so all other comparisons are considered non-significant.

‡Patients from France and Italy were excluded from the analysis.

§Analysed post hoc.

¶Analysed in patients with baseline oral glucocorticoid dosage ≥20 mg/day.

aCRR, alternative CRR; CRR, complete renal response; $CRR_{0.5}$, CRR with UPCR ≤0.5 mg/mg; n, number of patients meeting the criteria for a response; N, number of patients included in the analysis; UPCR, urine protein–creatinine ratio.

week 52) (online supplemental figure S11). This association was observed to a greater extent in patients with baseline 24-hour UPCR >3 mg/mg (who had higher clearance) compared with patients with baseline 24-hour UPCR ≤3 mg/mg (online supplemental figure S11).

Pharmacodynamics

The PD analysis included 137 IFNGS-high patients. A median PD neutralisation >80% was observed with anifrolumab IR across all visits (weeks 12, 24, 36 and 52). Sustained PD neutralisation to this degree was not observed with anifrolumab BR (figure 3D). Minimal PD neutralisation was observed in the placebo group.

Safety and tolerability

Table 3 shows the safety summary. The percentages of patients with any AE were 95.6%, 92.2% and 89.8% in the anifrolumab BR, anifrolumab IR and placebo groups, respectively. The AEs that were more common (≥5% difference) in the combined anifrolumab versus placebo groups were HZ, urinary tract infection and influenza. Serious AEs occurred in 22.2%, 17.6% and 16.3% of the anifrolumab BR, anifrolumab IR and placebo groups, respectively. HZ was the only serious AE reported in >1 patient per treatment group. There were no deaths during the treatment period. There was one fatal vascular neurological AE in the anifrolumab BR group during the follow-up. AEs leading to investigational product discontinuation occurred in 11.1%–12.2% of patients across groups.

Overall, AESIs occurred in 24.0% and 16.3% of patients in the combined anifrolumab and placebo groups, respectively. Of the AESIs, HZ and influenza occurred more commonly in the combined anifrolumab versus placebo group. HZ occurred in 20.0%, 13.7% and 8.2% of patients in the anifrolumab BR, anifrolumab IR and placebo groups, respectively. Of the 16 HZ cases in the combined anifrolumab group, the majority were of mild to moderate intensity, 6 were serious, and all were cutaneous (13 localised, 3 disseminated). HZ events tended to occur early in the trial (online supplemental figure S12) and were resolved with conventional treatment. The incidence of other AESIs was low across groups.

DISCUSSION

There is high unmet need in the treatment of LN. Despite recent advances, remission rates remain suboptimal,[22–24] and patients are at high risk of developing end-stage kidney disease[4–6] and drug-related toxicity, particularly relating to prolonged, high-dose glucocorticoid use.[19 25]

Here, we report the primary analysis results of the phase II TULIP-LN trial, which explored the safety and efficacy of two anifrolumab dosing regimens alongside standard therapy in patients with active LN. The primary endpoint was not met; however, UPCR improvement in the combined anifrolumab group versus placebo group was adversely impacted by the suboptimal anifrolumab exposure obtained with BR dosing (~50% lower than in non-renal SLE[21]). The suboptimal PK exposure with anifrolumab BR was likely related to increased

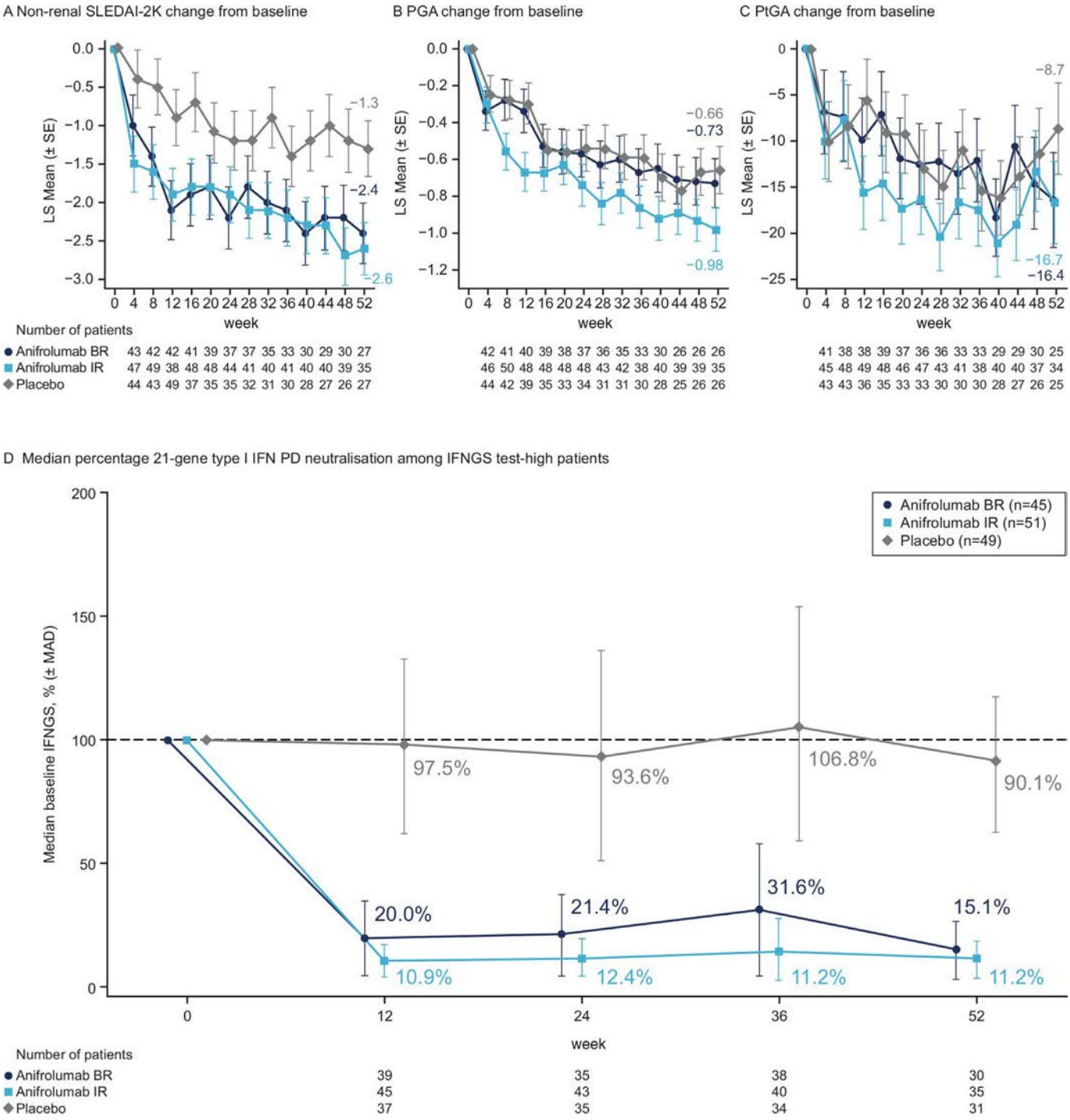

Figure 3 Measures of disease activity and IFNGS neutralisation over time. Number of patients with non-missing value at visit are presented. SLEDAI-2K, PGA and PtGA change from baseline were analysed using a mixed model for repeated measures, controlling for stratification factors, and based on observed data up to investigational product discontinuation. PD neutralisation was analysed descriptively. BR, basic regimen; IFN, interferon; IFNGS, interferon gene signature; IR, intensified regimen; LS, least squares; MAD, median absolute deviation; PD, pharmacodynamic; PGA, Physician's Global Assessment, PtGA, Patient's Global Assessment; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

clearance associated with proteinuria in LN[19 20]; indeed, we observed an association between the magnitude of decrease in anifrolumab clearance and the improvement in 24-hour UPCR over time. The suboptimal PK exposure obtained with the anifrolumab BR regimen was also reflected in the lower degree of 21-IFNGS neutralisation and relatively infrequent clinical responses observed with anifrolumab BR. The anifrolumab IR was required to attain serum exposure and PD neutralisation that was similar to levels observed in non-renal SLE.[26] As such, the anifrolumab IR was required to reach clinical efficacy, with clinically meaningful responses across renal endpoints, including proteinuria, multiple stringent CRR definitions (including requirements for UPCR ≤0.5 mg/mg or inactive urinary sediment), sustained oral glucocorticoid dosage reductions, disease activity measures and lupus serologies.

Table 3 AEs during the treatment period (mITT population)

Patients, n (%)	Anifrolumab combined (n=96)	Anifrolumab BR (n=45)	Anifrolumab IR (n=51)	Placebo (n=49)
Any AE	90 (93.8)	43 (95.6)	47 (92.2)	44 (89.8)
Any AE with outcome of death	0	0	0	0
Any SAE	19 (19.8)	10 (22.2)	9 (17.6)	8 (16.3)
Any AE leading to discontinuation of investigational product	11 (11.5)	5 (11.1)	6 (11.8)	6 (12.2)
Adverse events of special interest	23 (24.0)	12 (26.7)	11 (21.6)	8 (16.3)
Non-opportunistic serious infections*	1 (1.0)	0	1 (2.0)	3 (6.1)
Opportunistic infections†	1 (1.0)	1 (2.2)	0	1 (2.0)
Anaphylaxis	0	0	0	0
Infusion-related reactions	1 (1.0)	1 (2.2)	0	2 (4.1)
Malignancy	1 (1.0)	0	1 (2.0)	0
Herpes zoster‡	16 (16.7)	9 (20.0)	7 (13.7)	4 (8.2)
Tuberculosis/LTB	0	0	0	0
Influenza§	8 (8.3)	2 (4.4)	3 (5.9)	1 (2.0)
Vasculitis (non-SLE)	0	0	0	0
Major adverse cardiovascular events according to the CV-EAC	0	0	0	1 (2.0)
Any AEs ≥5% in the combined anifrolumab group				
Urinary tract infection	16 (16.7)	10 (22.2)	6 (11.8)	5 (10.2)
Herpes zoster	16 (16.7)	9 (20.0)	7 (13.7)	4 (8.2)
Nasopharyngitis	15 (15.6)	6 (13.3)	9 (17.6)	9 (18.4)
Upper respiratory tract infection	15 (15.6)	8 (17.8)	7 (13.7)	8 (16.3)
Bronchitis	11 (11.5)	4 (8.9)	7 (13.7)	6 (12.2)
Influenza§	8 (8.3)	2 (4.4)	6 (11.8)	1 (2.0)
Diarrhoea	7 (7.3)	3 (6.7)	4 (7.8)	10 (20.4)
Cough	7 (7.3)	4 (8.9)	3 (5.9)	4 (8.2)
Pharyngitis	7 (7.3)	3 (6.7)	4 (7.8)	2 (4.1)
Oral herpes	6 (6.3)	3 (6.7)	3 (5.9)	2 (4.1)
Headache	5 (5.2)	2 (4.4)	3 (5.9)	4 (8.2)
Herpes simplex	5 (5.2)	3 (6.7)	2 (3.9)	2 (4.1)
Nausea	5 (5.2)	1 (2.2)	4 (7.8)	2 (4.1)

AEs are coded using MedDRA V.22.1. Percentages are based on the 145 patients in the mITT who received ≥1 dose of anifrolumab or placebo. Any AE occurring from the day of the first dose to 28 days after the last dose was included.
*Excludes tuberculosis/latent tuberculosis and influenza.
†Excludes herpes zoster and visceral disseminated herpes zoster.
‡Includes visceral disseminated herpes zoster.
§In the anifrolumab IR group, the AESI incidence of influenza cases was derived from the AE category, as there were three recorded cases of influenza in the AESI category and six in the any AE category, owing to data collection differences.
AE, adverse event; AESI, AE of special interest; BR, basic regimen; CV-EAC, Cardiovascular Event Adjudication Committee; IR, intensified regimen; LTB, latent tuberculosis; MedDRA, Medical Dictionary for Regulatory Activities; mITT, modified intention-to-treat; SAE, serious adverse event; SLE, systemic lupus erythematosus.

Reduction of proteinuria is associated with reduced risk of end-stage kidney disease[27–29]; thus, it is an appropriate and objective surrogate endpoint for a proof-of-concept trial. Here, numerically greater improvements in 24-hour UPCR were observed early in the trial with both anifrolumab groups vs placebo; however, by week 52, all treatment groups had improvements in baseline 24-hour UPCR of approximately 70%. In the placebo group, the 24-hour UPCR improvement may have been overestimated, owing to large amounts of missing data generated from the high rate of investigational product discontinuation. These missing data were imputed into the primary analysis model; however, high levels of data imputation could confound the model-estimated treatment effect. In the cumulative UPCR analysis, treatment with both anifrolumab regimens numerically improved cumulative proteinuria over time more than placebo. By week 52, cumulative UPCR was ~30% and ~20% lower than placebo in the anifrolumab IR and BR groups, respectively. Cumulative UPCR signifies overall proteinuria improvement over time, so it may be less susceptible to short-term confounders, including collection errors, diet and exercise.[30 31]

Anifrolumab IR was also associated with clinically meaningful responses over placebo across CRR definitions as early as week 12, including the robust composite endpoint $CRR_{0.5}$, which is favoured for registrational clinical trials.[32 33] Anifrolumab IR yielded the strongest response (treatment difference 28%) for aCRR, a highly stringent endpoint requiring no haematuria (a pathognomonic marker of active glomerular inflammation[34]). More patients also achieved a sustained oral glucocorticoid dosage reduction and a CRR with a sustained dosage reduction with anifrolumab IR vs placebo, which merits further exploration, as reducing glucocorticoid dosages is a key treatment goal for patients with LN.[19 25]

The safety profile of anifrolumab in LN was generally consistent with SLE without active renal disease, including higher incidence of HZ with anifrolumab versus placebo.[10 35] Most AEs were mild or moderate in intensity, were not serious, and did not lead to investigational product discontinuation.[35] In alignment with previous observations,[36–38] the incidence of HZ was higher among patients with LN than those with non-renal SLE. This was likely related to LN requiring more potent background

immunosuppressive regimens, including glucocorticoids,[19] which are identified risk factors for HZ reactivation.[37 39] Consistently, HZ tended to occur early in the trial when glucocorticoids had not yet been tapered. Most HZ events were mild or moderate, cutaneous, and resolved with antivirals without investigational product discontinuation.

Limitations include that this was a proof-of-concept, dose-finding study, with a relatively small enrolment of patients. There was also a high rate of investigational product discontinuation; as discussed previously, this may have confounded the high 24-hour UPCR improvement estimate for placebo. Discontinuations could also have impacted binary response rates, as patients meeting the discontinuation criteria or using restricted medications were classified as non-responders, irrespective of disease activity improvements.

Overall, the TULIP-LN study results support further assessment of the efficacy and safety of anifrolumab IR in patients with active LN. The PK results suggest that three intensified doses of anifrolumab 900 mg improved clearance to a lower rate, enabling dosage tapering to the 300 mg every 4 weeks regimen indicated for patients with SLE without active renal disease.[40] In patients with active LN, the anifrolumab IR was required to obtain clinical efficacy; indeed, the anifrolumab IR was numerically superior to placebo for several clinically relevant endpoints, whereas the anifrolumab BR was not. As such, the results suggest that the anifrolumab IR is a more suitable dosing regimen than the anifrolumab BR to carry forward into future clinical investigations of anifrolumab to treat patients with active LN. Learnings from this trial will support dose selection and the development of trial designs for future studies of anifrolumab in LN.

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Ethics approval The study protocol was approved by each centre's ethics committee or institutional review board. The study was overseen by an external data and safety monitoring board. Please refer to uploaded file named '221659 RP06_IRB_EC Submission and Approval_20210818' for all Approval Numbers/IDs. Of note, France and Italy rejected Protocol Amendment number 3; therefore, patients from France and Italy are excluded from all relevant analyses. This is clearly specified in the manuscript where applicable. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. Data underlying the findings described in this manuscript may be obtained in accordance with AstraZeneca's data sharing policy described at https://astrazenecagrouptrials.pharmacm.com/ST/Submission/Disclosure. Deidentified participant data can be made available upon reasonable request to Catharina Lindholm (ORCHID ID: 0000-0002-0533-7185) or through the Vivli web-based data request platform. Reuse is permitted only with permission from AstraZeneca. The Clinical Study Protocol, Statistical Analysis Plan, and Clinical Study Report Synopsis are available at: https://astrazenecagrouptrials.pharmacm.com/ST/Submission/View?id=22674.

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CLINICAL SCIENCE

Development and validation of a patient-reported outcome measure for systemic sclerosis: the EULAR Systemic Sclerosis Impact of Disease (ScleroID) questionnaire

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ABSTRACT

Objectives Patient-reported outcome measures (PROMs) are important for clinical practice and research. Given the high unmet need, our aim was to develop a comprehensive PROM for systemic sclerosis (SSc), jointly with patient experts.

Methods This European Alliance of Associations for Rheumatology (EULAR)-endorsed project involved 11 European SSc centres. Relevant health dimensions were chosen and prioritised by patients. The resulting Systemic Sclerosis Impact of Disease (ScleroID) questionnaire was subsequently weighted and validated by Outcome Measures in Rheumatology criteria in an observational cohort study, cross-sectionally and longitudinally. As comparators, SSc-Health Assessment Questionnaire (HAQ), EuroQol Five Dimensional (EQ-5D), Short Form-36 (SF-36) were included.

Results Initially, 17 health dimensions were selected and prioritised. The top 10 health dimensions were selected for the ScleroID questionnaire. Importantly, Raynaud's phenomenon, impaired hand function, pain and fatigue had the highest patient-reported disease impact. The validation cohort study included 472 patients with a baseline visit, from which 109 had a test–retest reliability visit and 113 had a follow-up visit (85% female, 38% diffuse SSc, mean age 58 years, mean disease duration 9 years). The total ScleroID score showed strong Pearson correlation coefficients with comparators (SSc-HAQ, 0.73; Patient's global assessment, Visual Analogue Scale 0.77; HAQ-Disability Index, 0.62; SF-36 physical score, −0.62; each $p<0.001$). The internal consistency was strong: Cronbach's alpha was 0.87, similar to SSc-HAQ (0.88) and higher than EQ-5D (0.77). The ScleroID had excellent reliability and good sensitivity to change, superior to all comparators (intraclass correlation coefficient 0.84; standardised response mean 0.57).

Conclusions We have developed and validated the EULAR ScleroID, which is a novel, brief, disease-specific, patient-derived, disease impact PROM, suitable for research and clinical use in SSc.

Key messages

What is already known about this subject?
- Patient-reported outcome measures (PROMs) are important to integrate the patient's view into routine care.
- They are an integral part of clinical trials and required for registration of novel treatments.
- A brief and specific validated PROM for overall systemic sclerosis (SSc) is lacking.

What does this study add?
- It develops and validates the Systemic Sclerosis Impact of Disease (ScleroID), a disease-specific PROM that captures patient experience and SSc complexity in an easy to apply format for clinical care and clinical trials.

How might this impact on clinical practice or future developments?
- ScleroID can be used to integrate patient experience to improve decision making in clinical practice.
- Further studies are needed to validate ScleroID as a potential PROM for future clinical trials in SSc.

INTRODUCTION

Systemic sclerosis (SSc) is characterised by a chronic and frequently progressive course and by a high patient-to-patient variability.[1] SSc has one of the highest morbidities and case-specific mortalities among the connective tissue diseases.[2 3] Overall, general health (as measured by the Short Form-36 (SF-36) and EuroQol Five Dimensional (EQ-5D) questionnaires), as well as quality of life and functional abilities (as measured by the Health Assessment Questionnaire Disability Index, HAQ-DI) are significantly reduced in SSc.[4–6]

A disease-specific, patient-reported outcome measure (PROM) for use in clinical trials and in

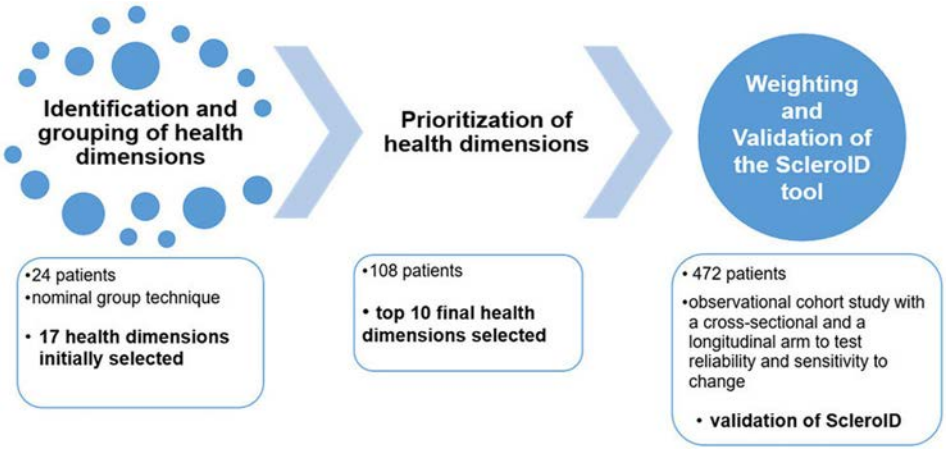

Figure 1 General ScleroID project workflow and procedure. ScleroID, Systemic Sclerosis Impact of Disease.

clinical practice in SSc that covers the different disease features of this multiorgan autoimmune disease is lacking.[7] The European Medicines Agency recommends that sufficient evidence needs to be provided on the patient benefit by PROMs before granting approval of a new therapeutic agent,[8] and PROMs need to be included as outcome measures in therapeutic randomised controlled trials (RCTs). Thus, the lack of sensitive, disease-specific PROMs covering the overall disease is currently one of the greatest challenges for drug development in this devastating disease. In addition, published data show that systematic use of PROMs in clinical practice improves patient-physician communication and decision making, as well as patients' satisfaction.[9]

Research in the field of other autoimmune diseases provides the basis for the successful development of disease-specific PROMs. For rheumatoid arthritis, the Rheumatoid Arthritis Impact of Disease (RAID) questionnaire,[10 11] and for psoriatic arthritis, the Psoriatic Arthritis Impact of Disease (PsAID) questionnaire,[12] were designed to capture the burden of disease that is most important to patients. Furthermore, the RAID has been successfully used to identify thresholds for symptom states acceptable for patients, as well as evaluating onset of response to medication.[13 14]

In this study, we aimed to develop a novel, patient-derived PROM for SSc that is able to cover the global disease burden—the EULAR Systemic Sclerosis Impact of Disease (ScleroID). Furthermore, we validated the ScleroID by the Outcome Measures in Rheumatology (OMERACT) filter in a large, multicentric, clinical cohort study.[15]

METHODS

The development of the European Alliance of Associations for Rheumatology (EULAR) ScleroID follows approaches used in the EULAR-endorsed RAID and PsAID questionnaires,

Table 1 Initially selected candidate health dimensions and their prioritisation ranking by importance

No	Health dimensions*	Mean rank	Median rank	Order by median rank	% patients giving rank 1 to the dimension	% patients giving rank 1–3 to the dimension	% patients giving rank 1–10 to the dimension
1	Raynaud	5.8	5	1	19.4	36.1	84.3
2	Hand function	6.7	5	1	8.3	25.0	78.7
3	Upper GI symptoms	7.2	6	2	7.4	24.1	73.1
4	Pain	6.9	6	2	10.2	25.9	75.9
5	Fatigue	6.7	6	2	9.3	26.9	78.7
6	Lower GI symptoms	7.8	7	3	10.2	24.1	69.4
7	Limitation of life choices and activities	8.3	8	4	4.6	20.4	66.7
8	Body mobility	8.7	8,5	5	2.8	11.0	65.7
9	Breathlessness	8.6	9	6	12.0	27.8	52.8
10	Digital ulcers	9.5	10	7	1.9	17.6	54.6
11	Anxiety	10.2	10	7	2.8	9.3	50.9
12	Dryness	10.1	10	7	1.9	9.3	54.6
13	Appearance	10.3	11	8	3.7	9.3	49.1
14	Concentration difficulties	10.9	12	9	1.9	9.3	39.8
15	Cough	11.3	13	10	1.9	10.2	38.9
16	Depression	11.6	13	10	0.9	7.4	35.2
17	Calcinosis	12.5	14	11	0.9	6.5	31.5

*Patients from the prioritisation cohort were asked to rank the dimensions in order of their importance by giving a rank from 1 (most important) to 17 (least important). Each rank could only be used once. The top 10 dimensions with the lowest median rank (highest importance) were selected for the questionnaire. The 10–12th dimension had an equal median rank but the 10th dimension had a higher role for more patients (% giving top rank, last two columns) and was consequently chosen in favour of dimensions 11 and 12. Dimensions included in the final ScleroID questionnaire are bolded.
GI, gastrointestinal; No, number; ScleroID, Systemic Sclerosis Impact of Disease.

Table 2 The ScleroID questionnaire

The EULAR ScleroID												
How much have the different aspects of systemic sclerosis affected you during the last week?												
Please mark your responses on the scale by choosing the appropriate no for each of the following dimensions:												
Raynaud's phenomenon:												
Circle the no that best describes the severity of your Raynaud's phenomenon during the last week:												
None	0	1	2	3	4	5	6	7	8	9	10	Extreme
Hand function:												
Circle the no that best describes your hand function limitations due to your systemic sclerosis during the last week:												
No limitation	0	1	2	3	4	5	6	7	8	9	10	Extreme limitation
Upper gastrointestinal tract symptoms (eg, swallowing difficulties, reflux, vomiting):												
Circle the no that best describes the severity of your upper gastrointestinal tract symptoms due to your systemic sclerosis during the last week:												
None	0	1	2	3	4	5	6	7	8	9	10	Extreme
Pain:												
Circle the no that best describes the pain you felt due to your systemic sclerosis during the last week:												
None	0	1	2	3	4	5	6	7	8	9	10	Extreme
Fatigue:												
Circle the no that best describes the impact of overall fatigue due to your systemic sclerosis during the last week:												
None	0	1	2	3	4	5	6	7	8	9	10	Extreme
Lower gastrointestinal tract symptoms (eg, bloating, diarrhoea, constipation, anal incontinence):												
Circle the no that best describes the severity of lower gastrointestinal tract symptoms during the last week:												
None	0	1	2	3	4	5	6	7	8	9	10	Extreme
Limitations of life choices and activities (eg, social life, personal care, work):												
Circle the no that best describes how severe the limitations of life choices and activities due to your systemic sclerosis were during the last week:												
No	0	1	2	3	4	5	6	7	8	9	10	Extreme
Body mobility:												
Circle the no that best describes how much your body mobility was affected due to your systemic sclerosis during the last week:												
Not affected	0	1	2	3	4	5	6	7	8	9	10	Extremely affected
Breathlessness:												
Circle the no that best describes how severe your breathlessness due to systemic sclerosis was during the last week:												
None	0	1	2	3	4	5	6	7	8	9	10	Extreme
Digital ulcers:												
Circle the no that best describes how much your digital ulcers affected you overall during the last week:												
None	0	1	2	3	4	5	6	7	8	9	10	Extreme

ScleroID, Systemic Sclerosis Impact of Disease.

as well as in the Pancreatic Cancer Disease Impact Score (PACADI),[10–12 16 17] with some modification given the differences between these diseases and SSc. Validation of the EULAR ScleroID follows the internationally recommended methodology of the OMERACT filter[15] (online supplemental file). This is a longitudinal, multicentric project, involving 11 European expert SSc centres and patient research partners. The project workflow and process are presented in figure 1.

Patient and public involvement

Patient research partners were involved in all the stages of the ScleroID project, starting with project design (KF and ATK), to the identification of the relevant health dimensions, and development and validation of the ScleroID including item reduction by weighting. These steps are detailed in the sections below. Furthermore, the dissemination of the study has been supported by the patient organisation Federation of European Scleroderma Associations (FESCA) by invited presentations of the preliminary results at patient congresses.

Part 1: development of the ScleroID questionnaire

Identification, prioritisation and selection of the health dimensions for the ScleroID

Initially, 24 patients with SSc participated in a nominal group technique exercise and selected candidate health dimensions with the highest impact on their disease status. First, the expert investigators (RD, MB and TH) presented a review of the literature on PROMs used in SSc. The patient representatives thereafter suggested health dimensions on which the disease has an important impact, according to their personal perception. On day one, 66 health dimensions were collected. On the second day, these were discussed and grouped by the patients according to the main concept that they are referring to, under moderation by TH. Finally, 17 candidate dimensions were unanimously selected (further details in online supplemental annex 2).

Subsequently, the identified health dimensions were evaluated by a larger group of SSc patients from all 11 participating centres. The objective of this exercise was to optimise face validity and to prioritise the dimensions. The health dimensions were translated by the investigators and patient research partners into each language (online supplemental file). Patients were presented with the list of candidate health dimensions in a random order and asked to rank them according to a decreasing order of importance. The top 10 dimensions based on median ranking were selected by the steering committee (MB, RD, KF, ATK, TH and OD) for the final ScleroID. The limitation to 10 dimensions was chosen based on ranking and aiming for a better feasibility of the final questionnaire and focussing on the most relevant health dimensions reported by the SSc patient research partners.

Development of the ScleroID questionnaire

The experts (MB, RD, TH and OD) developed one question with Numeric Rating Scales (NRS) to assess each of the selected top 10 health dimensions. The ScleroID questionnaire was subsequently translated into all applicable languages following the protocol detailed in online supplemental file.

Part 2: weighting of the dimensions and validation of ScleroID

Study design

A cross-sectional international observational cohort study with longitudinal reliability and sensitivity to change arms was performed. Patients above 18 years of age fulfilling the American College of Rheumatology/European Alliance of Associations for Rheumatology (ACR/EULAR) 2013 classification criteria for SSc were prospectively included.[18] Patients with severe comorbidities not related to SSc were excluded (eg, concomitant inflammatory disease, organ failure, recent acute cerebrovascular event, serious psychiatric or neurological disease). All patients signed written informed consent.

The sample target for the cohort study was 500 patients for the cross-sectional arm and 100/150 patients for reliability/sensitivity to change longitudinal arms, respectively, based on previous experiences with RAID and PsAID. As comparator questionnaires for the ScleroID, the most frequently used global PROMs applied in SSc were selected (online supplemental file).

Data collection

Clinical and demographic data were collected according to the European Scleroderma Trials and Research group (EUSTAR) standards[19] (online supplemental file). In addition, patients completed the ScleroID questionnaire, the selected comparators (SSc-HAQ, EQ-5D, SF-36), patient's global assessment on a Visual Analogue Scale (VAS), specific questions on the state of disease and a minimal clinically important difference question (online supplemental table S1) at all visits (online supplemental file).[20–25] For the weighting procedure, in order to assess the relative impact of the health dimensions, patients were asked to distribute 100 points between the 10 dimensions of the ScleroID according to the perceived impact on their health (online supplemental file). This was the basis for calculation of the ScleroID score (see statistical methods). Patients considered to be in a stable state by the physician and with no foreseeable change in treatment or medical intervention in the next 10 days following the baseline visit were included into the reliability arm, and asked to complete the reliability questionnaire at 7±3 days after the baseline visit (online supplemental annex). Patients considered to have active disease by the treating physician were included into the sensitivity to change arm and completed the respective questionnaire at the 12 months visit and/or at the 6 months visit, if available (online supplemental annex).

Statistical analysis

The calculation of the ScleroID score was based on the ranking of the weights, as performed in RAID, PsAID and PACADI.[10–12 16 17] Mean and median weights were calculated for each health dimension, after which mean and median ranks were computed for the whole cohort. These represent the basis for calculating the final weight, which is multiplied by the value on the NRS for each dimension/item and summed up for the final ScleroID score, which is then divided by 100.

The validation of ScleroID psychometric properties was performed according to the OMERACT filter, which assesses three main features: feasibility, truth and discrimination.[15] Feasibility addresses the applicability of the ScleroID questionnaire. Truth encompasses face validity (does the measure make sense), and content validity (eg, distribution of the score, floor/ceiling effect). As other measures of truth, internal consistency using Cronbach's alpha and construct validity (concurrent validity) with Pearson correlations to other scores (SSc-HAQ, SF-36, EQ-5D) were calculated. Construct validity was also investigated using a confirmatory factor analysis (online supplemental file). In addition, we assessed reliability and sensitivity to change. In the reliability arm, patients, who reported themselves as 'stable', were included in the test–retest reliability (reproducibility) analysis by assessing the intraclass correlation coefficient and agreement by Bland-Altman plot. In the sensitivity to change arm, patients reporting themselves as 'not stable' were included in the sensitivity to change (responsiveness) analysis by the standardised response mean (SRM, which is the difference in the baseline and follow-up mean values divided by the SD of the change scores). CIs were obtained by bootstrapping.

RESULTS

Part 1: development of the ScleroID questionnaire

Identification and prioritisation of health dimensions for the ScleroID

In the initial nominal group exercise, 24 patient research partners selected 17 health dimensions reflecting the impact of SSc (table 1). An additional cohort of 108 patients (online supplemental table S2) subsequently prioritised these health dimensions. The selected health dimensions and their prioritisation are summarised in table 1.

Selection of health dimensions and development of the ScleroID questionnaire

The steering committee agreed unanimously to include the ten health dimensions rated with the highest priority into the ScleroID questionnaire. One question with appropriate anchors to assess each of the selected ten health dimensions was developed by the steering committee (MB, RD, KF, ATK, TH and OD). These questions formed the ScleroID questionnaire (table 2), which was also agreed on by the patient research partners.

Part 2: weighting and validation of the ScleroID questionnaire

Cohort study

In total, 472 SSc patients from nine countries (France, Italy, Hungary, Poland, Romania, Spain, Sweden, Switzerland, UK) were included in the cross-sectional cohort study.

Most patients were female (84.8%), more than one-third had diffuse cutaneous SSc (dcSSc, 37.5%) and the median age was 57 years. The various disease manifestations, including lung fibrosis (42.6%), pulmonary arterial hypertension (7%), gastrointestinal (GI) involvement (>60% of patients with oesophageal symptoms), articular involvement (4.4% with synovitis) and digital ulcers (24.0% with previous ulcers, 13.0% with current ulcers) were well represented, reflecting a typical SSc population (table 3).

Weighting of the health dimensions and calculation of the ScleroID score

Overall, valid data on weighting was provided by 446 (94%) patients, and 462 (98%) patients provided complete data on the ScleroID questionnaire.

The health dimensions which were assigned the highest weights (and thus highest impact) by the patients were Raynaud's phenomenon, fatigue, hand function and pain, followed by

Table 3 Characteristics of the patients with SSc included in the weighting and validation cohort study

Characteristics	Overall	% of missingness
Age, years, median (IQR)	57 (48–65)	1.1
Female gender (n, %)	396 (84.8)	1.1
Time since RP onset, years, median (IQR)	11 (5.8–20)	26.3
Time since first non-RP manifestations, years, median (IQR)	9 (4.7–15)	5.5
Diffuse cutaneous SSc (n, %)	152 (37.5)	14.2
Limited cutaneous SSc (n, %)	253 (62.5)	14.2
mRSS, median (IQR)	4 (0–8)	26.5
Presence of Raynaud's phenomenon (n, %)	332 (94.6)	25.6
Digital ulcers (n, %)	47 (13)	23.5
Joint contractures (n, %)	124 (35.7)	26.5
Joint synovitis (n, %)	15 (4.4)	28.4
Oesophageal symptoms (dysphagia, reflux) (n, %)	232 (60.3)	18.4
Stomach symptoms (early satiety, vomiting) (n, %)	61 (17.6)	26.5
Intestinal symptoms (diarrhoea, bloating, constipation) (n, %)	135 (33.8)	15.5
Malabsorption syndrome (n, %)	18 (7.4)	48.7
Dyspnoea, NYHA stages III and IV (n, %)	27 (9.6)	40.7
FVC, % predicted, median (IQR)	95 (82–108)	40.5
FVC <80% predicted (n, %)	58 (20.6)	40.5
DLCO/SB, % predicted, median (IQR)	69 (55–81)	44.9
DLCO/SB, <70% predicted (n, %)	133 (51.2)	44.9
Lung fibrosis detected by HRCT (n, %)	78 (42.6)	61.2
Pulmonary hypertension (n, %)	19 (6.6)	39.4
PAPsys, mm Hg, median (IQR)	28 (24–32)	54.4
LVEF, %, median (IQR)	60 (55–65)	35.4
ANA positive (n, %)	319 (96.7)	30.1
ACA positive (n, %)	118 (36.5)	31.6
Anti-Scl-70 AB positive (n, %)	112 (35.2)	32.6
Anti-RNA Polymerase III AB positive (n, %)	21 (7.6)	41.1
ESR, mm/h, median (IQR)	17 (10–30)	25.2
CRP, mg/L, median (IQR)	2 (0.9–5)	35
Immunosuppression (n, %)	59 (21.2)	41.1

Definitions of organ manifestations according to EUSTAR.[19]
ACA, anticentromere antibodies; ANA, antinuclear antibodies; CRP, C reactive protein; DLCO/SB, diffusing capacity of the lung for carbon monoxide/single breath; ESR, erythrocyte sedimentation rate; EUSTAR, European Scleroderma Trials And Research; FVC, forced vital capacity; HRCT, high resolution CT; LVEF, left ventricular ejection fraction; mRSS, modified Rodnan Skin Score; NYHA, New York Heart Association; RP, Raynaud's phenomenon; Scl70, anti-Scl70 antibodies, anti-topoisomerase I antibodies; SSc, systemic sclerosis.

upper and lower GI symptoms (table 4), confirming the results from the prioritisation.

The mean ranks given in table 4 were rescaled to sum up to 1 for the final weights. The ScleroID was calculated as a composite score of the selected 10 dimensions. For each dimension, the NRS score was multiplied by the specific weight for this item and the weighted scores were summed up (see example in table 5).

Performance of ScleroID by the OMERACT filter

Feasibility

The ScleroID showed feasibility in the application, given the low proportion of missing data: ten patients (2.1%) had missing items, compared with 36 and 37 patients with missing data for SF-36 physical/mental component summary (PCS), 8 for EQ-5D, 12 for HAQ-DI and 16 for SSc-HAQ (online supplemental table S3). The majority of participants (462 or 98%) had complete data on the ScleroID questionnaire. Missing data were evenly distributed among the ScleroID items (no item had significantly higher missing values).

In daily practice, single items of questionnaires are frequently missing. We therefore assessed how imputation of single items affects the overall ScleroID score. When one missing item of the ScleroID score was imputed by the mean of the remaining cohort for this item, the error was minimal (up to 0.29/10 or <10%, (online supplemental table S4)).

Truth

Face validity was ensured by the involvement of patient research partners in all steps of the ScleroID development.[26]

The ScleroID score range is 0–10, the actual median and IQR in our patients was 3.2 (1.9–4.7) at baseline. The median and IQ for lcSSc patients was 3.3 (2.0–4.7) and for difusse cutaneous SSc (dcSSc) patients 3.3 (1.7–4.8; online supplemental figure S2). In total, eight patients recorded a ScleroID score of 0, while the highest observed value was 9.4. There was no relevant floor or ceiling effect, which would be assumed if >15% of patients scored either the minimum or maximum value ([27] online supplemental figure S2). The ScleroID questionnaire showed a good construct validity when correlated with the comparators (SSc-HAQ r=0.73; EQ-5D r=−0.48; Patient's global assessment, VAS r=0.77; HAQ-DI r=0.62; SF-36 PCS r=−0.62; each p<0.001, table 6).

The internal consistency as another measure of construct validity was also strong: Cronbach's alpha for the ScleroID was 0.87, similar to the SSc-HAQ (0.88) and higher than for the EQ-5D (0.77, online supplemental table S2). We also performed a confirmatory factor analysis which suggested a bifactor model (one general factor with additional two or three factors) with good model fit indices (online supplemental table S6 and figure S2). The omega indices, which are thought to

Table 4 Weighting of the health dimensions according to their perceived impact by the patients participating in the cross-sectional cohort study (n=472)

Dimension	Weight mean (SD)	Rank mean (SD)	Top ranked	Upper 25%	Bottom 25%	Lowest ranked
Raynaud	20.9 (18.9)	7.8 (2.6)	39.0	65.9	28.0	16.7
Fatigue	12.9 (10.6)	7.6 (2.0)	23.7	58.5	25.6	18.2
Hand function	12.1 (10.4)	7.3 (2.3)	19.5	55.9	36.2	21.2
Pain	10.4 (8.7)	7.0 (2.3)	16.7	46.0	42.2	23.5
Upper.GI symptoms	8.0 (8.2)	6.4 (2.4)	12.3	37.3	50.6	36.0
Life choices	7.9 (8.2)	6.6 (2.3)	12.1	35.8	52.1	37.9
Lower GI symptoms	7.6 (9.1)	6.2 (2.5)	11.4	36	56.1	42.8
Body mobility	7.0 (6.7)	6.4 (2.3)	8.1	38.6	54.0	39.2
Dyspnoea	6.8 (8.8)	6.1 (2.4)	9.3	33.7	64.4	46.2
Digital ulcers	5.9 (9.8)	5.6 (3.0)	17.2	32.2	68.6	61.4

Column 'weight' gives the mean (SD) of the weight given to each dimension, column "Rank" gives the mean (SD) ranking of each dimension according to the patient distributed weights. The remaining four columns give the percentage of times the dimension was ranked as most important (top ranked), the percentage of times it was ranked as least important (lowest ranked), as well as in the upper and lower quartiles of importance.
GI, gastrointestinal.;

Table 5 Computation of the ScleroID score

Element	Raynaud	Fatigue	Hand function	Pain	Life choices	Upper GI symptoms	Body mobility	Lower GI symptoms	Dyspnoea	Digital ulcers
ScleroID weights	0.117	0.114	0.109	0.104	0.098	0.096	0.095	0.093	0.091	0.083
Example NRS scores	9	3	4	0	7	2	6	4	0	3
weights(x)scores	0.117×9	0.114×3	0.109×4	0.104×0	0.098×7	0.096×2	0.095×6	0.093×4	0.091×0	0.083×3
=	1.053	0.342	0.436	0	0.686	0.192	0.57	0.372	0	0.249
ScleroID =	3.9									

Example of computation of the ScleroID score for a given patient. The final score is computed using a weighted sum over the NRS (0–10) scores given to each dimension by the patient. The weights sum to 1, and are proportional to the mean ranks given to each dimension.
GI, gastrointestinal tract; NRS, Numeric Rating Scale; ScleroID, Systemic Sclerosis Impact of Disease.

be superior to Cronbach's alpha,[28 29] suggested not only good model fit for the bifactor models (online supplemental table S7), but also supported our claim for sufficient unidimensionality to justify the use of a sum score (see also online supplemental file).

Test–retest reliability

In total, 109 patients were included in the longitudinal reliability arm and completed a second visit at 7±3 days after baseline. The ScleroID had a very good test–retest reliability, with an intraclass correlation coefficient of 0.84 (ranging 0.61–0.79 for the individual items), superior to all comparators (online supplemental table S8); see also Bland-Altman plot for agreement in online supplemental figure S5).

Sensitivity to change

A total of 113 patients were included and had a median follow-up visit at 12.2 (IQR 11.5–13.1) months. The sensitivity to change for the ScleroID was estimated using the SRM between baseline and follow-up, using only those patients (n=37) reporting disease status as not-stable (table 7). The SRM is computed for all patients regardless of whether they report improved/worsened disease state, and then separately for those with improved and worsened state (table 7). The ScleroID performed better than all other comparator PROMs in indicating overall change. This performance was even better in patients who experienced improvement (table 7).

Table 6 Construct validity analysis by correlation between ScleroID and other established PROMs

Variable	Pearson correlation coefficient*
Physician's Global Assessment	0.28 (0.05)
Patient's Global Assessment	0.77 (0.03)
SF-36 Physical Component Score	−0.62 (0.03)
SF-36 Mental Component Score	−0.47 (0.03)
HAQ-DI	0.62 (0.03)
SSc-HAQ	0.73 (0.02)
EQ-5D (UK-weighted)	−0.48 (0.04)
VAS-GIT	0.38 (0.05)
VAS-Dyspnoea	0.38 (0.04)
VAS-Raynaud	0.42 (0.04)
VAS-Ulcers	0.37 (0.05)

*Bootstrap standard errors (SEs) of estimated correlation given in brackets.
EQ-5D, EuroQol Five Dimensional Questionnaire; GIT, gastrointestinal tract; HAQ-DI, Health Assessment Questionnaire Disability Index; PROMs, patient-reported outcome measures; ScleroID, Systemic Sclerosis Impact of Disease; SF-36, Short Form (36) Health Survey; SSc, systemic sclerosis; VAS, Visual Analogue Scale.

DISCUSSION

PROMs are being developed to capture the patient's aspects of a disease, that is, the specific patient experience beyond the disease manifestations that are in the physician's focus, which are typically lethal or associated with high morbidity. Especially in SSc, which has a high morbidity and mortality as well as a high work disability, there is a discordance between the patient's experience and the physician's assessment, exemplified by differences in the patient's and physician's global assessment.[30–32] This was also observed in this study, underlining the need to implement PROMs in the clinical assessment and shared decision making. Most PROMs used in SSc are legacy questionnaires adapted from other diseases and not SSc-specific instruments.

Hence, specific PROMs are needed, although some have tried to incorporate the patient's view.[7 33]

We have developed and validated the ScleroID questionnaire as a global measurement tool to assess the disease burden in SSc patients. The questionnaire is simple and easy to apply, has high internal consistency and shows good correlation with the patient global assessment and the SSc-HAQ. Although weighting reflects patient experience, it does not significantly change the overall score. It is planned to develop a calculator (or app) to provide final scores. The ScleroID health dimensions have a high face validity due to the inclusion of SSc patient research partners throughout the development and validation process. Notably, main dimensions of the ScleroID questionnaire such as dyspnoea, pain, digital ulcers, GI symptoms or fatigue were also associated with a high self-reported disability and high disease burden in other reports from the literature.[5 34]

The ScleroID questionnaire has a very good retest reliability, which is even better than comparators and has better sensitivity to change than the comparators used. This is especially important as a high percentage of patients are relatively stable, but progression of the disease drives mortality and morbidity.[35] In addition, other frequently used major outcomes of SSc studies, such as the mRSS, show a relatively low sensitivity to change, which might partially explain the many randomised clinical trials with borderline significance using the mRSS as a primary outcome.[36]

Comparison to other PROMs

In contrast to other validated PROMs that have not been developed specifically for SSc (such as Patient-Reported Outcomes Measurement Information System-29; PROMIS-29)[37–39] or have only been adapted to SSc (such as the Scleroderma Health Assessment Questionnaire (SHAQ))[39 40], the ScleroID questionnaire was specifically developed, with involvement of SSc patient research partners. Although other specific PROMs for SSc have been developed, the Symptom Burden Index and the Systemic Sclerosis Questionnaire (SySQ) did not involve the target

Table 7 Sensitivity to change of the ScleroID compared with other PROMs

Variable	SRM (all)	95% CI (all)	SRM (improved)	95% CI (improved)	SRM (worsened)	95% CI (worsened)
ScleroID	0.57 (36)	0.31 to 0.86	0.76 (20)	0.42 to 1.23	−2.31 (4)	−25.14 to −1.35
Raynaud	0.08 (37)	−0.26 to 0.4	0.21 (20)	−0.25 to 0.68	−1.50 (4)	– to −1.17
Hand function	−0.20 (36)	−0.57 to 0.11	−0.22 (20)	−0.77 to 0.22	−0.78 (4)	−3.5 to −0.5
Pain	0.01 (37)	−0.23 to 0.45	0.04 (20)	−0.39 to 0.51	0.00 (4)	−1.5 to 1.5
Fatigue	0.24 (37)	−0.08 to 0.54	0.40 (20)	0 to 0.79	−1.306 (4)	– to −0.5
Upper GI symptoms	0.56 (37)	0.33 to 0.81	0.58 (20)	0.25 to 0.99	– (4)	–
Lower GI symptoms	0.44 (37)	0.09 to 0.82	0.43 (20)	−0.03 to 1.07	– (4)	–
Life Choices	0.53 (37)	0.25 to 0.87	0.77 (20)	0.33 to 1.51	0.50 (4)	0.5 to 1.5
Body mobility	0.35 (37)	0.03 to 0.63	0.54 (20)	0.14 to 1	0.00 (4)	−1.5 to 1.5
Dyspnoea	0.50 (37)	0.2 to 0.85	0.65 (20)	0.25 to 1.24	0.00 (4)	−1.5 to 1.5
Digital ulcers	−0.09 (36)	−0.43 to 0.23	0.00 (20)	−0.62 to 0.39	−0.5 (4)	−1.5 to −0.5
Patient's Global Assessment	0.29 (36)	−0.04 to 0.66	0.57 (20)	0.22 to 1.02	−0.20 (4)	−1.5 to 1.5
Physician's Global Assessment	0.09 (29)	−0.26 to 0.47	0.31 (17)	−0.18 to 0.9	−0.5 (4)	−1.5 to −0.5
SF-36 Physical Component Score	−0.2 (37)	−0.53 to 0.08	−0.45 (20)	−0.85 to −0.07	10.96 (4)	9.25 to 128.35
SF-36 Mental Component Score	−0.08 (37)	−0.4 to 0.26	−0.18 (20)	−0.64 to 0.31	−0.24 (4)	−1.22 to 2.65
HAQ-DI	−0.01 (36)	−0.39 to 0.32	0.10 (19)	−0.34 to 0.61	−0.78 (4)	−2.6 to −0.5
SSc HAQ	0.15 (34)	−0.23 to 0.45	0.24 (18)	−0.26 to 0.69	−0.46 (4)	−5.5 to 0.5
EQ-5D	0.41 (37)	0.09 to 0.74	0.33 (20)	−0.09 to 0.74	1.42 (4)	1.25 to 9.94

EQ-5D, EuroQol Five Dimensional; GI, gastrointestinal; HAQ-DI, Health Assessment Questionnaire Disability Index; PROMs, patient-reported outcome measures; ScleroID, Systemic Sclerosis Impact of Disease; SF-36, Short Form (36) Health Survey; SRM, standardised response mean; SSc, systemic sclerosis.

population for dimension/item generation. The Scleroderma Assessment Questionnaire (SAQ), which is based on the SysQ, had only partial involvement of patients.[41 42] However, these questionnaires have only been partially validated, mostly lacking a discriminant validity analysis, and are partly not validated in English (SysQ and SAQ). The recently published PROM Cochin Scleroderma Functional scale 17, a 17-item PROM that focused on mobility and general task aspects of SSc, was also developed with involvement of SSc patients.[43] It has been evaluated in a smaller cohort than the ScleroID and in French only, with data on discriminant validity (sensitivity to change) still missing.

Limitations of the study

Although patients with diverse disease manifestations participated in the nominal group exercise, disease-related or demographic data were not prospectively collected at this early stage. Patients included in the cross-sectional analysis had to fulfil the ACR/EULAR 2013 classification criteria for SSc but there were no recommendations concerning disease subtype or organ involvement. The final selection of participants by the centres has an impact on the weighting of the ScleroID dimensions and the cross-sectional part included mainly patients with long-standing disease. However, our cohort reflects other observational cohorts such as the EUSTAR registry, etc, indicating that it is a representative SSc population. Although SSc patients often acquire expert knowledge about their disease and are aware that the questionnaire evaluates SSc-related burden, it might be difficult at times to distinguish symptoms related to SSc from common, unrelated symptoms, for example, as in the case of GI problems. This is however common to all PROMs.

Another potential limitation is the relative paucity of patients who experience change of their disease status, who then enter the sensitivity to change analysis. As this change was anchored by the patients themselves, there were no prior data to guide selection of these patients.

The ScleroID was designed as an overall measure of disease impact. It was derived from patients under routine clinical care and as such, it is still to be validated in clinical trials aiming at overall disease modification. If the ScleroID questionnaire can also be used for clinical trials focusing on organ-specific disease progression is subject to further analysis.

In summary, the ScleroID questionnaire is a unique, easy to apply, SSc-specific PROM that has been successfully validated in a large European clinical cohort using multiple translations. It should be further validated for clinical trials and in large registries and has the potential to measure disease impact that will be more meaningful for patients and health authorities than currently used approaches.

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TRANSLATIONAL SCIENCE

Large-scale analysis of longitudinal skin gene expression in systemic sclerosis reveals relationships of immune cell and fibroblast activity with skin thickness and a trend towards normalisation over time

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ABSTRACT

Objectives Determine relationships between skin gene expression and systemic sclerosis (SSc) clinical disease features, and changes in skin gene expression over time.

Methods A total of 339 forearm skin biopsies were obtained from 113 SSc patients and 44 matched healthy controls. 105 SSc patients had a second biopsy, and 76 had a third biopsy. Global gene expression profiling was performed, and differentially expressed genes and cell type-specific signatures in SSc were evaluated for relationships to modified Rodnan Skin Score (mRSS) and other clinical variables. Changes in skin gene expression over time were analysed by mixed effects models and principal component analysis. Immunohistochemical staining was performed to validate conclusions.

Results Gene expression dysregulation was greater in SSc patients with affected skin than in those with unaffected skin. Immune cell and fibroblast signatures positively correlated with mRSS. High baseline immune cell and fibroblast signatures predicted higher mRSS over time, but were not independently predictive of longitudinal mRSS after adjustment for baseline mRSS. In early diffuse cutaneous SSc, immune cell and fibroblast signatures declined over time, and overall skin gene expression trended towards normalisation. On immunohistochemical staining, most early diffuse cutaneous SSc patients with high baseline T cell and macrophage numbers had declines in these numbers at follow-up.

Conclusions Skin thickness in SSc is related to dysregulated immune cell and fibroblast gene expression. Skin gene expression changes over time in early diffuse SSc, with a tendency towards normalisation. These observations are relevant for understanding SSc pathogenesis and could inform treatment strategies and clinical trial design.

Key messages

What is already known about this subject?

- Skin gene expression is dysregulated in patients with systemic sclerosis (SSc), but is heterogeneous. The relationships between clinical disease features and skin gene expression are incompletely defined, and the extent to which skin gene expression changes over time within individuals is unclear.

What does this study add?

- A large-scale analysis of longitudinal skin gene expression in a diverse group of well-characterised SSc patients validated that immune cell and fibroblast signatures are positively associated with skin thickness in SSc.
- Skin gene expression trended towards normalisation over time in early diffuse cutaneous SSc.
- Immune cell and fibroblast gene expression signatures were predictive of longitudinal modified Rodnan Skin Score (mRSS), but were not independently predictive after adjustment for baseline mRSS.

How might this impact on clinical practice or future developments?

- The relationships of immune cell and fibroblast gene expression signatures with skin thickness and the trend towards normalisation over time in early diffuse cutaneous SSc can inform clinical trial design and treatment strategies in SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a multisystem autoimmune and fibrotic disease associated with high morbidity and mortality.[1] Several studies have shown dysregulated skin gene expression in SSc, with a common theme of molecular heterogeneity.[2–11] Variable degrees of immune cell and fibroblast activity have been observed in the skin of a large percentage of SSc patients, while a subgroup of patients' skin gene expression profiles resemble those of healthy controls (HCs).

Clinical trial design and individual patient management decisions in SSc are hampered by incomplete understanding of pathogenesis and variability in clinical progression and treatment responses. Recent studies have demonstrated

prognostic significance of the baseline modified Rodnan Skin Score (mRSS)[12 13] and disease duration[12–14] for future mRSS progression, with one study also showing independent predictive significance of RNA Polymerase III antibody.[13] There have also been reports that skin gene expression might predict mRSS progression[8 15] or response to immunosuppressive therapy.[16–18]

Three areas of uncertainty regarding SSc skin gene expression are: (1) the relationships between skin gene expression and clinical disease features such as mRSS, disease duration and autoantibodies, (2) the predictive significance of skin gene expression beyond the information provided by these clinical data and (3) the extent to which skin gene expression changes over time within individual patients. To address these questions we investigated longitudinal skin gene expression profiles, and their relationships to clinical disease features, in a large and diverse group of SSc patients.

METHODS

Patients and HC subjects

A total of 113 SSc patients were recruited from the Genetics vs Environment in Scleroderma Outcomes Study (GENISOS) cohort in Houston, Texas, USA, along with 44 local matched HCs. Patients fulfilled the 2013 ACR/EULAR SSc classification criteria,[19] and underwent initial skin biopsy within 6 years of disease onset (defined as the first non-Raynaud's symptom characteristic of SSc). One hundred and five SSc patients underwent a second biopsy (mean of 0.8 years after initial biopsy), 76 underwent a third biopsy (mean of 1.9 years after initial biopsy), and one underwent a fourth biopsy. mRSS, skin score at the biopsy site and immunosuppressant use were recorded at each biopsy, and most SSc patients had interval mRSS measurements between and after skin biopsies. Classification of diffuse or limited cutaneous involvement was based on skin involvement at the time of initial biopsy. Of note, 3 out of 43 SSc patients with limited cutaneous involvement at the time of initial biopsy subsequently developed diffuse cutaneous involvement at the time of a follow-up biopsy. Autoantibodies were determined in the laboratory of the UT Health Science Center Houston Rheumatology Division (described in online supplemental methods).

Skin biopsy and analyses

Two 3 mm punch biopsies were obtained from the forearm a few millimetres apart. One was used for RNA extraction, one fixed in formalin for immunohistochemical (IHC) staining. For longitudinal biopsies, the same forearm was sampled one inch from the original biopsy. Details regarding gene expression analyses and IHC staining are described in online supplemental methods.

Analysis of cell type-specific expression

Cell type-specific gene expression analysis was performed using the method we have used previously,[6 10 20] with 15 skin-associated cell types. To identify cell type-specific genes, linear models with moderated t-statistics were used to compare expression in each cell type to the other 14 cell types (R package: limma).[21] The 250 genes with lowest p values and increased expression in the target cell type were identified, and of these, we selected the 125 genes with highest fold-change (target cell type/14 other cell types). This provided a ranked set of 125 cell type-specific signature genes for each of the 15 cell types, which were used to calculate signature scores for SSc patient biopsies. For each gene, the average expression difference between SSc and HC samples was estimated, yielding $\log_2$-scaled fold-change estimates. For a given cell type, the signature score was equal to the (weighted) average $\log_2$-scaled fold-change estimate (SSc/HC) among the 125 signature genes. The average was calculated with greater weight assigned to those signature genes more strongly elevated in the target cell type as compared with the other 14 cell types. For a given patient and cell type, a signature score was significantly elevated if fold-change estimates for the 125 signature genes were higher than those of non-signature genes ($p<0.05$, Wilcoxon rank sum test).

Statistical analyses

Associations between cell type signatures and clinical features in SSc patients were analysed by Spearman's rank order correlation. Changes among continuous variables over time were analysed by mixed effects linear regression modelling. Models predicting longitudinal mRSS course based on initial cell type signature included all mRSS values up to 2 years after the initial biopsy. Principal component analyses (PCA) were performed using either all genes differentially expressed in SSc compared with HC (false discovery rate, FDR <0.05) or only those differentially expressed genes (DEGs) with a fold-change >1.5 or <0.67 in SSc compared with HC. Additional details are provided in online supplemental methods.

RESULTS

Demographics

Demographics and clinical features of HCs and SSc patients at the time of initial, second and third biopsies are shown in table 1. Online supplemental table 1 shows demographics and clinical features of diffuse cutaneous and limited cutaneous SSc (lcSSc) patients separately. The majority of patients had declining or stable mRSS over time after initial biopsy, while some had mRSS increases (online supplemental table 2).

Skin gene expression profiles of SSc patients at initial biopsy compared with HCs

A total of 2157 genes were significantly differentially expressed between SSc patients and HCs (1429 upregulated, 728 downregulated) using a FDR cut-off of 0.05 (figure 1A). Lists of DEGs are included in online supplemental data file (https://go.uth.edu/scleroderma-data). The most over-represented canonical pathways in SSc skin based on Ingenuity Pathway Analysis were hepatic fibrosis, agranulocyte adhesion and diapedesis, and granulocyte adhesion and diapedesis (figure 1B). The top upregulated transcriptional regulators were STAT1, IRF7 and IRF1, while the top upregulated cytokines/growth factors were transforming growth factor β1, interferon (IFN)α2 and IFNγ. These results were similar to those of a non-overlapping group of previously biopsied SSc patients in GENISOS.[6]

Similar to our prior findings,[6] 4314 genes were differentially expressed in SSc patients with affected forearm skin compared with HCs, while only 29 genes were differentially expressed in SSc patients with unaffected forearm skin (local skin score of 0) compared with HCs (figure 1C). A total of 1933 genes were differentially expressed between patients whose biopsies were from affected skin compared with those with biopsies from unaffected skin. A total of 4202 genes were differentially expressed in diffuse cutaneous SSc (dcSSc) patients compared with HCs, while only 57 genes were differentially expressed in lcSSc patients compared with HCs. 3389 genes were differentially expressed between dcSSc and lcSSc patients (figure 1C). In a sensitivity analysis using a less stringent FDR cut-off of 0.1, results were similar (online supplemental figure 1).

Table 1 Demographics and clinical characteristics of SSc patients and matched HCs

Characteristic	HC (n=44)	SSc initial biopsy (n=113)	SSc second biopsy (n=105)	SSc third biopsy (n=76)
Age (years) at biopsy, mean (SD)	50.4 (11.7)	48.9 (13.3)	50.0 (13.4)	50.9 (13.5)
Race/ethnicity, n (%)				
White	25 (56.8)	69 (61.1)	65 (61.9)	48 (63.2)
Black	11 (25.0)	17 (15.0)	15 (14.3)	12 (15.8)
Hispanic	6 (13.6)	20 (17.7)	18 (17.1)	10 (13.2)
Asian	2 (4.5)	7 (6.2)	7 (6.7)	6 (7.9)
Female, n (%)	32 (72.7)	89 (78.8)	83 (79.0)	64 (84.2)
Disease duration in years, mean (SD)		2.6 (1.4)	3.4 (1.5)	4.3 (1.5)
time since initial biopsy in years, mean (SD)			0.8 (0.4)	1.9 (0.5)
Diffuse skin involvement, n (%)		70 (61.9)	64 (61.0)	46 (60.5)
mRSS, mean (SD)		16.9 (11.8)	15.4 (11.8)	12.2 (10.0)
Local skin score, mean (SD)		1.2 (1.1)	1.0 (1.0)	0.9 (1.0)
Biopsy from clinically affected skin, n (%)		75 (66.4)	61 (58.1)	39 (51.3)
SSc-associated autoantibody, n (%)				
Anti-topoisomerase I		20 (17.7)	19 (18.1)	12 (15.8)
Anti-RNA polymerase III		23 (20.4)	21 (20.0)	15 (19.7)
Anti-centromere		15 (13.3)	14 (13.3)	11 (14.5)
Mycophenolate, n (%)		28 (24.8)	38 (36.2)	32 (42.1)
Methotrexate, n (%)		23 (20.4)	22 (21.0)	15 (19.7)
Other immunosuppressant, n (%)*		6 (5.3)	6 (5.7)	5 (6.6)

*Azathioprine, cyclophosphamide or biologic.
HC, healthy control; mRSS, modified Rodnan Skin Score; SSc, systemic sclerosis.

In a PCA based on genes that were differentially expressed by >1.5-fold or <0.67-fold in SSc compared with HC, biopsies from affected skin mostly clustered apart from HC skin, whereas biopsies from unaffected skin clustered nearer to HC (figure 1D). Similarly, biopsies from dcSSc clustered apart from HC, whereas biopsies from lcSSc clustered nearer to HC.

Of note, there were 925 DEGs between patients with early dcSSc (within 3 years of disease onset) compared with later dcSSc (>3 years since disease onset). In the PCA, early dcSSc clustered farther from HC skin than later dcSSc.

PCA results were similar when all DEGs were analysed (online supplemental figure 2).

Immune cell and fibroblast signatures and their relationships to clinical disease features at initial biopsy

Cell type-specific analysis revealed that 67% of patients had upregulation of the fibroblast signature compared with HCs (figure 2). Fifty-nine per cent and 51% of SSc patients had elevated M1 and M2 macrophage signatures, respectively, while a minority of SSc patients had adaptive immune cell signatures. Consistent with our prior studies,[6 10] the highest fibroblast signatures were observed in patients with elevated immune cell signatures, suggesting co-occurrence of immune cell activity and fibroblast dysregulation.

Immune cell and fibroblast signatures were higher in SSc patients with affected skin compared with those with unaffected skin (figure 2 and online supplemental table 3), and in patients with higher overall mRSS (figure 2). Consistently, there were significant positive associations of immune cell and fibroblast signatures with overall mRSS and local skin score at the biopsy site (table 2).

Immune cell and fibroblast signatures correlated inversely with disease duration, particularly in patients with diffuse cutaneous involvement in which M1 macrophage, M2 macrophage and fibroblast signatures showed statistical significance (online supplemental table 4). Consistently, these signatures were significantly higher in early dcSSc patients compared with later dcSSc patients (online supplemental table 5).

We previously observed associations between skin immune cell signatures and skin thickness progression rate (STPR) prior to initial biopsy in early dcSSc patients.[10] In the current study, there were not significant associations between STPR and immune cell signatures when analysing all dcSSc patients of up to 3 years duration. However, when restricting the analysis to dcSSc patients of <18 months duration (reasoning that many patients have a peak in mRSS by this time,[14] there were significant positive associations between immune cell signatures and STPR (online supplemental table 6). This finding suggests that the relationships between skin immune cell activity and STPR predominates early in dcSSc, prior to or around the time in which patients have a peak in mRSS.

Patients positive for RNA Pol III antibody had higher immune cell and fibroblast signatures than those positive for Topoisomerase-I or Centromere antibodies or negative for all three antibodies (online supplemental table 7). Most of the differences were only statistically significant comparing Centromere to RNA Pol III, but the fibroblast signature was significantly higher in the RNA Pol III patients compared with all of the other subgroups. However, we noticed that RNA Pol III patients had the shortest disease duration at initial biopsy, which might have a confounding effect. After adjustment for disease duration, there was no significant difference between fibroblast signatures in RNA Pol III vs Topoisomerase-I patients, while there remained significant differences between RNA Pol III patients and those with Centromere antibody or negative for all three (online supplemental table 8).

Without adjustment for other variables, immune cell and fibroblast signatures tended to be higher in patients taking immunosuppression compared with those that were not (online supplemental table 9). This finding is likely explained by the fact

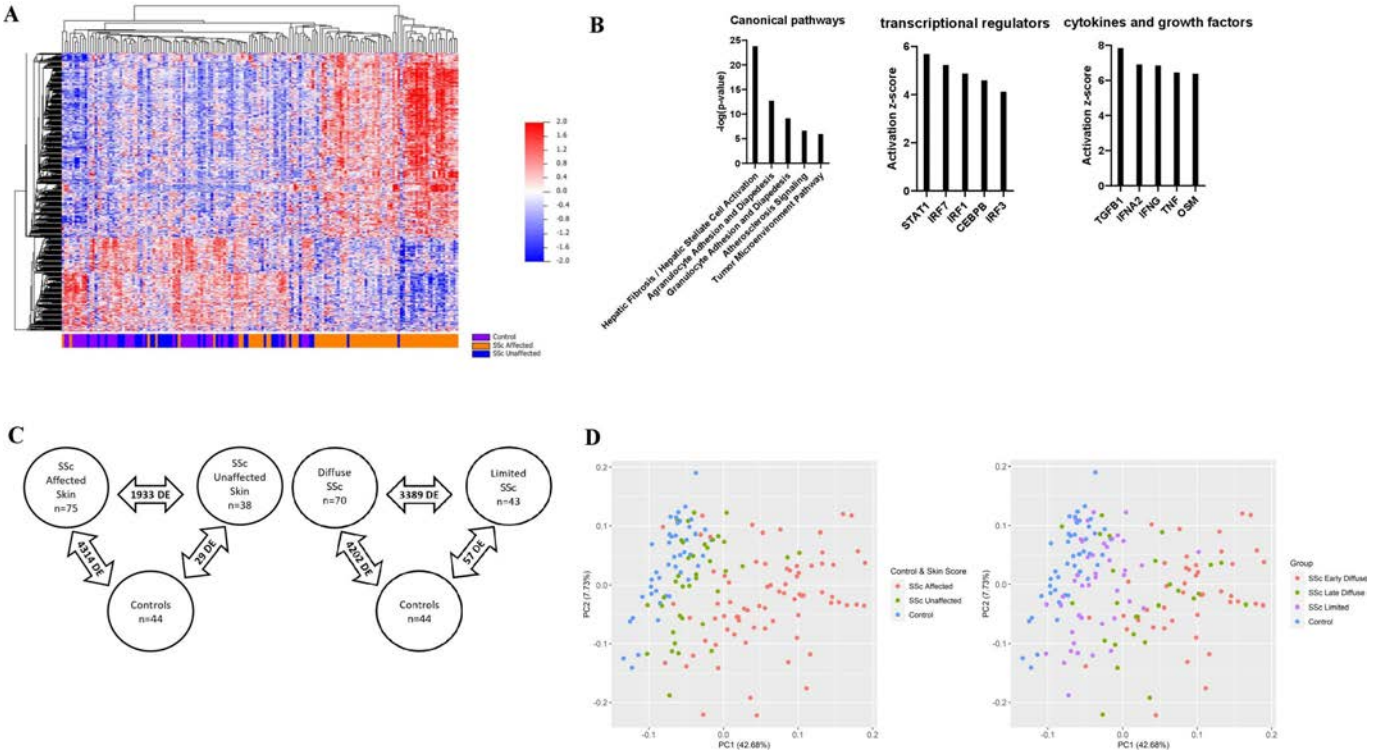

Figure 1 Differentially expressed genes in SSc initial biopsies compared with HCs. (A) Heat map of differentially expressed genes. (B) Top five overrepresented pathways (left), predicted upstream transcriptional regulators (middle) and predicted upstream cytokines and growth factors (right) in SSc compared with HC as determined by ingenuity pathway analysis of differentially expressed genes. (C) Numbers of differentially expressed genes (FDR <0.05 in SAM analysis) in SSc patients with affected skin at the biopsy site compared with those with unaffected skin at the biopsy site and HCs (left) or between dcSSc, lcSSc and HCs (right). (D) Principal component analysis of genes differentially expressed by >1.5 fold or <0.67 fold in SSc compared with HC, highlighting controls, SSc with unaffected skin, and SSc with affected skin (left) or highlighting controls, limited cutaneous SSc, early diffuse cutaneous SSc (defined here as within 3 years of disease onset), or late diffuse cutaneous SSc (defined here as more than 3 years since disease onset). dcSSc, diffuse cutaneous SSc; FDR, false discovery rate; HC, healthy control; lcSSc, limited cutaneous SSc; SSc, systemic sclerosis; TNF, tumour necrosis factor.

that immunosuppression use was more prevalent in patients with diffuse cutaneous involvement, who also had higher immune cell and fibroblast signatures. After adjustment for mRSS and disease duration, there were no significant relationships between immunosuppression use and baseline immune cell or fibroblast signatures (online supplemental table 10).

Prediction of longitudinal mRSS by baseline immune cell and fibroblast signatures

Mixed effects linear regression modelling of follow-up mRSS as a function of baseline cell type signature score showed that elevated immune cell and fibroblast signatures were predictive of higher subsequent longitudinal mRSS. Considering that cell type signatures correlated with the baseline mRSS, we next investigated whether the predictive significance of cell type signatures was independent of baseline mRSS. After adjustment for baseline mRSS, the cell type signatures were not independently predictive of subsequent longitudinal mRSS measurements (table 3).

Trend towards normalisation of skin gene expression over time in early dcSSc

Consistent with our cross-sectional results, longitudinally obtained immune cell and fibroblast signatures correlated positively with the concurrently obtained longitudinal mRSS measurements (online supplemental table 11).

We analysed changes in skin gene expression in longitudinally collected biopsies using mixed effects linear regression models of cell type signatures as a function of time since initial biopsy. In the SSc cohort as a whole, there were no significant changes in immune cell or fibroblast signatures over time (online supplemental table 12). However, when disease classification (limited vs diffuse) was included in the models as an interaction term with time since initial biopsy, significant interactions were observed, with immune cell and fibroblast signatures declining over time in dcSSc (online supplemental table 13). Looking at interaction between autoantibody and time since initial biopsy, more decline was observed in immune cell and fibroblast signatures over time among RNA Pol III patients compared with other autoantibody subsets, although only a few of these interactions were statistically significant (online supplemental table 14). No significant interaction was observed between immunosuppression use and time since initial biopsy (online supplemental table 15).

Given our findings that baseline immune cell and fibroblast signatures were highest in patients with diffuse skin involvement early in disease course, we performed a subanalysis of the 44 dcSSc patients of <3 years disease duration at initial biopsy. This subgroup is also of particular interest because a cut-off of 3 years disease duration has been an inclusion criterion for some clinical trials targeting skin disease in dcSSc.[18 22] Supporting our cross-sectional results, M1 macrophage, M2 macrophage, natural killer cell and fibroblast signatures significantly decreased over time in early dcSSc (table 4, online supplemental figure 3). T cell and B Cell signatures also

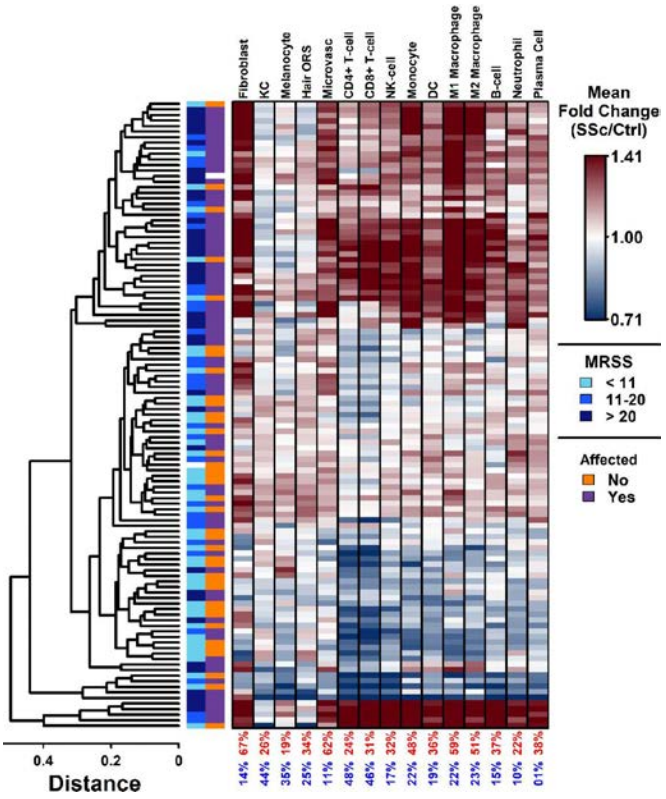

Figure 2 Cell type signatures in skin of SSc patients compared with HCs. Cell type signature scores for each SSc baseline sample (n=113). Scores represent the average fold-change (SSc/HC) for 125 cell type-specific signature genes (see methods). Bottom margin values indicate the percentage SSc biopsies with upregulated (red) and downregulated (blue) signatures compared with HCs. Patients were clustered based on signature scores (average linkage, Euclidean distance). The blue boxes to the left of the cell type signature scores indicate the mRSS, and the purple/orange boxes indicate affected (skin score of 1, 2 or 3) vs unaffected (skin score of 0) skin at the site of the biopsy, with legends at the right of the figure. White boxes indicate no skin scores recorded at the time of the biopsy. DC, dendritic cell; HC, healthy control; KC, keratinocyte; mRSS, modified Rodnan Skin Score; NK, natural killer; ORS, outer root sheet; SSc, systemic sclerosis.

tended to decline. By contrast, keratinocyte and hair outer root sheet signatures tended to increase over time. Among these 44 patients, no significant interactions were observed between autoantibody or immunosuppression with time since initial biopsy (data not shown), which might be due to further sample partitioning in this subgroup analysis.

Table 2 Associations of immune cell and fibroblast signatures with skin thickness scores in SSc initial biopsies

	mRSS	Local Skin Score
M1 macrophage	0.51 (<0.001)	0.46 (<0.001)
M2 macrophage	0.48 (<0.001)	0.42 (<0.001)
CD4 cell	0.38 (<0.001)	0.35 (<0.001)
CD8 cell	0.40 (<0.001)	0.38 (<0.001)
B cell	0.31 (<0.001)	0.33 (<0.001)
NK cell	0.41 (<0.001)	0.41 (<0.001)
Fibroblast	0.50 (<0.001)	0.42 (<0.001)

Spearman's rank order correlation coefficient (p value in parentheses).
mRSS, modified Rodnan Skin Score; NK, natural killer; SSc, systemic sclerosis.

To assess change in global dysregulation of skin gene expression over time in early dcSSc, we performed PCA of the aforementioned SSc transcript signature (genes that were differentially expressed in SSc compared with HC) in initial and third biopsies from early dcSSc patients. While both groups of biopsies from SSc patients (initial and follow-up) clustered separately from HC, follow-up SSc biopsies clustered nearer to HC than did initial SSc biopsies (figure 3 and online supplemental figure 4).

Decline in immune cell numbers from initial to follow-up SSc biopsies on histology

To further test the hypothesis that skin immune cell activity tends to decline over time in early dcSSc, we performed IHC staining of CD3 and CD68 in initial and follow-up biopsies from 17 dcSSc patients with <2 years disease duration at initial biopsy (patient characteristics are shown in online supplemental table 16. As previously shown,[10] CD3-positive cell counts based on IHC positively correlated with T cell gene expression signatures, and CD68-positive cell counts based on IHC positively correlated with macrophage gene expression signatures (online supplemental figure 5). In paired analyses, CD3-positive and CD68-positive cell counts declined on average from initial to follow-up biopsies, although the value for CD68 was not statistically significant. Looking at the cell counts categorically as high (at or above the baseline median value) or low (below the baseline median value), six out of nine SSc patients (66.7%) with high baseline CD3-positive cell counts had declines to below the median at follow-up, whereas only one out of eight patients (12.5%) with a low baseline CD3-positive cell count had an increase to above the median at follow-up. The same trend was observed for CD68-positive cells (figure 4).

DISCUSSION

Working with a diverse, well-characterised cohort of SSc patients, we have performed to our knowledge the largest longitudinal skin gene expression study in SSc. The large sample size, coupled to close clinical follow-up, allowed for retesting of hypotheses regarding relationships of skin gene expression to clinical disease features, assessment of baseline skin gene expression as a predictor of subsequent mRSS, and within-patient change in skin gene expression over time.

We found a greater extent of dysregulated skin gene expression in patients with affected skin at the biopsy site compared with those with unaffected skin, and positive associations of immune cell and fibroblast signatures with skin thickness scores. These associations confirm our prior cross-sectional results,[6 10] which are also consistent with the analogous finding that higher mRSS was associated with inflammatory skin gene expression in other SSc cohorts.[17 23] Our results solidify the hypothesis that the severity of skin involvement in SSc is related to dysregulated skin gene expression generally, and more specifically to inflammatory and fibrotic gene expression profiles.

One important area of uncertainty in SSc is the extent to which skin gene expression changes over the course of disease, particularly in patients with severe skin involvement. Several prior cross-sectional studies suggest that the extent of dysregulated skin gene expression is greater early in the course of dcSSc compared with later disease. Specifically, patients with later-stage SSc tended to have a normal-like skin gene expression profile compared with those with early-stage SSc,[6] immune cell signatures were higher in early compared with later-stage dcSSc,[10] inflammatory skin gene expression was associated with shorter disease duration in a compendium of datasets from multiple

Table 3 Predictive significance of baseline cell type signature for subsequent longitudinal mRSS

	Without adjustment			With adjustment for baseline mRSS		
	Coefficient	95% CI	P value	Coefficient	95% CI	P value
M1 macrophage	10.2	6.3 to 14.0	**<0.01**	0.2	−1.8 to 2.1	0.86
M2 macrophage	13.0	7.6 to 18.5	**<0.01**	0.4	−2.2 to 3.0	0.75
CD4 cell	11.3	5.1 to 17.5	**<0.01**	0.9	−1.8 to 3.6	0.51
CD8 cell	8.8	3.9 to 13.7	**<0.01**	0.4	−1.8 to 2.5	0.74
B cell	12.8	5.8 to 19.7	**<0.01**	1.3	−1.7 to 4.3	0.40
NK cell	11.7	5.4 to 18.1	**<0.01**	0.5	−2.2 to 3.3	0.70
Fibroblast	12.7	7.9 to 17.6	**<0.01**	1.4	−1.0 to 3.7	0.27

p values <0.05 are in bold.
mRSS, modified Rodnan skin score; NK, natural killer.

cohorts,[9] and the inflammatory intrinsic subset was associated with shorter disease duration in dcSSc patients.[17 23] Moreover, PCA of dysregulated genes showed some separation of skin biopsies from early compared with later dcSSc patients, with later dcSSc trending closer to HC and lcSSc.[11] Consistent with these cross-sectional analyses, a tendency towards less inflammatory and/or more normal-like skin gene expression has been observed in some longitudinally biopsied dcSSc patients in observational cohorts[10 11 16 17] and clinical trials.[9 23] In the current study, our cross-sectional findings redemonstrate greater skin gene expression dysregulation in early compared with later dcSSc, and higher immune cell and fibroblast signatures associated with shorter disease duration. In direct support of the hypothesis that skin gene expression changes towards a less inflammatory, more normal-like state over time in early dcSSc, we observed decline in immune cell and fibroblast gene expression signatures and reduced overall dysregulation of gene expression in follow-up compared with initial biopsies in a large group of early dcSSc patients. We acknowledge that these findings contrast with previous reports in which significant gene expression changes were not found in SSc patients who underwent serial biopsies during a clinical trial of Rituximab[24] or in PCA of longitudinal biopsies in an observational cohort.[11] We believe that our study was better suited to detect changes in gene expression over time for three reasons: (1) We included a larger sample size, (2) Our study included biopsies taken 1–2 years after initial biopsy (in contrast to 1 year or less for most of the longitudinal biopsies in the aforementioned studies), allowing more time for gene expression to change and (3) We used a narrower definition of early dcSSc (within 3 years of onset) compared with Clark *et al*[11] (within 5 years of onset); this difference is noteworthy because some patients appear to have already undergone a plateau and decline in inflammatory skin gene expression in the first 3 years. Taken together with the aforementioned cross-sectional data, our longitudinal data indicate that inflammatory and fibrotic skin gene expression tends to peak early in dcSSc, then to decline towards a more normal-like state over time.

Table 4 Change in cell type signature over time in early diffuse cutaneous SSc patients

	Coefficient	95% CI	P value
M1 macrophage	−0.09	−0.16 to −0.02	**0.02**
M2 macrophage	−0.07	−0.12 to −0.02	**0.01**
CD4 T cell	−0.03	−0.08 to 0.02	0.19
CD8 T cell	−0.05	−0.12 to 0.01	0.09
B cell	−0.04	−0.08 to 0.02	0.12
NK cell	−0.05	−0.10 to −0.001	**0.046**
Fibroblast	−0.06	−0.11 to −0.01	**0.02**
Keratinocyte	0.03	−0.003 to 0.06	0.08
Melanocyte	0.01	−0.02 to 0.04	0.42
Hair ORS	0.03	−0.01 to 0.06	0.12
Microvasc	−0.04	−0.08 to −0.01	**0.01**
Monocyte	−0.07	−0.13 to −0.004	**0.04**
Dendritic cell	−0.03	−0.07 to 0.02	0.21
Neutrophil	−0.03	−0.07 to 0.01	0.19
Plasma cell	−0.03	−0.06 to 0.004	0.09

p values <0.05 are in bold.
NK, natural killer; ORS, outer root sheet; SSc, systemic sclerosis.

A peak of inflammatory and fibrotic gene expression early in the course of dcSSc is consistent with the clinical finding that mRSS often reaches a plateau before the 3 years mark,[13 14] and would support the premise of enriching or stratifying for early disease in clinical trials targeting inflammatory pathways.

Prediction of mRSS trajectory using clinical and/or molecular data has the potential to improve patient selection for clinical trials and to aid individual patient management decisions. We found that baseline immune cell and fibroblast signatures were predictive of longitudinal mRSS over time, but did not have independent predictive significance after adjustment for baseline mRSS. This is not surprising in light of the relationship between skin gene expression and mRSS, and the reproducible finding that the baseline mRSS itself has predictive significance for mRSS trajectory over time.[12 13] The relationship between skin gene expression and mRSS warrants attention in efforts to use skin gene expression profiles as prognostic or predictive tools. It would make sense to judge skin gene expression-based predictors of skin trajectory by the value they add to the readily available clinical metric of baseline mRSS.

Among autoantibody subgroups, RNA Pol III was associated with the highest immune cell and fibroblast signatures at initial biopsy, and a tendency for these signatures to decline more rapidly over time. These findings are consistent with recent literature suggesting a difference in gene expression profiles related to autoantibody subtype,[11 25] and also suggest a possible gene expression correlate of the clinical finding that RNA Pol III is associated with a more rapid, severe peak in mRSS followed by more rapid decline over time.[13] However, we note that some of the differences observed between autoantibody subgroups were modest and might have been influenced by differing disease durations at initial biopsy. Validation of differing skin gene expression profiles and trajectories by autoantibody subgroup will require further study with larger sample sizes.

Strengths of this study include the large sample size of longitudinal biopsies and close clinical follow-up. Skin scoring over time was performed by the same experienced SSc expert (MDM and SA), avoiding inter-rater variability in this outcome measure. IHC staining of immune cell markers in concurrently

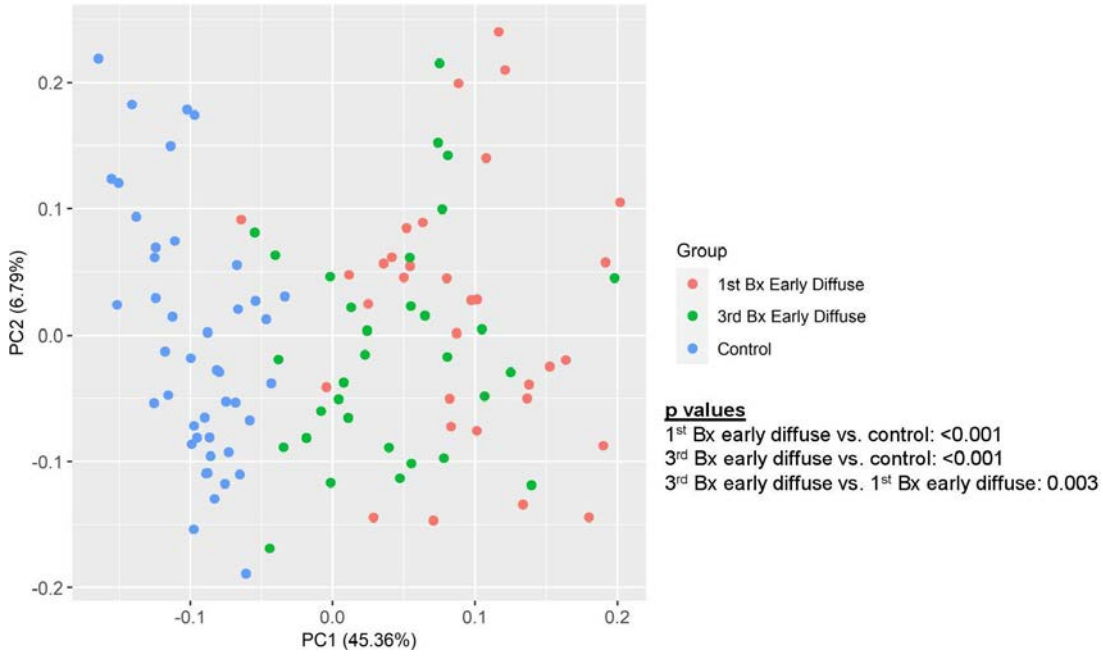

Figure 3 Comparison of dysregulated gene expression in follow-up versus initial biopsies from early diffuse cutaneous SSc. PC analysis of genes differentially expressed by >1.5 fold or <0.67 fold in SSc compared with HC, highlighting controls and initial versus third biopsies from early dcSSc patients. P values for comparison between groups were determined as described in online supplemental methods. Bx, biopsy; dcSSc, diffuse cutaneous SSc; HC, healthy control; PC, principal component; SSc, systemic sclerosis.

collected skin samples provided additional support for the gene expression-based results.

This study has limitations. As is typical of observational studies, conclusions about the effects of medications are inherently limited by the lack of randomised treatment assignments. As with all gene expression studies examining bulk tissue samples, conclusions about cell type-specific gene expression profiles are based on inferences from prior gene expression datasets. Single cell analyses are beginning to provide a more granular understanding of the cellular composition and cell-specific gene expression profiles in SSc skin.[26–28] The relatively low prevalence of anti-centromere antibody in this study may

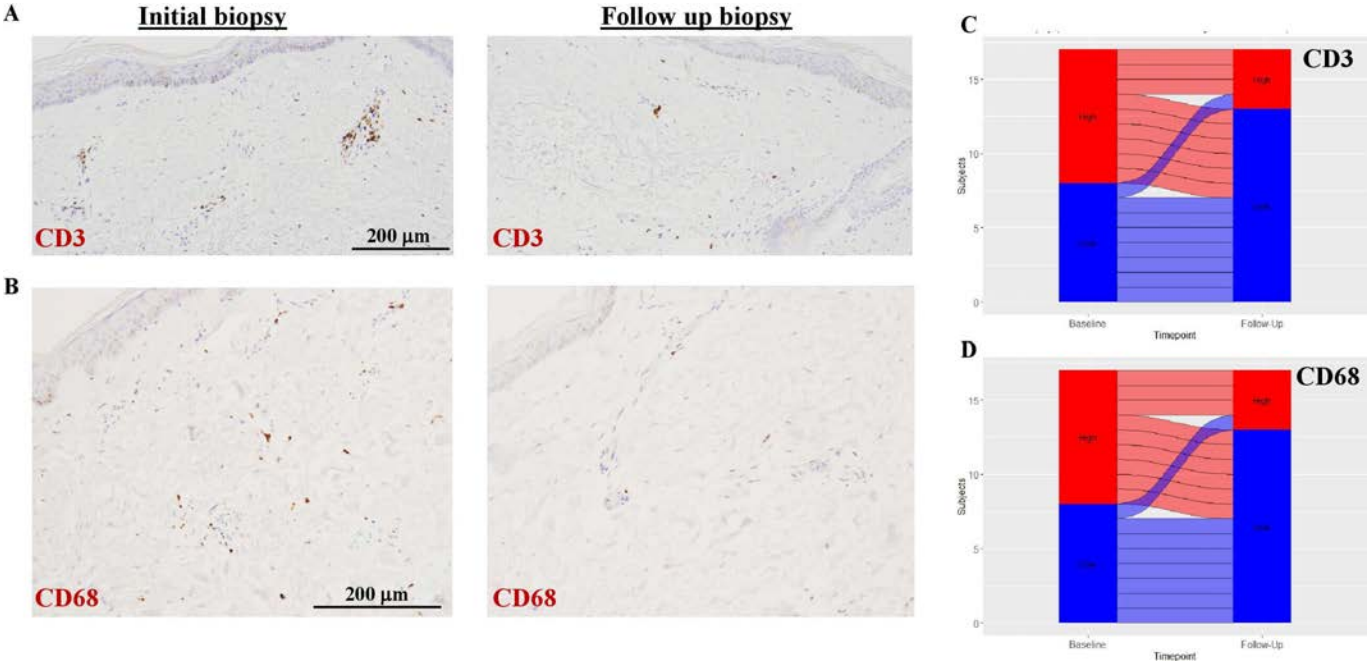

Figure 4 Immunohistochemical staining of CD3 and CD68 in initial and follow-up skin biopsies from early diffuse cutaneous SSc. Representative images of CD3 (A) and CD68 (B) in initial and follow-up biopsies from an early dcSSc patient. (C) Flow diagram of initial and follow-up skin biopsies from 17 early dcSSc patients, with red representing samples with CD3-positive cell counts equal to or greater than the baseline median value (high), and blue representing cell counts less than the baseline median value (low). Each row represents one patient. (D) Same as (C), but for CD68-positive cell counts. dcSSc, diffuse cutaneous SSc; SSc, systemic sclerosis.

reflect a referral bias, in which SSc patients with severe skin or internal organ involvement are more likely to be referred to our scleroderma specialty clinic than centromere positive patients; this might affect the generalisability of our results.

In conclusion, our findings show relationships of immune cell and fibroblast gene expression profiles with the severity of skin disease in SSc and demonstrate a trend towards normalisation of skin gene expression over time within early dcSSc patients. These results can inform treatment strategies and clinical trial design in SSc.

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Contributors SA, BS, MAL, MDM and GS designed the study. All authors were involved in analysis and interpretation of the data. All authors were involved in manuscript preparation and approved the final version of the manuscript. SA was responsible for the overall content as guarantor and accepts full responsiblity for the conduct of the study, had access to the data, and controlled the decision to publish.

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Competing interests MDM reports support from Mitsubishi-Tanabe, Boehringer Ingelheim, EICOS and Corbus to her institution outside the submitted work, and fees from Medtelligence, Actelion Pharma, Mitsubishi-Tanabe, Boehringer Ingelheim and EICOS to her outside the submitted work. SA reports grant support from Momenta, Boehringer Ingelheim, Janssen, and the Scleroderma Research Foundation to his institution outside the submitted work; consulting fees from Boehringer Ingelheim, Novartis, Corbus, CSL Behring, Abbvie, AstraZeneca to him outside the submitted work; honorarium from Boehringer Ingelheim for lectures and payment from the American College of Rheumatology to him outside the submitted work. BS, MAL, WRS, GS, MW, TMT, JC and CPV have no relevant competing interests to declare.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by UT Health Science Center at Houston, Institutional Review Board, Committee for the Protection of Human Subjects. Protocol#: HSC-MS-06-0063. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Gene expression, demographic and clinical data were uploaded to NCBI's Gene Expression Omnibus (GEO), accession number GSE181549. The data can be accessed using the token: mdqtwqkqldwtjml.

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TRANSLATIONAL SCIENCE

Blocking GM-CSF receptor α with mavrilimumab reduces infiltrating cells, pro-inflammatory markers and neoangiogenesis in ex vivo cultured arteries from patients with giant cell arteritis

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ABSTRACT

Background Effective and safe therapies are needed for the treatment of patients with giant cell arteritis (GCA). Emerging as a key cytokine in inflammation, granulocyte-macrophage colony stimulating factor (GM-CSF) may play a role in promoting inflammation in GCA.

Objectives To investigate expression of GM-CSF and its receptor in arterial lesions from patients with GCA. To analyse activation of GM-CSF receptor-associated signalling pathways and expression of target genes. To evaluate the effects of blocking GM-CSF receptor α with mavrilimumab in ex vivo cultured arteries from patients with GCA.

Methods Quantitative real time PCR, in situ RNA hybridisation, immunohistochemistry, immunofluorescence and confocal microscopy, immunoassay, western blot and ex vivo temporal artery culture.

Results GM-CSF and GM-CSF receptor α mRNA and protein were increased in GCA lesions; enhanced JAK2/STAT5A expression/phosphorylation as well as increased expression of target genes CD83 and Spi1/PU.1 were observed. Treatment of ex vivo cultured GCA arteries with mavrilimumab resulted in decreased transcripts of CD3ε, CD20, CD14 and CD16 cell markers, and reduction of infiltrating CD16 and CD3ε cells was observed by immunofluorescence. Mavrilimumab reduced expression of molecules relevant to T cell activation (human leukocyte antigen-DR [HLA-DR]) and Th1 differentiation (interferon-γ), the pro-inflammatory cytokines: interleukin 6 (IL-6), tumour necrosis factor α (TNFα) and IL-1β, as well as molecules related to vascular injury (matrix metalloprotease 9, lipid peroxidation products and inducible nitric oxide synthase [iNOS]). Mavrilimumab reduced CD34 + cells and neoangiogenesis in GCA lesions.

Conclusion The inhibitory effects of mavrilimumab on multiple steps in the GCA pathogenesis cascade in vitro are consistent with the clinical observation of reduced GCA flares in a phase 2 trial and support its development as a therapeutic option for patients with GCA.

Key messages

What is already known about this subject?

- GM-CSF transcripts were detected in temporal arteries from patients with giant cell arteritis (GCA) more than two decades ago.
- More recently, GM-CSF protein has been shown to be produced and secreted by peripheral blood mononuclear cells from patients with active GCA and detected in GCA-involved temporal arteries by immunohistochemistry.
- Expression of GM-CSF receptor and its functional role in GCA lesions has not been previously explored.

What does this study add?

- The study demonstrates expression of GM-CSF and its receptor in distinct cell subsets in GCA lesions.
- Moreover, GM-CSF receptor signalling is activated, and expression of typical target genes is increased.
- Exposure of ex-vivo cultured arteries to mavrilimumab reduces CD16 and CD3ε cell infiltration and reduces key molecules involved in T cell activation and differentiation, expression of pro-inflammatory cytokines, markers of vascular injury and neoangiogenesis.
- Taken together, these data point towards a relevant role of GM-CSF in the development of vascular inflammation and injury in GCA.

How might this impact on clinical practice or future developments?

- The clear impact of mavrilimumab on key steps in the pathogenesis of GCA supports its further development as a therapeutic option for patients with GCA.

INTRODUCTION

Giant cell arteritis (GCA) is a chronic inflammatory condition affecting large and medium arteries in individuals older than 50 years. Common manifestations include headache, scalp tenderness, polymyalgia rheumatica and systemic symptoms.[1 2] Inflammation-induced vascular remodelling results in ischaemic complications or aneurysms.[3]

High-dose glucocorticoids (GCs) dramatically improve symptoms of GCA, but relapses occur in 34%–75% of patients when GCs are tapered,[4–6]

leading to prolonged treatment and frequent GC-associated side effects.[7 8] Blocking the interleukin 6 (IL-6) receptor with tocilizumab (TCZ) demonstrated efficacy in reducing relapses, sparing GC,[9 10] and improving quality of life.[11] However, more than 40% of patients treated with TCZ are unable to maintain GC-free remission and about 60% of responders relapse on discontinuation,[12] indicating heterogeneity in response and underlining the need for alternative therapeutic options. TCZ also inhibits synthesis of acute-phase reactants, even without full suppression of disease activity, rendering their use unreliable for monitoring of disease flare.[13 14]

The search for additional therapeutic targets in GCA is hampered by the limited understanding of pathogenesis. Studies indicate that genetics, ageing and immune responses against unknown antigen(s) likely play a major role.[15 16] Dendritic cells activated by innate immune mechanisms may drive adaptive immunity by stimulating T lymphocytes and promoting their differentiation into Th1 and Th17 effector cells.[17–24] Concomitant and subsequent activation of macrophages amplifies inflammatory loops, leading to vascular injury and remodelling.[25–27]

GM-CSF is a pro-inflammatory cytokine produced by fibroblasts, epithelial, endothelial, myeloid and T cells on stimulation with other cytokines or pathogen-associated molecular pattern molecules.[28–30] GM-CSF has a seminal role in disease progression in animal models of inflammatory conditions.[28–30] GM-CSF receptor is composed of an alpha-chain conferring specificity and a signalling beta-chain shared with other cytokine receptors (IL-3, IL-5 and IL-34).[28–30] On GM-CSF binding, the receptor beta-chain predominantly signals through JAK2–STAT5 pathway. GM-CSF acts primarily on myeloid cells, promoting activation of dendritic cells and macrophages and differentiation of monocytes into dendritic cells, but other cell types may also respond.[28–30] GM-CSF mRNA has been detected in arterial lesions of GCA, and GM-CSF protein production by circulating peripheral blood mononuclear cells from GCA patients is increased compared with healthy controls.[22 24] According to its known biological functions, GM-CSF may have a role in promoting and amplifying vascular inflammation and injury in GCA.

Mavrilimumab is a fully human IgG4 monoclonal antibody able to neutralise GM-CSF effects by binding to the GM-CSF receptor alpha chain (GM-CSFRα).[31] In a phase 2b trial in patients with rheumatoid arthritis, mavrilimumab showed comparable efficacy to anti-TNFα blocker golimumab and superior efficacy compared with placebo, as well as a good safety profile.[32–34] The putative role of GM-CSF in critical steps of GCA pathogenesis suggests therapeutic potential for mavrilimumab in this disease, supported by a recent phase 2 trial.[35]

This study aimed to investigate the expression of GM-CSF and GM-CSFRα in inflamed arteries from patients with GCA, to detect activation of GM-CSFR-related signalling pathways and modulation of downstream gene expression, and to investigate the impact of GM-CSFRα blockade with mavrilimumab on inflammation in ex vivo cultured arteries from patients with GCA.

PATIENTS AND METHODS

Patients

The study investigated samples from four different patient groups according to the processing of their biospecimens (clinical characteristics of patients, controls and their samples: online supplemental table S1).

Temporal artery culture

Details have been previously described[36] and are available in online supplemental methods.

In situ RNA hybridisation

RNAScope (RS) (ACDbio, Abingdon, UK) in situ hybridisation was performed on formalin-fixed paraffin-embedded (FFPE) sections of GCA and control temporal artery biopsies to detect transcripts of specific genes, including GM-CSF, GM-CSFRα, CD83 and Spi1 (PU.1). After fixation and sectioning, tissue was permeabilised and probed with target-specific double Z probes specific to single target mRNA, and hybridisation signals were further amplified for detection. Visualised with a microscope, each red dot represents a single target mRNA molecule. Expression score was calculated as RS score (dots/cell) multiplied by positivity score (% cells positive with >1 dot/cell) (online supplemental table S2).

Candidate gene expression analysis

Candidate genes relevant to the immunopathogenesis of GCA were selected according to the current pathogenesis model[15 16] and known effects of GM-CSF in experimental systems.[28 29] Transcripts were detected by quantitative real-time PCR, details of RNA extraction, reverse transcription and fluorescence quantification are provided in the online supplemental methods (online supplemental table S3).

Immunohistochemistry

Two micrometre thick temporal artery sections from FFPE samples were used for immunohistochemistry. After 20-minute antigen retrieval with citrate buffer (pH 6), samples were immunostained with specific antibodies, using the Leica Microsystems' Bond-max automated immunostainer and the Bond Polymer Refine Detection System (Leica Microsystems), developed with diaminobenzidine and counterstained with haematoxylin (antibodies used, dilutions and optimised incubation times: online supplemental table S4-C). Positive and negative control tissues for protocol optimisation were selected from Human Protein Atlas (www.proteinatlas.org) and obtained from Institut d'Investigacions Biomèdiques August Pi i Sunyer Biobank.

Immunofluorescence

Immunofluorescence staining and imaging were performed with fresh-frozen or cultured temporal artery sections (online supplemental methods and online supplemental table S4-A).

Protein detection by western blot

Fresh-frozen temporal artery biopsies (TABs) from three patients with GCA and three controls were processed as described in online supplemental table S4-B.

Detection of proteins in the supernatants of cultured arteries and patient sera

Cytokines, chemokines or membrane-bound molecules released into artery culture supernatants were detected by immunoassay (online supplemental table S5).

Statistical analysis

Non-parametric Mann-Whitney U test and Wilcoxon matched-pairs signed rank test were used for unpaired and paired data analysis, respectively, using Graphpad Prism software.

RESULTS

GM-CSF and GM-CSFRα expression is increased in GCA lesions

GM-CSF and GM-CSFRα transcripts were increased in homogenised temporal artery biopsies from patients with GCA, whereas GM-CSF mRNA was virtually undetectable, and GM-CSFRα expression was very low in control arteries (figure 1A,B). Transcripts for GM-CSF or GM-CSFRα mRNA were clearly detectable by in situ RNA hybridisation in all arterial layers of GCA biopsies, whereas virtually no signal for either gene product was detectable in control arteries (figure 1C–E).

Immunostaining confirmed the presence of GM-CSF and GM-CSFRα protein on infiltrating inflammatory cells and endothelial cells in GCA arteries. In contrast, no GM-CSF protein and only low levels of GM-CSFRα protein were detected in control arteries (figure 1F,G).

Cell subsets potentially expressing GM-CSF and GM-CSFRα in GCA lesions were explored. As illustrated by immunofluorescence in figure 2, GM-CSF was mainly observed in macrophages and luminal endothelial cells and, to a lesser extent, in T cells, intimal myofibroblasts, and endothelial cells from vasa vasorum and neovessels. GM-CSFRα was detected mainly in macrophages, giant cells, endothelial cells and intimal myofibroblasts.

Serum GM-CSF concentration at diagnosis was 0.061±0.02 pg/mL (average±SEM) in patients with GCA and 0.035±0.02 pg/mL in controls (p=0.889).

GM-CSF receptor-driven signalling pathways are activated in GCA lesions, and expression of molecules regulated by this pathway is increased

After observing higher expression of GM-CSF and GM-CSFRα in GCA-involved arteries, signalling molecules downstream of GM-CSFR were examined. As shown in figure 3A,B and online supplemental figure S1, JAK2 and STAT5A, the main signalling proteins activated by GM-CSFR engagement, were phosphorylated in GCA lesions, and transcripts regulated by STAT5, such as Spi1 (PU.1) and CD83, were significantly increased in GCA arteries (figure 3C–G). CD83 and PU.1 protein, absent in controls, were clearly expressed in GCA arteries (figure 3H,I). PU.1 was detected in the nuclei, consistent with its function as transcription factor and suggestive of nuclear translocation on activation of upstream signalling. CD83 staining was more diffuse, possibly due to detection of its soluble form in addition to the membrane molecule.

GM-CSFRα inhibiting monoclonal antibody mavrilimumab reduces lymphocyte and myeloid cell markers in ex vivo cultured arteries from patients with GCA

To determine the contribution of GM-CSF to the above results and to assess the effects of GM-CSF pathway blockade on vascular inflammation, GCA arteries were cultured with anti-GM-CSFRα, mavrilimumab, for 5 days. Compared with placebo, treatment with mavrilimumab resulted in reduced phospho-STAT5 in lesions (figure 4A,B) and in lower mRNA expression of Spi1 (PU.1), a transcription factor that, along with STAT5, mediates GM-CSF effects (figure 4C).[28–30] Furthermore, treatment with mavrilimumab resulted in significantly lower mRNA levels for T cell marker CD3ε, B cell marker CD20, monocyte marker CD14 and myeloid cell marker CD16 mRNAs (figure 4D). By contrast, no consistent changes were observed with transcripts for the macrophage marker CD68. Accordingly, fewer CD16 + and CD3ε + infiltrating cells and no change in CD68 + cells were observed by immunofluorescence (figure 4F). The reduction in CD20 transcripts, however, did not result from decreased numbers of B cells in tissue during the duration of mavrilimumab exposure (figure 4E,F).

Mavrilimumab reduces expression of molecules involved in T cell activation and related to the Th1 differentiation pathway in ex vivo cultured arteries from patients with GCA

To further delineate the effects of mavrilimumab, expression of human leukocyte antigen-DR (HLA-DR) and CD83, relevant molecules to antigen presentation and T cell activation, was examined. Mavrilimumab significantly reduced HLA-DR and CD83 transcripts (figure 5A). Interestingly, concentration of the soluble, shed form of CD83, with counter-regulatory functions, did not decrease in the supernatant (figure 5A). HLA-DR reduction was also observed at the protein level (figure 5A).

To determine whether these effects resulted in decreased differentiation of T cells towards the Th1 or Th17 lineage, select markers were explored. Transcripts of master regulators of Th1 and Th17 differentiation, *TBX21* (T-bet) and *RORC* (RORγ), respectively, trended lower (figure 5B,C). Cytokines/chemokines related to Th1 differentiation pathway (interferon-γ (IFNγ) and CXCL10) trended lower (mRNA level) or were significantly lower (protein level) (figure 5B). IL-17A mRNA was virtually undetected in cultured arteries (data not shown), and IL-23p19 had disparate response among donors (figure 5C).

Mavrilimumab decreases pro-inflammatory cytokines in ex vivo cultured arteries from patients with GCA

Mavrilimumab elicited a significant reduction in the production and release of pro-inflammatory cytokines IL-6, TNFα and IL-1β, mostly but not exclusively produced by macrophages (figure 6A). Mavrilimumab also decreased markers of M2-like phenotype, including the mannose receptor CD206 and the scavenger receptor CD163 (figure 6B). A trend towards an increase in the anti-inflammatory cytokine IL-10 (mRNA and protein) was also observed (figure 6B).

Further supporting these results, recombinant human GM-CSF increased expression of the main transcripts decreased by mavrilimumab (online supplemental figure S2)

Mavrilimumab decreases mediators of vascular injury in ex vivo cultured arteries from patients with GCA

Mavrilimumab decreased transcript and protein concentrations of the elastinolytic matrix metalloprotease 9 (MMP-9), whereas mRNA and protein of its natural inhibitor tissue inhibitor of metalloproteinases 1 (TIMP-1) remained unchanged, resulting in a significant decrease in proteolytic MMP-9/TIMP-1 balance (figure 7A,B). Mavrilimumab also reduced oxidative damage, as demonstrated by decreased presence of lipid peroxidation products (4-hydroxynonenal (HNE) protein adducts) in cultured arteries exposed to mavrilimumab as compared with placebo (figure 7C). *NOS2* (inducible nitric oxide synthase [iNOS]) mRNA expression also trended lower (figure 7D).

Mavrilimumab reduces tissue angiogenesis in ex vivo cultured arteries from patients with GCA

Mavrilimumab reduced vascular endothelial growth factor A (VEGFA) mRNA in cultured arteries and VEGFA protein expression in tissue by immunofluorescence (figure 8A–C). However, no changes in VEGFA protein in the supernatant was observed, possibly due to its matrix-binding capacity and its autocrine/paracrine function.[37] Based on the reduction of this important angiogenic factor, we explored the effects of mavrilimumab on endothelial cell markers and angiogenesis. Mavrilimumab did

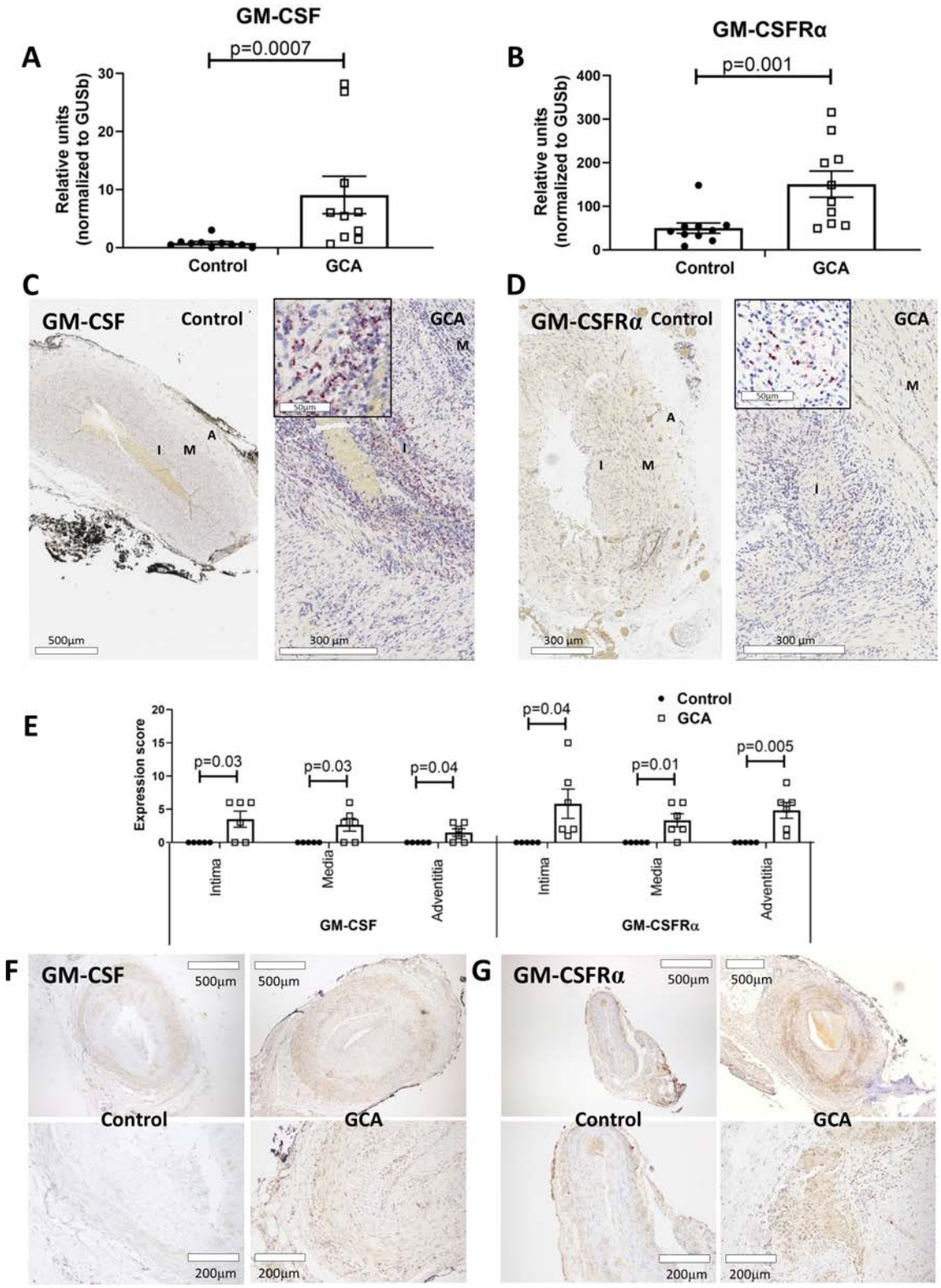
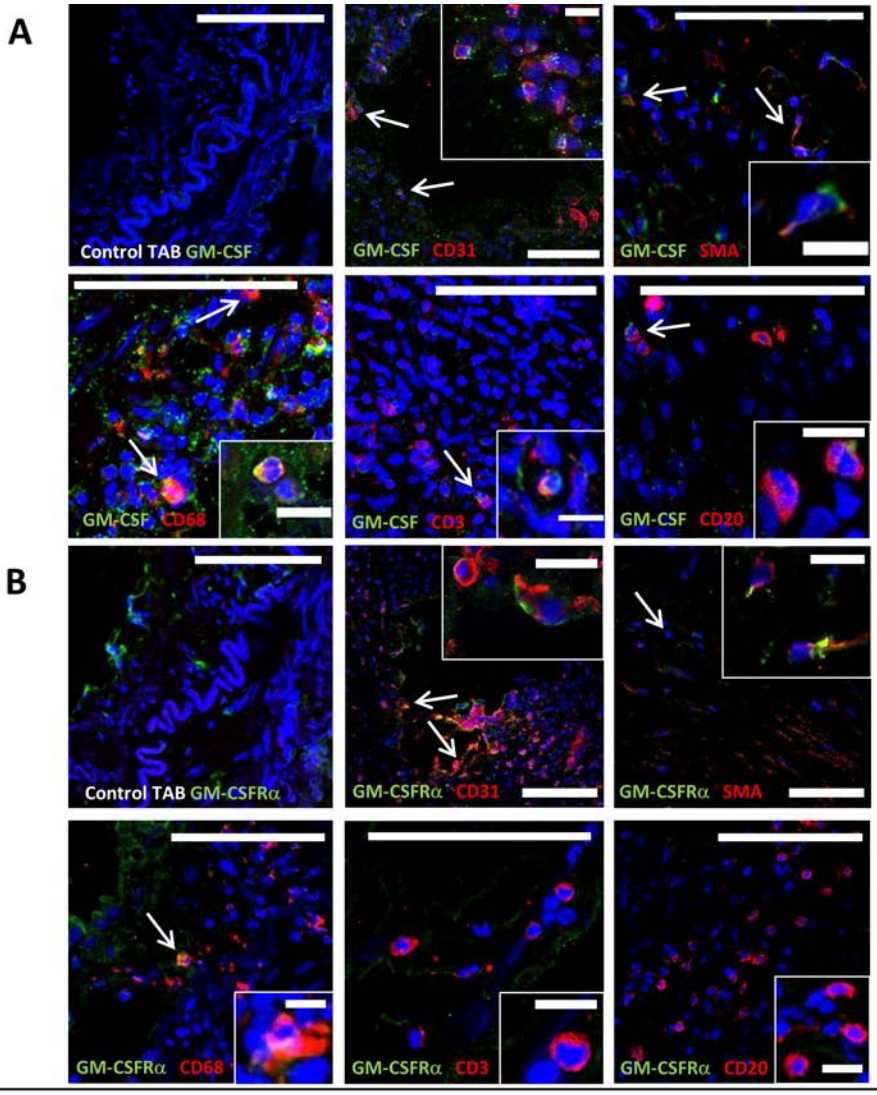


Figure 1 Granulocyte-macrophage colony stimulating factor (GM-CSF) and GM-CSFRα expression in GCA lesions. Concentrations of GM-CSF (A) and GM-CSFRα mRNA (B) measured by qRT-PCR in fresh-frozen histologically negative arteries (controls) (n=10) vs GCA-positive arteries (n=10). Results are expressed in relative units normalised to the housekeeping transcript *GUSB*. GM-CSF (C) and GM-CSFRα (D) RNA hybridisation signals (red dots) on control temporal arteries and GCA-involved arteries. (E) Quantitation of RS signal (expression score) in different arterial layers in 6 GCA-involved and 5 control arteries. Immunostaining with anti-GM-CSF (F) and anti-GM-CSFRα (G) antibodies (brown colour) of FFPE normal or GCA-involved arteries (representative of 5 controls and 12 GCA arteries). A, adventitia layer; FFPE, formalin-fixed paraffin-embedded; GCA, giant cell arteritis; GM-CSFRα, GM-CSF receptor alpha chain; I, intima layer; M, media layer; qRT-PCR, quantitative real-time PCR; RS, RNAScope.



C

	Control		GCA	
	GM-CSF	GM-CSFRα	GM-CSF	GM-CSFRα
Luminal endothelium	-	+	+++	++
Neovessels / Adventitial endothelium	-	-	+	+
VSMC	-	-	+	+/-
Neointimal myofibroblasts	-	-	++	++
Macrophages	-	-	+++	++
B cells	-	-	+	+/-
T cells	-	-	++	-

Figure 2 GM-CSF and GM-CSFRα expression by immune and resident cells. Merged double immunofluorescence staining with anti-GM-CSF (A) or anti-GM-CSFRα (B) antibodies (both in green) and cell surface markers CD68 (macrophages), CD31 (endothelial cells), CD3 (T lymphocytes), CD20 (B lymphocytes) and SMA (identifying vascular smooth muscle cells and myofibroblasts) (all in red) of fresh-frozen temporal arteries from patients with GCA or controls (first panel). Nuclei are stained with DAPI (blue). Co-expression (orange/yellow) is pointed with arrows and insets show magnified double-positive cells (scale bars in figures measure 100 µm and 15 µm for insets). (C) Summary panel of GM-CSF and GM-CSFRα expression by different cell types in three GCA-involved temporal arteries detected by immunofluorescence as in A and B. +++: 50%–100% positive cells; ++: 20%–40% positive cells; +: less than 20% positive cells; +/–: scattered cells; –: negative. DAPI, 4′,6-diamidino-2-phenylindole; GCA, giant cell arteritis; GM-CSF, granulocyte-macrophage colony stimulating factor; GM-CSFRα, GM-CSF receptor alpha chain; SMA, smooth muscle actin; TAB, temporal artery biopsy; VSMC, vascular smooth muscle cells.

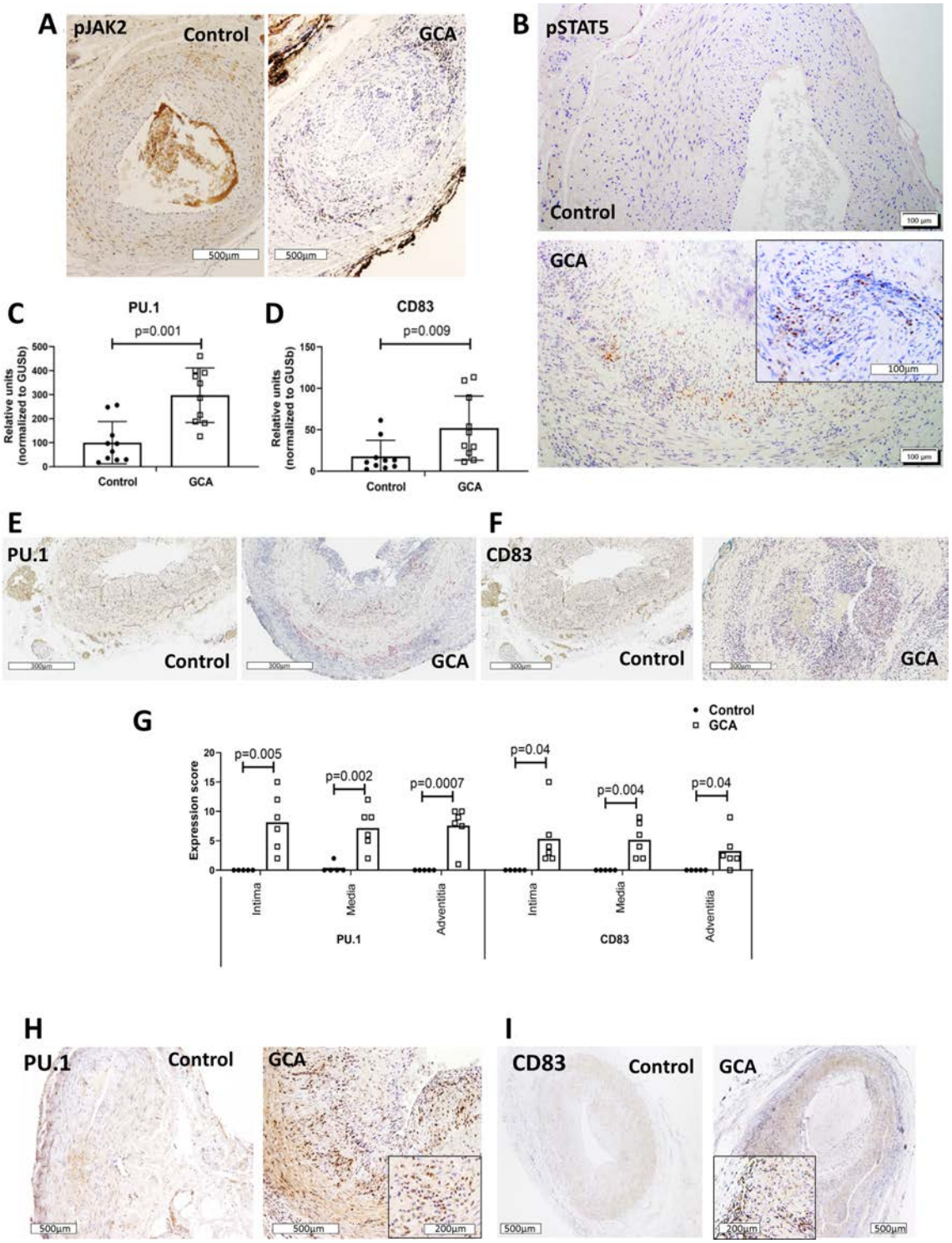


Figure 3 Activation of GM-CSFR-driven signalling pathways and target gene expression in GCA lesions. Immunostaining of histologically negative temporal artery biopsies (control) and GCA-involved arteries with anti-phospho-JAK2 (A) or anti-phospho-STAT5 (B) antibody (brown colour). Representative of 12 GCA and 5 control arteries. mRNA concentrations of PU.1 (C) and CD83 (D), in fresh-frozen control and GCA arteries (n=10 each group). PU.1 (E) and CD83 (F) RS images with positive red staining on control (n=5) and GCA temporal arteries (n=6), with their corresponding quantitation (G) in the intima, media and adventitia layers of the artery wall. Immunohistochemistry with anti-PU.1 (H) and anti-CD83 (I) antibodies on FFPE control and GCA arteries (brown). Representative of 12 GCA arteries and 5 controls. Magnification of each figure is indicated individually. FFPE, formalin-fixed paraffin-embedded; GCA, giant cell arteritis; GM-CSF, granulocyte-macrophage colony stimulating factor; GM-CSFRα, GM-CSF receptor alpha chain; RS, RNAScope.

not elicit changes in constitutive endothelial cell marker vWF or CD31 mRNAs but a decrease in CD34 mRNA, expressed by neovessels and haematopoietic stem cells (HSC) was observed (figure 8D).[38 39] Immunofluorescence showed a reduction in CD31 + and CD34+ neovessels within inflammatory lesions on exposure to mavrilimumab (figure 8E,F). Scattered CD34 +

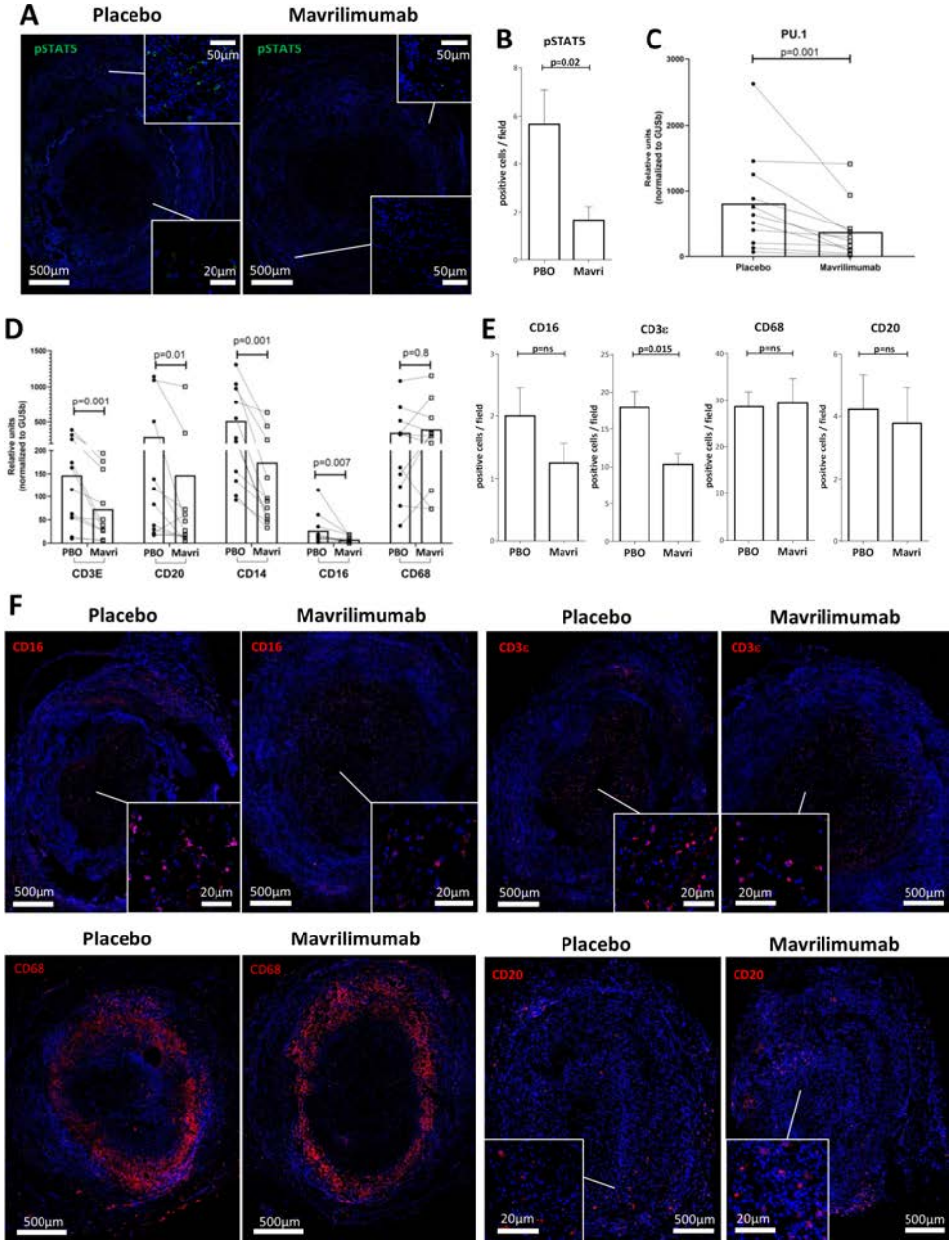


Figure 4 Effect of mavrilimumab on inflammatory infiltrates in ex vivo cultured arteries from patients with GCA. (A) Immunofluorescence staining with anti-phospho-STAT5 antibody (green) of a GCA artery cultured with placebo or mavrilimumab. (B) Quantification of positive cells per field A; this experiment was performed three times with similar results. (C) mRNA Spl1/PU.1 transcripts in 11 cultured GCA-affected temporal arteries in the presence of placebo or mavrilimumab. (D) Transcript levels for cell markers CD3ε, CD20, CD14, CD16 and CD68 in 11 cultured GCA-involved temporal arteries exposed to placebo or mavrilimumab. (E) Quantification of cells per field that are positive for anti-CD16, anti-CD3Ɛ, anti-CD68, and anti-CD20. (F) Immunofluorescence staining of cultured GCA-involved arteries in the presence of placebo or mavrilimumab with anti-CD16, anti-CD3Ɛ, anti-CD68, and anti-CD20 (red colour) and DAPI (blue). Representative of 3 GCA cultured arteries. Panel E is the quantification of panel F. DAPI, 4′,6-diamidino-2-phenylindole; GCA, giant cell arteritis.

cells not aligned around a lumen were also observed in lesions and were reduced by mavrilimumab.

DISCUSSION

This study demonstrates expression of GM-CSFRα, the target of mavrilimumab, within the lesions of GCA-affected arteries and confirms the increased production of GM-CSF previously reported.[24 40 41] Macrophages were the main cell type immunostained for GM-CSF and GM-CSFRα in inflamed arteries. Luminal endothelial cells and, to a lesser extent, intimal myofibroblasts and endothelial cells from vasa vasorum and neovessels also expressed GM-CSF along with a small subset of T cells, presumably ThGM-CSF cells.[30] GM-CSFRα was expressed mainly by macrophages, endothelial cells and intimal myofibroblasts, suggesting that these cell types would be the most responsive to GM-CSF.

Contrary to a report in granulomatosis with polyangiitis,[42] but similar to findings in other inflammatory conditions,[28–30] GM-CSF was barely detectable in serum from patients with GCA, with no differences from healthy individuals. This supports a paracrine function of GM-CSF in the inflammatory microenvironment and limits the utility of serum GM-CSF as a biomarker of disease activity.

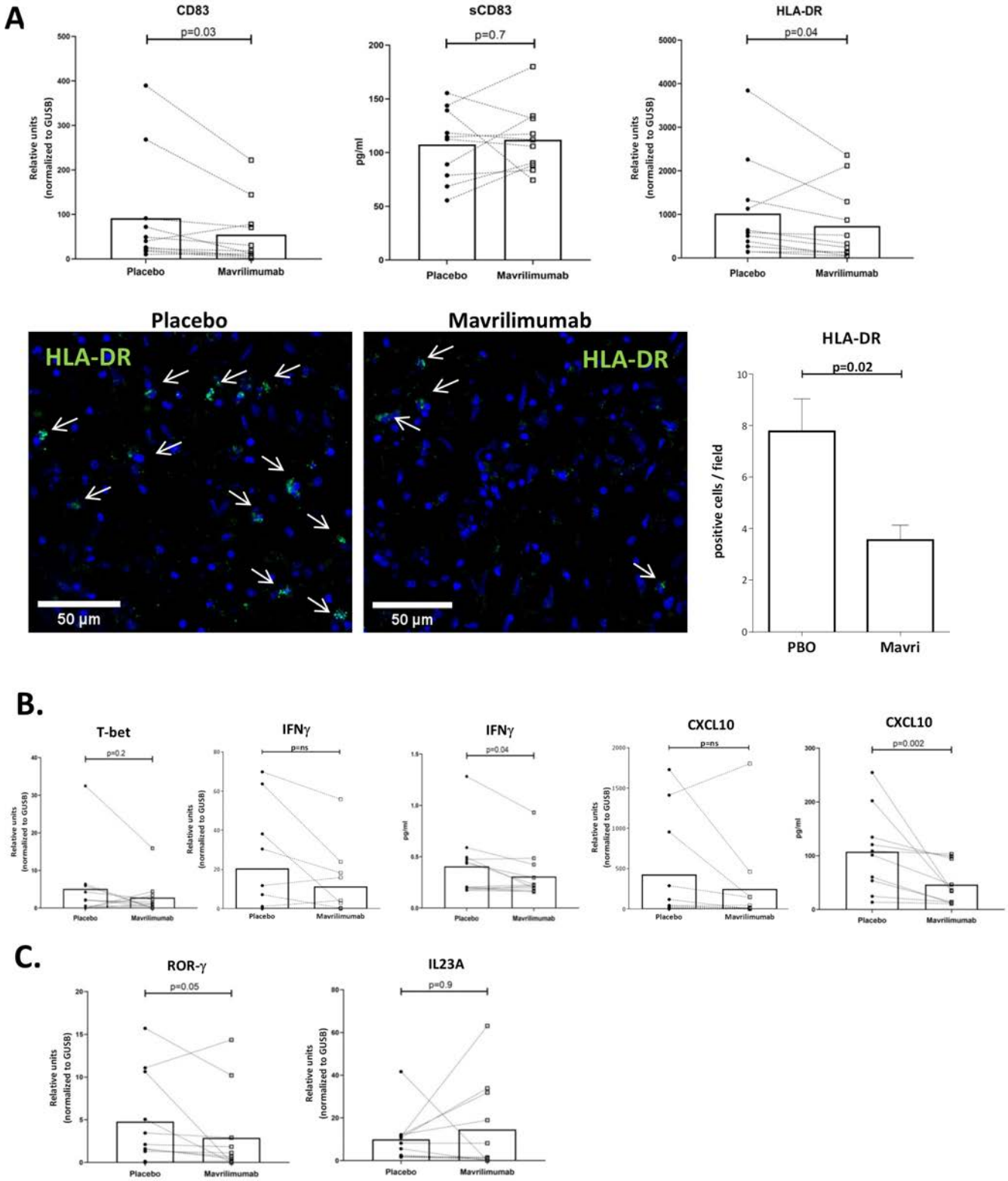


Figure 5 Mavrilimumab decreases molecules related to T lymphocyte activation and differentiation. (A) mRNA transcripts of CD83 (left) and HLA-DR (right) expressed in relative units and normalised to housekeeping gene *GUSB* in GCA-positive temporal arteries (n=11) cultured with placebo or mavrilimumab. Soluble CD83 measured (pg/mL) in supernatants of nine GCA cultured arteries exposed to placebo or mavrilimumab (central panel). Image shows HLA-DR expression by immunofluorescence in a GCA artery cultured with placebo or mavrilimumab. Images show detailed zoom amplification by confocal microscope with arrows indicating green HLA-DR-positive cells. Nuclei are stained with DAPI (blue). The graph on the right show the number of HLA-DR-positive cells per field in 9 fields per section. Immunofluorescence was performed in two GCA cultured arteries, with consistent results. (B) mRNA transcripts of *TBX21* (T-bet), *IFNG* (IFNγ) and CXCL10 in GCA arteries cultured with placebo or mavrilimumab (n=11). IFN-γ and CXCL-10 proteins were also measured in artery culture supernatants of the same specimens. Results are expressed in pg/mL. (C) *RORC* (ROR-γ) and *IL-23A* mRNA measurement in cultured GCA arteries treated with placebo or mavrilimumab. DAPI, 4′,6-diamidino-2-phenylindole; GCA, giant cell arteritis; HLA-DR, human leukocyte antigen-DR; IFN, interferon.

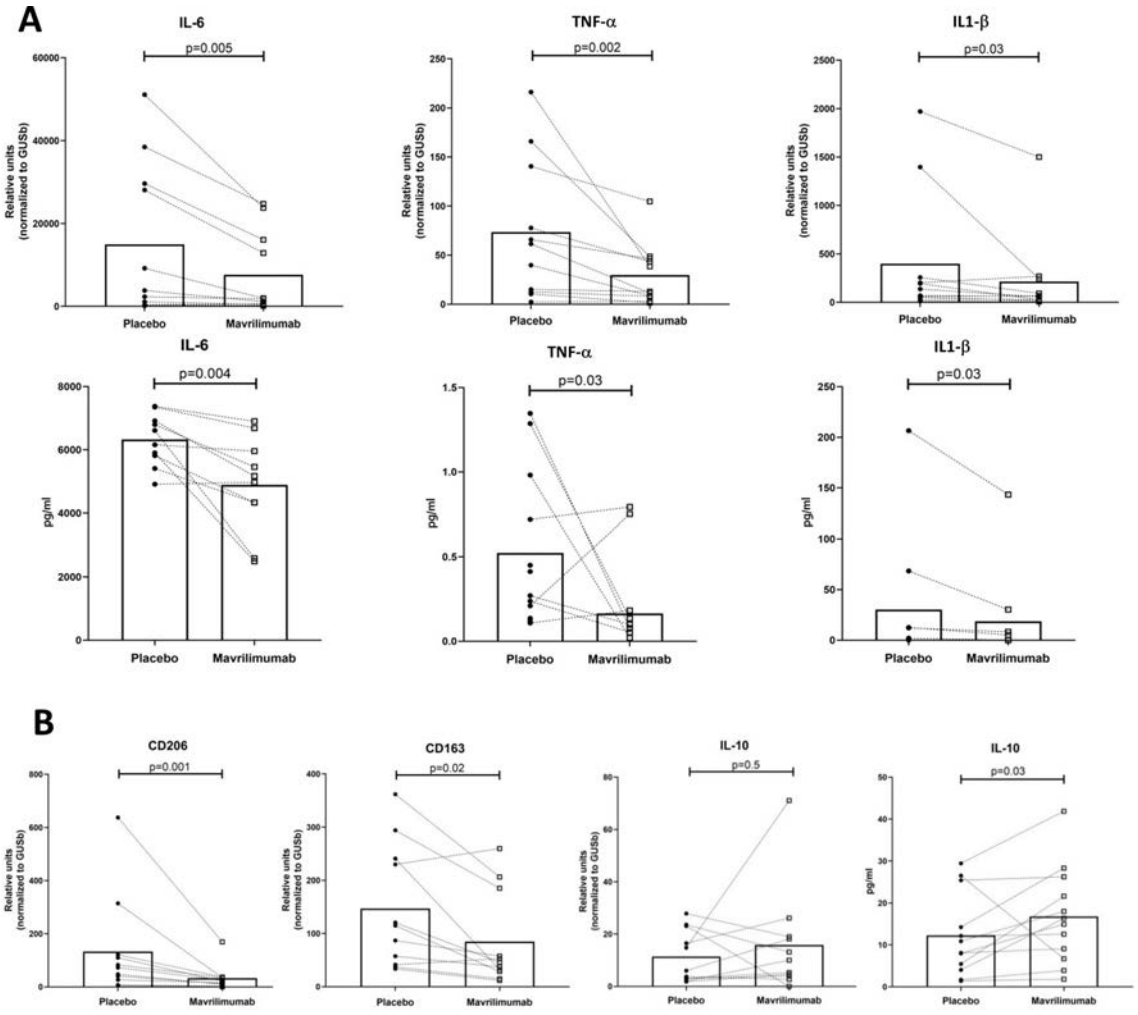


Figure 6 Mavrilimumab impacts macrophage functions. (A) Transcript levels of IL-6 (left), TNFα (central) and IL-1β (right) in GCA-positive arteries (n=11) exposed to placebo or mavrilimumab (mRNA, relative units). IL-6, tumour necrosis factor α (TNFα) and IL-1β proteins (pg/mL) were also measured in GCA artery culture supernatants of the same samples. (B) CD206, CD163 and IL-10 mRNA transcript levels in the same GCA arteries exposed to mavrilimumab or placebo. IL-10 protein (pg/mL) was also detected in the supernatant (right panel). GCA, giant cell arteritis; IL, interleukin.

Detection of JAK2 and STAT5A phosphorylation in GCA lesions, along with increased expression of paradigmatic STAT5-regulated molecules, such as CD83 and transcription factor Spi1/PU.1,[43] suggested activation of GM-CSF receptor-driven signalling pathways. Increased expression of additional relevant STAT5 or PU.1 regulated molecules, including major histocompatibility complex (MHC) class II molecule HLA-DR, adhesion molecules intercellular adhesion molecule 1 (ICAM-1) or vascular cell adhesion molecule 1 (VCAM-1), macrophage marker CD163, pro-inflammatory cytokines, such as IL-1 and TNFα, and metalloproteases such as MMP-9, has been previously demonstrated in GCA.[44–48] Although these pathways can be activated by other cytokines, these data suggest active GM-CSF signalling in GCA arteries and a contribution of GM-CSF to the increased expression of key molecules involved in the pathogenesis of GCA.

To confirm the participation of GM-CSFR-mediated signalling in the increased expression of these and additional relevant molecules and inflammatory cell markers, cultured temporal arteries from patients with histopathologically proven GCA were exposed to mavrilimumab. Treatment with mavrilimumab resulted in significantly decreased transcripts of lymphoid markers, including B lymphocyte surface molecule CD20 and T lymphocyte surface glycoprotein CD3ε. A significant decrease in classical monocyte marker CD14 and myeloid cell marker CD16 mRNAs was also observed. In contrast, there was no consistent change in the expression of CD68, a scavenger receptor widely expressed by macrophages.

Mavrilimumab decreased expression of molecules produced by dendritic cells and B cells, which are essential for antigen-presenting function/T cell activation, such as CD83 and HLA-DR.[49 50] This likely resulted in decreased Th1 differentiation, as indicated by reduced expression of Th1-related molecules, including IFNγ, TNFα and IFNγ-induced molecules such as CXCL10. Molecules related to Th17 differentiation, IL-1β and IL-6 were also decreased, but a more direct impact on IL-17 production could not be assessed. Although we and others have previously shown increased IL-17 expression in affected temporal arteries from patients with GCA,[18 21–23] baseline expression of IL-17 was very low in cultured arteries, possibly related to previous GC treatment in the majority of patients[18] or to the possible impact of culture on certain molecules.[36]

Mavrilimumab had a significant impact on pro-inflammatory functions of macrophages and endothelial cells, including expression of IL-1β, TNFα and IL-6, and expression of adhesion molecules for leucocytes. It also tended to increase expression and release of the anti-inflammatory cytokine IL-10, produced by regulatory T cells and B cells and M2-type macrophages.[51] Mavrilimumab reduced MMP-9 expression with no change in

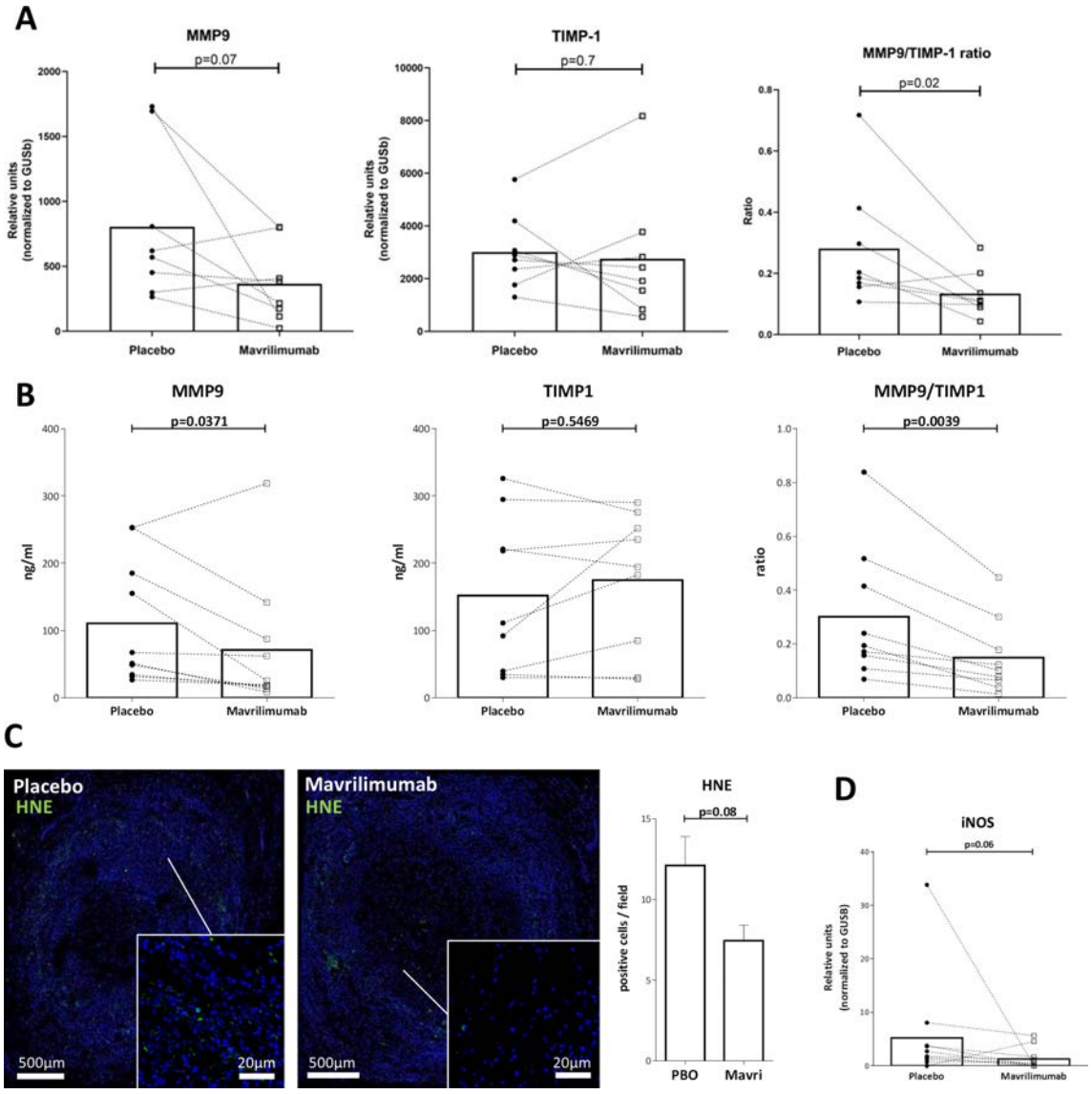


Figure 7 Effect of mavrilimumab on molecules related to vascular injury. (A) Transcripts of MMP-9, tissue inhibitor of metalloproteinases 1 (TIMP-1) and MMP-9/TIMP-1 mRNA ratio in 8 GCA-positive temporal arteries cultured with placebo or mavrilimumab. (B) MMP-9, TIMP-1 protein concentration and MMP-9/TIMP-1 protein ratio in the corresponding supernatants (ng/mL). (C) Immunofluorescence staining of HNE (green) with nuclei (in blue) in a GCA-involved artery cultured with placebo or mavrilimumab, and its quantitation (right panel). Immunofluorescence was performed in two GCA cultured arteries, with consistent results. (D) *NOS2* transcripts in 11 cultured GCA arteries exposed to placebo or mavrilimumab. GCA, giant cell arteritis; HNE, 4-hydroxynonenal; MMP-9, matrix metalloprotease 9.

expression of its natural inhibitor TIMP-1, thereby suggesting a shift in the MMP-9 proteolytic balance.[47] Proteolytic enzyme MMP-9 has elastinolytic activity and may contribute to elastin degradation since it is expressed and activated in GCA lesions and in aortic tissue.[52] MMP-9 may also contribute to GM-CSF-induced aneurysm formation, shown in an animal model.[53] Macrophages present in GCA lesions have oxidative capacity as indicated by the presence of lipid peroxidation products (HNE) in GCA lesions.[27] Treatment with mavrilimumab decreased HNE presence in cultured arteries indicating that mavrilimumab decreases oxidative damage in inflamed arteries.

The tuning in macrophage function induced by mavrilimumab does not parallel classical M1 (pro-inflammatory) or M2 (anti-inflammatory, reparative) phenotypes. Mavrilimumab reduced M1 markers, including HLA-DR and iNOS, and tended to increase M2 cytokine IL-10. However, mavrilimumab also reduced CD206 and CD163, which have been considered markers of M2 phenotype.[54] It is important to remark that this distinction has been established mostly in in vitro differentiated macrophages or in murine models. In humans, plasticity of macrophages is far more complex.[54] For example, macrophages co-expressing CD206 and MMP-9 have been observed in GCA lesions[41] and a population of pro-inflammatory CD14+ HLA-DR^{high} CD206^{+} macrophages has been identified in human viral hepatitis.[55] Overall, mavrilimumab decreased the inflammatory and destructive potential of macrophages.

GM-CSF influences endothelial cell behaviour and stimulates angiogenesis in experimental systems.[56] Accordingly, mavrilimumab reduced microvessel density in GCA lesions. In addition to its potential direct effects, our results indicate that GM-CSF regulates VEGFA production. Since CD34 is expressed not only by endothelial cells from neovessels but also by HSC, which have recently been identified in chronic inflammatory lesions and promoted by GM-CSF,[57 58] we cannot exclude the possibility

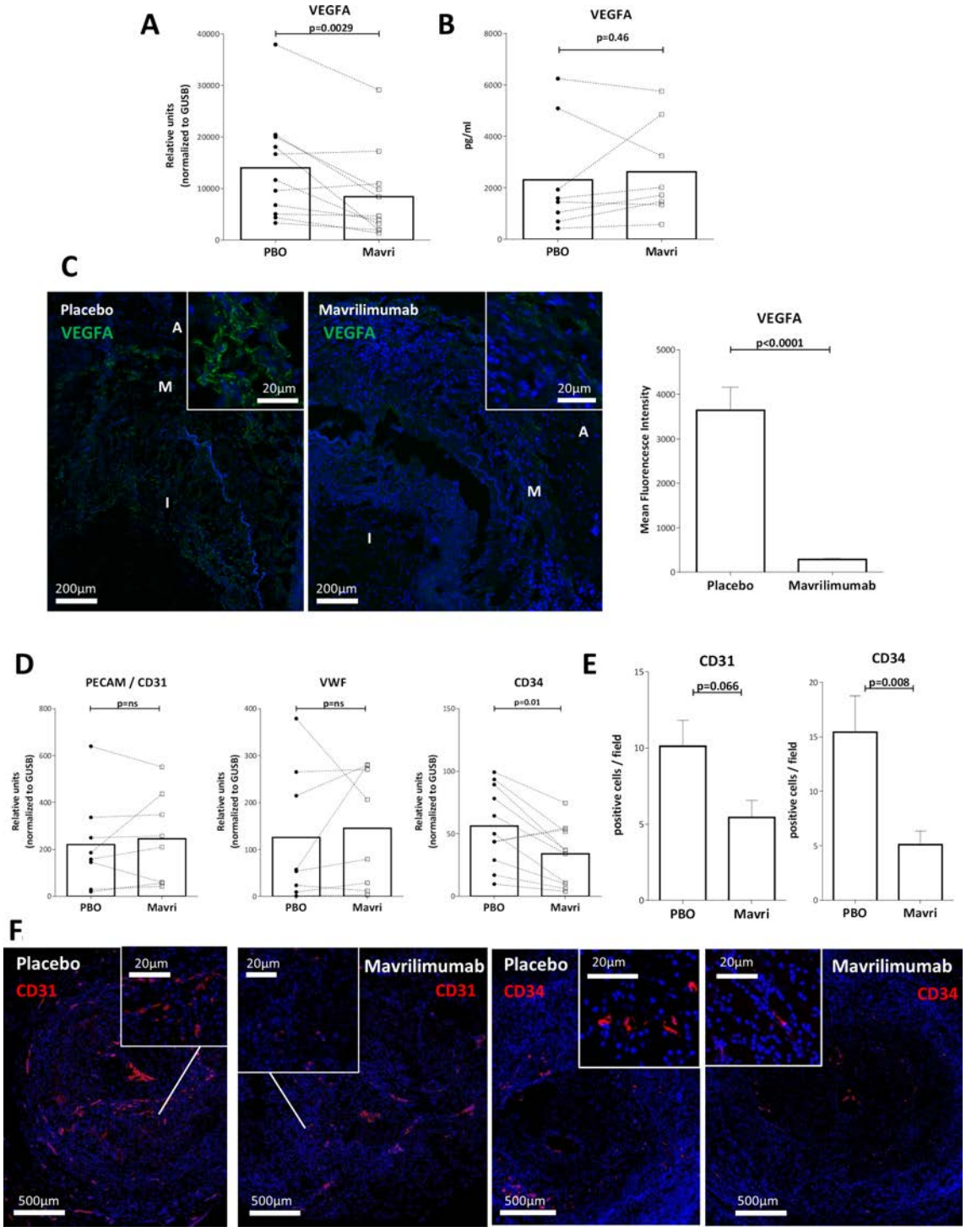


Figure 8 Mavrilimumab effect on angiogenesis. (A) Detection of vascular endothelial growth factor A (VEGFA) transcripts in 11 GCA-positive temporal arteries cultured with placebo or mavrilimumab. (B) Detection of VEGFA protein (pg/mL) in supernatants of eight respective arteries cultured with placebo or mavrilimumab. (C) Immunofluorescence with anti-VEGFA antibody of a GCA artery cultured with placebo or mavrilimumab (I, intima; M, media; A, adventitia). The graph on the right shows quantification of mean fluorescence intensity of the entire artery wall. (D) Measurement of PECAM-1 (n=8), vWF (n=8) and CD34 (n=11) transcripts in GCA cultured temporal arteries treated with placebo or mavrilimumab (relative units, normalised to housekeeping *GUSB*). (E) Quantification (positive cells per field) of immunofluorescence. Immunofluorescence was performed on two cultured biopsies with consistent results. (F) Immunofluorescence with anti-CD31 or anti-CD34 antibody of a GCA artery cultured with placebo or mavrilimumab. Inset images show zoom amplifications of positive (red) cells in areas of interest across the neointimal layer. Panel E is the quantification of panel F. GCA, giant cell arteritis.

that some detected CD34 + cells were ectopic HSC. Mavrilimumab reduction of ectopic HSC may be a potential new relevant effect of mavrilimumab. Since neoangiogenesis is prominent in GCA lesions, and newly formed capillaries express adhesion molecules and recruit inflammatory leucocytes into arteries,[45 57] mavrilimumab could indirectly reduce leucocyte recruitment by decreasing neoangiogenesis in addition to its direct effects on myeloid and other cells

Our study has limitations, including the relatively small number of cases investigated, inherent to the low incidence of GCA and the need of viable fresh tissue. In addition, our model explores changes induced by mavrilimumab in a target organ isolated from a functional immune system. However, the effects of mavrilimumab observed were consistent with the known functions of GM-CSF obtained in a variety of experimental systems. Furthermore, due to the small amount of available tissue, our experiments were limited to a single time-point. We cannot exclude that effects could be more prominent at other time points. Finally, most arteries were obtained from patients who had previously received GC treatment, as currently advised by international guidelines on GCA suspicion.[59] Previous GC exposure reduces baseline expression of a variety of molecules, including GM-CSF.[36 60] It would be possible that using treatment-naïve samples, changes would have been more prominent. However, this setting better reflects the real world and mavrilimumab still adds to potential GC effects on key inflammatory molecules.

In summary, this study reveals for the first time, functional changes induced by mavrilimumab in a classical target tissue of GCA. Mavrilimumab impacts inflammatory pathways considered relevant to the pathogenesis of vascular inflammation and injury, and the results from a recent phase 2 trial in which mavrilimumab was superior to placebo (both with 26-week prednisone taper) in reducing the risk of GCA flare and maintaining sustained remission[35] validated the role of GM-CSF in GCA.

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Contributors MCC and JFP are responsible for the overall content as the guarantor. MCC and JFP designed and supervised the work. MC-B, RA-R, FK and AD performed the experiments. GE-F, RR-G and JM-H performed accurate patient selection and collected biological samples. SM, AJ, AD'A and KB supervised experiments. MCC, MC-B and RA-R made the initial manuscript draft. All authors contributed intellectual input, revised data and revised and approved the manuscript.MCC dedicates her contribution to the Department of Oncology, Hospital Clínic, Barcelona, particularly to the Oncology specialists Montserrat Muñoz, Meritxell Molla and Immaculada Alonso for their excellent professional care and encouragement throughout the development of this study. Without their support, her contribution would have not been possible.

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Data availability statement Data are available upon reasonable request. The individual anonymised data supporting the analyses contained in the manuscript will be made available upon reasonable written request from researchers whose proposed use of the data for a specific purpose has been approved. Data will not be provided to requesters with potential or actual conflicts of interest, including individuals requesting access for commercial, competitive or legal purposes. Data access may be precluded for studies in which clinical data were collected subject to legal, contractual or consent provisions that prohibit transfer to third parties. All those receiving access to data will be required to enter into a Data Use Agreement, which shall contain terms and conditions that are customary for similar agreements and similar companies in the industry. For requests, please email JFP, Kiniksa Pharmaceuticals's Chief Medical Officer, at jpaolini@kiniksa.com.

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CLINICAL SCIENCE

Exercise and education versus saline injections for knee osteoarthritis: a randomised controlled equivalence trial

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ABSTRACT

Objective To compare the efficacy of an exercise and education programme with open-label placebo given as intra-articular injections of inert saline on pain and function in individuals with knee osteoarthritis (OA).

Methods In this open-label, randomised controlled trial, we recruited adults aged ≥50 years with symptomatic and radiographically confirmed knee OA in Denmark. Participants were randomised 1:1 to undergo an 8-week exercise and education programme or four intra-articular saline injections over 8 weeks. Primary outcome was change from baseline to week 9 in the Knee Injury and Osteoarthritis Outcome Score (KOOS) questionnaire pain subscale (range 0 (worst)–100 (best)). Prespecified equivalence margins of ±8 KOOS pain points were chosen for the demonstration of comparable efficacy. Key secondary outcomes were the KOOS function and quality of life subscales, and patients' global assessment of disease impact.

Results 206 adults were randomly assigned: 102 to exercise and education and 104 to intra-articular saline injections. For the primary outcome, the least squares mean changes in KOOS pain were 10.0 for exercise and education and 7.3 for saline injections (difference 2.7 points, 95% CI −0.6 to 6.0; test for equivalence p=0.0008). All group differences in the key secondary outcomes respected the predefined equivalence margins. Adverse events and serious adverse events were similar in the two groups.

Conclusion In individuals with knee OA, an 8-week exercise and education programme provided efficacy for symptomatic and functional improvements equivalent to that of four open-label intra-articular saline injections over 8 weeks.

Trial registration number NCT03843931.

Key messages

What is already known about this subject?

- Exercise and education are recommended as the primary symptom management strategies for knee osteoarthritis (OA).
- No adequate placebo-controlled studies of exercise and education alone for knee OA exist.
- The isolated clinical effect of exercise and education has not been separated from that of a placebo intervention.

What does this study add?

- An exercise and education programme was equally effective as open-label application of inert intra-articular saline injections in providing symptomatic and functional improvements in individuals with knee OA.

How might this impact on clinical practice or future developments?

- These findings raise important questions about mechanisms of action as well as the continued widespread recommendation of exercise and education in the management of knee OA.

INTRODUCTION

Knee osteoarthritis (OA) is a highly and increasingly prevalent musculoskeletal condition causing pain, physical disability and reduced quality of life.[1] Exercise and education are recommended as the primary symptom management strategies based on numerous clinical trials.[2–4] In previous studies, multimodal physiotherapy (exercise, education, advice, gait aid, massage, taping and mobilisation) for knee and hip OA did not provide benefits over inert sham treatments.[5 6] However, no adequate placebo-controlled studies of exercise and education alone for knee OA exist probably due to unclear mechanisms of action, difficulties with blinding and the complexity of the intervention. Hence, the effect of exercise and education has not been separated from contextual factors, placebo and regression to the mean phenomena.

Recent advances in open-label placebo research have shown that considerable placebo responses can be elicited by inert substances if applied adequately.[7 8] Open-label placebo provides an opportunity to compare exercise and education with an inert comparator and thereby mitigate some of the inbuilt challenges with blinded comparator groups in clinical trials of exercise and education. Intra-articular saline injection is one such inert treatment and is commonly used as a comparator in clinical trials for knee OA. In indirect comparisons, the symptom response to saline injection was comparable to that of exercise in knee OA.[9–11]

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In this trial, we took advantage of the potential of open-label application of inert treatments as comparator and conducted a randomised controlled trial where the aim was to assess if the efficacy of an exercise and education programme is equivalent to open-label placebo given as intra-articular injections of inert saline on pain and function in individuals with knee OA.

METHODS

Study design

We conducted an open-label, single centre randomised controlled trial with two parallel intervention groups. Evaluations and assessments took place in the OA outpatient's clinic at Bispebjerg-Frederiksberg Hospital, Copenhagen, Denmark, at baseline and at 9 and 12 weeks. Questionnaires were answered on a touch screen in the clinic. Links to online questionnaires were emailed weekly from baseline to week 8. The trial design and the trial protocol appears in online supplemental figure S1. The study was registered prospectively at www.ClinicalTrials.gov on 18 February 2019.

Participants

Between 30 July 2019 and 17 September 2020, participants were recruited from the OA outpatient's clinic at Bispebjerg-Frederiksberg Hospital. All participants provided written informed consent before participation.

Inclusion criteria were age ≥50 years, body mass index (BMI) of ≤35 kg/m^2, meeting the American College of Rheumatology clinical classification of knee OA,[12] average knee pain during weight-bearing activities in the last week of ≥4/10, radiographically verified tibiofemoral OA (Kellgren-Lawrence grade ≥2).[13] Major exclusion criteria were intra-articular treatments of any kind in either knee and participation in exercise therapy within 3 months of the baseline visit (for details, see online supplemental file).

Potential participants were informed about the trial by an investigator who obtained written informed consent and coordinated trial inclusion. Information was delivered neutrally, ensuring that descriptions of both interventions were promoted equally including that the investigators had no treatment preference (clinical equipoise).[11] Saline injections were described as inert, yet with potential beneficial effects that may compare to those of exercise and education. The participants were informed that 'active ingredients' in both interventions are unverified and involves the sum and interaction of many factors.[11] The most symptomatic knee at baseline was chosen as the study knee.

Interventions

Full details of the interventions are in the published protocol[11] and the online supplemental file.

The *exercise and education programme* consisted of the Good Life with osteoArthritis in Denmark (GLAD) programme.[14] GLAD is an 8-week structured treatment programme consisting of patient education and supervised neuromuscular exercise for people with symptomatic knee or hip OA.[14] In this trial, GLAD was delivered by GLAD-certified physiotherapists at the department of physiotherapy at Bispebjerg-Frederiksberg Hospital.

Two group-based educational sessions lasting about 1.5 hours were provided, addressing knowledge on knee OA, treatment options with a focus on exercise and its benefits, and advice about self-management.[14] The exercise part of GLAD was delivered as 12 1-hour, group-based, individually supervised sessions, two times per week for 6 weeks. Satisfactory treatment adherence was defined as attendance to at least one educational (50%) and eight exercise sessions (75%).

Intra-articular saline injections of 5 mL isotonic solution of sodium chloride in sterile water (0.9%=9 mg/mL) were given into the study knee at weeks 1, 3, 5 and 7 after baseline. Injections were performed using ultrasound imaging guidance[15] (Logic E9; General Electrics Medical System, Milwaukee, Wisconsin, USA) by two physiotherapists with 7 and 15 years of experience in ultrasound-guided intra-articular injections, under supervision and regulation by a senior rheumatologist (HB). The procedure was documented in real time, ensuring correct deposition of the bolus in the joint cavity. No local analgesics were used during the procedure. If excessive joint fluid was detected, it was aspirated if possible and deemed clinically relevant, prior to injection of the saline. Satisfactory treatment adherence was defined as reception of at least three injections (75%).

For all participants, mild analgesics (paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs) and acetylsalicylic acid) were allowed, while initiation of opioids, glucocorticoids and off-protocol intra-articular injections were not allowed.

Primary outcome

The primary outcome was the change from baseline in the pain subscale of the Knee injury and Osteoarthritis Outcome Score questionnaire (KOOS)[16] at week 9. KOOS consists of five subscales: pain, physical function, knee-related quality of life, sports and recreation, and symptoms. Each subscale consists of multiple items with scores ranging from 0 to 100 (worst to best).

Key secondary and secondary outcomes

Key secondary outcomes were changes from baseline in the KOOS physical function and knee-related quality of life subscales, and the participant's global assessment of the impact of OA on overall life assessed using a 100 mm Visual Analogue Scale (VAS) (higher is worse). Other secondary outcomes included changes from baseline in the KOOS sports and recreation and symptoms subscales, and physical performance by the 4×10 m fast walk test (seconds),[17] stair ascend and descend test (seconds),[17] and the number of chair stands in 30 s,[17] as well as treatment response according to the Outcome Measures in Rheumatology-Osteoarthritis Research Society International(OMERACT-OARSI) criteria.[18]

Safety and exploratory outcomes

Safety outcomes included swollen study knee (present/absent) by examination of palpable knee effusion judged by a rheumatologist.[19] Also, study knee effusion was visualised (present/absent) by ultrasound and aspirated joint fluid was recorded (millilitre). The exploratory outcomes included use of acetaminophen and NSAIDs recorded at baseline and week 9, and the Intermittent and Constant Osteoarthritis Pain questionnaire[20] with two subscores, constant pain and intermittent pain on 0–100 scales (best to worst). Further, average morning pain during the past week was assessed using a 100 mm VAS (higher is worse). Adverse and serious adverse events were registered at clinical visits and by spontaneous reports from the participants (see protocol).

Randomisation and blinding

Before randomisation, demographic information and all baseline measures were obtained.

Participants were assigned 1:1 to either exercise and education or saline injections according to a computerised randomisation

list based on permuted random blocks of variable size (2–6) generated before enrolment. Allocation sequence was developed by the trial biostatistician not actively involved in the conduct of the trial. Allocation was stratified by BMI of ≥30 kg/m^2, swollen study knee on palpation,[19] evidence of bilateral tibiofemoral OA (Kellgren-Lawrence grade ≥2) and participation in sports activities as a young adult (20s). Allocation was concealed until an investigator pressed 'randomise' in the electronic trial management system.

As this was an open-label trial neither health professionals delivering the interventions, nor participants were blinded to treatment allocation. Outcome assessors were blinded to allocation where possible, and participants were requested not to disclose allocation during assessments.

Sample size

The sample size was calculated for test of equivalence of the groups at 90% power and an alpha level of 0.05 using two one-sided tests (one-sided alpha of 0.025) with equivalence margins of ±8 KOOS pain points, assuming a mean difference of 0 and a common SD of 15 points. From this, a total sample size of 154 participants was required. To account for dropout, the sample size was a priori increased to 200 participants.

Statistical analysis

The analysis was performed according to the a priori statistical analysis plan that was publicly available online (www.clinicaltrials.gov) before the last participant's last visit (see online supplemental file).

The primary analysis was performed using the intention-to-treat (ITT) population; patients were assessed and analysed as members of their randomised groups, irrespective of adherence to the treatments. Continuous outcomes were analysed as change from baseline using repeated measures mixed linear models, including participants as random effects, with fixed effect factors for group and week (including all timepoints to respect the ITT principle) and the corresponding interaction, while adjusting for baseline values (to increase precision) and the stratification factors (as part of the design). Results are reported as least squares means and SEs, and differences between least squares means are reported with two-sided 95% CIs. The group difference in the primary outcome was assessed for equivalence by a two one-sided test of equivalence with alpha 0.025 assessing if the 95% CI respects the predefined equivalence margin of ±8 KOOS pain points. No explicit adjustments for multiplicity were applied; rather the key secondary outcome measures were analysed in a prioritised order. Missing data were handled implicitly in the ITT analysis by the mixed linear models.[21] Sensitivity analyses[22] were performed for the primary and key secondary outcomes at week 9 by repeating the primary analyses on the per-protocol population predefined as participants with satisfactory adherence and without major protocol deviations. Further, analysis of covariance with multiple imputation of missing data adjusted for stratification factors and baseline values was performed followed by analysis of covariance with a baseline observation carried forward imputation of missing data. If the primary analysis and the sensitivity analyses confirm each other, confidence in the results is increased both regarding equivalence and superiority claims. All analyses were performed in SAS V.9.4.

Patient and public involvement

Two patient research partners (one female and one male) were involved in designing and preparing the study, including review and revision of the protocol and patient information. They acknowledged the idea and purpose of the study and participated in discussions of ethics, design, choice of outcomes, relevance and feasibility of the trial. They worked voluntarily and have been offered coauthorship of trial-related publications. Both declined coauthorship of the present publication. Hence, they did not review this manuscript.

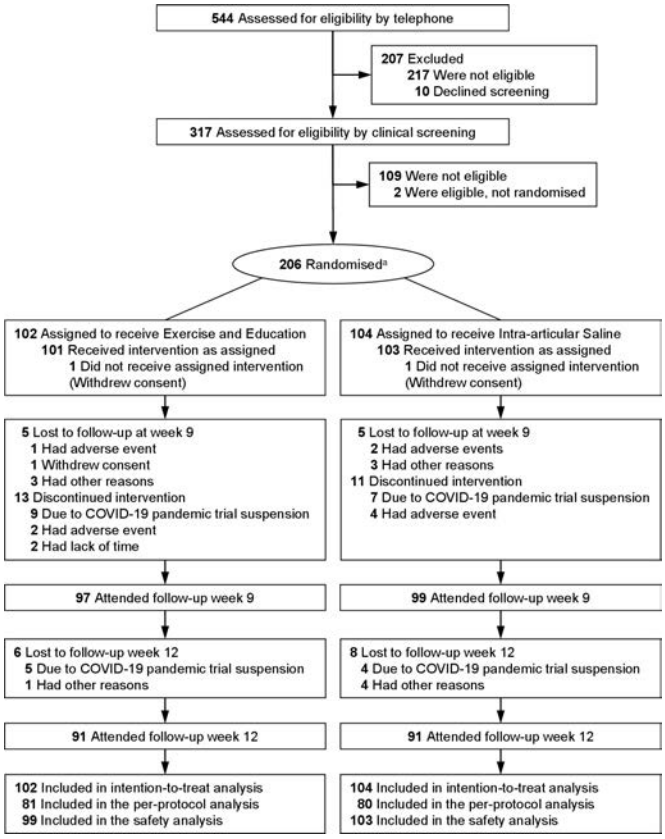

Figure 1 Flowchart of participants throughout the study. A stratified block randmisation method, stratified by BMI ≥30 kg/m2 (yes/no), swollen study knee upon palpation, evidence of bilateral tibiofemoral osteoarthritis assessed as Kellgren-Lawrence grade ≥2, and participation in sports activities as a young adult (20s).

RESULTS

Participants

From 30 July 2019 through 17 September 2020, 317 individuals were screened for eligibility (figure 1); 109 were ineligible for inclusion; and 2 eligible individuals chose not to be randomised. Thus, 206 subjects underwent randomisation; 102 (49.5%) were assigned to exercise and education and 104 (50.5%) to intra-articular saline. The mean age was 68.4 years; 54% were men; and the mean BMI was 27.3. Baseline characteristics were similar in the two groups (table 1). Participants in the exercise and education group attended on average 11.1 (79.3%) sessions out of possible 14 (range 0–14) sessions. Participants in the saline group received on average 3.4 (84.9%) injections out of possible 4 (range 0–4).

Primary outcome

The mean changes (±SE) in KOOS pain score from baseline to week 9 were 10.0±1.5 in the exercise and education group and 7.3±1.5 in the intra-articular saline group (group difference: 2.7 points, 95% CI −0.6 to 6.0; p=0.1122 for test of

Table 1 Baseline characteristics of the participants

	Exercise and education	Intra-articular saline
	n=102	n=104
Characteristics		
Age (years)	70.1±8.3	66.7±8.2
Male sex, n (%)	57 (55.9)	55 (52.8)
Body mass (kg)	80.7±14.2	81.5±13.9
Height (cm)	172.0±9.5	172.1±9.5
BMI*	27.2±3.7	27.4±3.6
Kellgren-Lawrence score, n (%)†		
2	25 (24.5)	31 (29.8)
3	36 (35.3)	30 (28.9)
4	41 (40.2)	43 (41.3)
Stratification factors, n (%)		
BMI ≥30	25 (24.5)	25 (24.0)
Swollen study knee	35 (34.3)	37 (35.6)
Bilateral tibiofemoral OA (K/L ≥2)	92 (90.2)	93 (89.4)
Active as a young adult	66 (64.7)	68 (65.4)
KOOS‡		
Pain††	56.3±14.9	57.6±13.1
Physical function in activities of daily living‡‡	65.7±15.0	67.8±14.7
Quality of life‡‡	39.7±15.5	40.8±14.6
Symptoms	64.0±17.1	62.8±16.3
Physical function in sports and recreation	35.2±21.3	31.8±19.6
Patient Global Assessment (mm)§‡‡	61.4±20.9	59.2±20.5
Morning pain (mm)¶	44.7±25.4	44.5±23.4
ICOAP scores**		
Constant pain subscore	23.6±28.4	15.4±23.8
Intermittent pain subscore	40.8±22.3	42.9±18.1
Total score	33.0±19.2	30.4±15.0
Performance tests		
4×10 m fast walk test (s)	26.6±5.3	25.5±6.5
30 s chair stand test (repetitions)	12.3±3.6	12.4±3.6
Stair climbing test (s)	15.2±5.9	13.9±8.0
Clinical assessment		
Swollen study knee, clinical, n (%)‡‡	35 (34.3)	37 (35.6)
Analgesics use		
Paracetamol or NSAID user, n (%)	34 (33.3)	43 (41.4)

Plus–minus values are means±SD unless otherwise stated.
*The BMI is the weight in kilogram divided by the square of the height in metre.
†Scores on the Kellgren-Lawrence scale range from 0 to 4, with a score of 2, 3 or 4 indicating definite OA and higher scores indicating more severe disease.
‡Scores on KOOS subscales range from 0 (worst) to 100 (best).
§Patient Global Assessment is a VAS relating to the degree of the patient's perceived impact of knee OA on overall life (with scores ranging from 0 to 100); higher scores indicate higher disease impact.
¶Morning pain is a VAS relating to the degree of the patient's perceived averaged morning knee pain during the last week (with scores ranging from 0 to 100); higher scores indicate more pain.
**Scores on ICOAP ranges from 0 (no pain) to 100 (extreme pain).
††Primary outcome measure.
‡‡Key secondary outcome measure.
BMI, body mass index; ICOAP, Intermittent and Constant Osteoarthritis Pain; K/L, Kellgren-Lawrence; KOOS, Knee Injury and Osteoarthritis Outcome Score; NSAID, non-steroidal anti-inflammatory drug; OA, osteoarthritis; VAS, Visual Analogue Scale.

superiority). The 95% CI of the group difference in change in KOOS pain from baseline to week 9 respected the predefined equivalence margin of ±8 points (p=0.0008 for equivalence, table 2). The trajectories of the KOOS pain subscale are illustrated in figure 2.

Secondary and other outcomes

In the key secondary outcomes, the estimated treatment differences between groups at week 9 were 0.8 points (95% CI −2.3 to 4.0) for KOOS function score; 1.8 points (95% CI −1.5 to 5.2) for KOOS quality of life score; and 5.7 mm (95% CI −11.3 to −0.1) for Participant Global Assessment (table 2). The key secondary outcomes all respected the predefined criteria for equivalence (see statistical analysis plan), although the group difference in the Participant Global Assessment was statistically in favour of the exercise and education group.

Numbers, rates and severity of adverse events and their relationship to trial treatment were similar across groups (table 3). Serious adverse events rate was similar, and none were related to the treatments.

Finally, the results in the primary and key secondary outcomes appeared stable (unchanged) at week 12 (online supplemental table S1). There were no differences between groups in the other secondary, safety and exploratory outcomes at week 9 (table 2) and week 12 (online supplemental table S1). The overall pattern of results for all outcomes was not changed in the sensitivity analyses (online supplemental tables S2-S4).

DISCUSSION

This study found that an exercise and education programme provided improvements in knee pain equivalent to that of inert intra-articular saline in the short term (9–12 weeks) in individuals with knee OA. The 95% CI of the group difference in KOOS pain change from baseline to week 9 was within our predefined equivalence margin of ±8 points. The key secondary and other secondary outcomes that evaluated patient-reported outcomes and physical performance corroborate the results of the primary outcome and met the predefined criteria for equivalence based on minimal clinically important differences. Treatment adherence was similar in the two groups, as were adverse and serious adverse events. None of the serious adverse events appeared related to the study treatment.

Over the past decades, more than 100 clinical studies on exercise for knee OA have shown beneficial effects as compared with no-treatment control groups,[23] which has resulted in strong recommendations of exercise as primary management strategy of knee OA.[2–4] However, comparison to no-treatment control groups induces a significant risk of bias and precludes assessment of the contribution of contextual factors, placebo and regression to the mean phenomena. This study is the first to compare a widely implemented exercise and education programme with an open-label placebo, and the results show that the exercise and education programme provides equal effects as an open-label application of intra-articular saline known to be associated with contextual factors and placebo responses.[9 10] Few studies have applied inert or sham comparators and those that do suggest that multimodal physical therapy (mixing exercise and other physiotherapeutic techniques) does not confer additional benefits in hip and knee OA.[5 6] Recently, the Strength Training for Arthritis Trial (START) study[24] showed that 18 months of muscle strengthening exercise for patients with knee OA were not more effective than an attention control group, suggesting that improvements in OA pain secondary to exercise are mainly driven by placebo response phenomena, contextual factors, natural course of the disease and regression to the mean, also suggested by others.[25] Our study corroborates this as the neuromuscular exercise and education intervention we delivered did not provide benefits that exceed those of inert saline injections.

In line with this, a possible explanation for the beneficial effects of exercise and education relates to the considerable contact time with clinicians (up to 15 hours with a physical therapist over 8 weeks), which is known to augment improvement in outcomes.[26–28] Likewise, the invasiveness of the procedures associated with intra-articular injections is known to provide strong placebo responses,[28–30] and it is possible that the placebo response to intra-articular saline is higher than

Table 2 Primary, secondary, safety and exploratory outcomes at primary endpoint, week 9 in the intention-to-treat population

	Exercise and education (n=102)	Intra-articular saline (n=104)	Estimated treatment difference	
	LSMean (SE)	LSMean (SE)	ΔLSMean (95% CI)	P value
Primary outcome				
Change in KOOS pain score, equivalence test†	10.0±1.5	7.3±1.5	2.7 (–0.6 to 6.0)	0.0008
Change in KOOS pain score, superiority test†				0.1122
Key secondary outcomes				
Change in KOOS function score	6.9±1.4	6.0±1.4	0.8 (–2.3 to 4.0)	
Change in KOOS quality of life score	8.0±1.5	6.2±1.5	1.8 (–1.5 to 5.2)	
Change in PGA–VAS (mm)	–19.8±2.6	–14.1±2.5	–5.7 (–11.3 to –0.1)	
Other secondary outcomes				
Change in KOOS sports and recreation score	8.0±2.1	9.0±2.1	–1.0 (–5.5 to 3.5)	
Change in KOOS symptoms score	6.2±1.6	8.0±1.5	–1.8 (–5.2 to 1.6)	
OMERACT-OARSI responders, n (%)*‡	44 (42.9)	32 (31.0)	9.6 (–6.6 to 24.1)¶	
Change in 4×10 m fast walk test (s)	–0.5±0.3	–0.5±0.3	0.1 (–0.5 to 0.7)	
Change in 30 s chair stand test (repetitions)	0.4±0.2	–0.1±0.2	0.5 (–0.1 to 1.0)	
Change in stair climbing test (s)	–1.2±0.3	–0.6±0.3	–0.5 (–1.2 to 0.1)	
Safety outcomes				
Swollen study knee, clinical, n (%)‡	40 (38.9)	32 (30.7)	8.2 (–11.2 to 27.8)¶	
Study knee effusion, ultrasound, n (%)‡	35 (34.4)	24 (23.2)	9.4 (–8.6 to 28.2)¶	
Study knee aspiration volume (mL)§	18.5±6.0	25.6±9.3	–7.1 (–24.3 to 10.1)	
Exploratory outcomes				
Change in average morning pain–VAS score (mm)	–14.9±2.5	–18.7±2.4	3.8 (–1.8 to 9.4)	
Change in ICOAP total score	–8.3±1.7	–8.3±1.6	0.0 (–3.7 to 3.7)	
Change in ICOAP constant pain subscore	–9.8±2.4	–6.7±2.3	–3.1 (–8.5 to 2.3)	
Change in ICOAP intermittent pain subscore	–8.1±2.2	–9.6±2.1	1.5 (–3.4 to 6.3)	
Paracetamol and NSAID discontinued, n (%)‡	11 (10.3)	10 (10.1)	–0.6 (–8.3 to 27.3)¶	
Treatment adherence				
Treatment adherence (%), mean (SD)	79.3 (29.0)	84.9 (24.7)	5.5 (–12.9 to 1.9)**	
Treatment adherers, n (%)	85 (83.3)	87 (83.7)	–0.3 (–13.3 to 7.8)¶	

Values are LSMean±SE unless otherwise stated.
*OMERACT-OARSI responder score is a single dichotomous variable based on changes after treatment in three symptomatic domains (pain, function and patient's global assessment).
†Primary outcome was analysed using both a test for equivalence and a test for superiority.
‡Missing data in binary outcomes were handled using an extreme-set multiple imputation technique followed by applying Rubin's rule to both the observed and four extreme case scenarios: (1) data as observed, (2) worst–worst case, (3) worst–best case, (4) best–worst case and (5) best–best case scenario.
§Aspiration only performed in case of effusion detected on ultrasound.
¶Adjusted risk difference with 95% CI (%).
**Mean difference (95% CI).
ICOAP, Intermittent and Constant Osteoarthritis Pain; KOOS, Knee Injury and Osteoarthritis Outcome Score; LSMean, least squares mean; NSAID, non-steroidal anti-inflammatory drug; PGA, Patient Global Assessment; VAS, 100 mm Visual Analogue Scale.

that of exercise and education. On the other hand, the within-group effect size for saline injections in this trial was slightly smaller than those reported in clinical trials where saline was used as a placebo comparator,[9 10] likely due to the open-label design of this trial compared with the double-blinded methodology in the other trials. In contrast, the within-group effect size for exercise and education is similar to those reported in previous clinical trials.[31]

Limitations and strengths of this study

There are limitations to this study. First, the dissimilarities of the two interventions can be argued to limit their comparability. However, due to the inherent unblindable nature and unknown 'active components' of exercise and education, it is not possible to deliver a completely inactive version. We sought to bypass this by applying an open-label study design and comparing exercise and education to intra-articular saline that is commonly used as placebo comparator and easier to monitor than oral or topical placebos. Despite this, the separation of specific and contextual effects of both treatments remains unclear. Second, we only assessed short-term efficacy. However, a similar exercise intervention with longer duration (12 weeks)[32] provided a similar response as the present, and the 18 months of efficacy of exercise were not superior to attention control in the recent START study.[24] The GLAD programme includes an encouragement of the patients to continue exercise on their own, and it is suggested that the effects are sustained for up to 1 year.[33] On the other hand, the effects of intra-articular saline have also been suggested to be sustainable in the long term (6–12 months),[9] and our results add to the discussion of the inertness of saline injections, as potential physiological effects have been suggested.[9 10]

The strengths of this trial include the relatively large sample size and the equivalence design, which increase the precision of the estimated group differences. A rather conservative equivalence margin of ±8 points for the KOOS pain subscale was chosen as this is the suggested threshold for minimal clinically important difference.[34] A less conservative ±10-points

Table 3 Adverse events in the safety population defined as participants in the intention-to-treat population who have received at least one intra-articular injection (intra-articular saline group) or attended at least one exercise session (exercise and education group)

	Exercise and education (n=99)	Intra-articular saline (n=103)
Exposure time (patient weeks)	1131	1175
AE, n patients (%)	34 (34%)	40 (39%)
AE, n events (rate–events per patient week)	49 (0.04)	48 (0.04)
AEs leading to discontinuation, n patients (%)	2 (2%)	4 (4%)
Maximum reported severity of AEs, n (%)		
Mild, n patients	16 (16%)	22 (21%)
Moderate, n patients	12 (12%)	14 (14%)
Severe, n patients	6 (6%)	4 (4%)
AEs, relationship to trial treatment, n events (rate–events per patient week)		
Not related	12 (0.01)	3 (0.003)
Probably not related	9 (0.01)	18 (0.015)
Probably related	28 (0.02)	27 (0.02)
AEs, classification, n events (rate–events per patient week)		
Infections and infestations	3 (0.003)	0 (0)
General disorders and administrative site conditions	5 (0.004)	6 (0.005)
Musculoskeletal and connective tissue disorders	34 (0.03)	38 (0.03)
Skin and subcutaneous tissue disorders	0 (0)	3 (0.003)
Injury, poisoning and procedural complications	7 (0.006)	1 (0.001)
SAE, n patients (%)	5 (5%)	5 (5%)
SAE, n events (rate–events per patient week)	9 (0.008)	5 (0.004)
SAEs leading to discontinuation, n patients (%)	0 (0%)	0 (0%)
SAEs, relationship to trial treatment, n events (rate–events per patient week)		
Not related	1 (0.001)	3 (0.003)
Probably not related	8 (0.007)	2 (0.002)
Probably related	0 (0)	0 (0)
Deaths, n events (rate–events per patient week)	0 (0)	0 (0)

The severity of an AE refers to the maximum intensity of the event. An event was considered severe (compared with mild or moderate) if it interfered substantially with the patients' usual activities. An AE was classified as serious if it was fatal or life-threatening, required inpatient hospitalisation, caused significant disabling, or required medical intervention to prevent permanent impairment or damage.
AE, adverse event; SAE, serious adverse event.

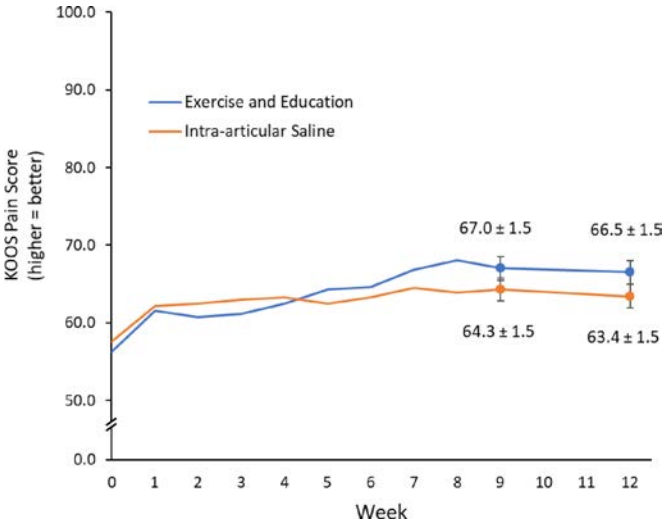

Figure 2 Trajectories of the KOOS pain subscale in the intention-to-treat population. High values represent less pain; low values represent more pain. Data points represent least squares means; error bars, SE. KOOS, Knee Injury and Osteoarthritis Outcome Score.

margin has been used previously to indicate absence of a clinically meaningful difference between anterior cruciate ligament reconstruction and structured rehabilitation.[35] Further, baseline characteristics of our participants and changes in the exercise and education group are on par with those reported from 28 370 patients following implementation of GLAD in real-world clinical practice on three different continents.[36] Also, we delivered the exercise and education intervention according to the GLAD standards. This altogether documents the fidelity of our trial intervention and worldwide generalisability of the results.

CONCLUSION

Among individuals with knee OA, an 8-week exercise and education programme provided efficacy for symptomatic and functional improvements equivalent to that of open-label application of intra-articular inert saline injections. These findings raise important questions about mechanisms of action as well as the continued widespread recommendation of exercise and education in the management of knee OA.

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Contributors EAB and MH had full access to all of the data in the study and take responsibility for the data and the accuracy of the data analysis. Concept and design: EAB, RC, HB, LEK and MH conceived and designed the trial and the protocol that was reviewed and adjusted following important scientific and practical advice from KE, CB, DJH and RA. Drafting of the manuscript: EAB and MH. Statistical analysis: EAB, RC and MH. Obtained funding: EAB, HB and MH. Administrative, technical or material support: EAB, RC, KE, AO, JG-M, HB and MH. Supervision: DJH, RA, HB and MH. Acquisition, analysis or interpretation of data, and critical revision of the manuscript for important intellectual content: all authors. MH is the guarantor.

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TRANSLATIONAL SCIENCE

Digoxin targets low density lipoprotein receptor-related protein 4 and protects against osteoarthritis

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ABSTRACT

Objectives Dysregulated chondrocyte metabolism is closely associated with the pathogenesis of osteoarthritis (OA). Suppressing chondrocyte catabolism to restore cartilage homeostasis has been extensively explored, whereas far less effort has been invested toward enhancing chondrocyte anabolism. This study aimed to repurpose clinically approved drugs as potential stimulators of chondrocyte anabolism in treating OA.

Methods Screening of a Food and Drug Administration-approved drug library; Assays for examining the chondroprotective effects of digoxin in vitro; Assays for defining the therapeutic effects of digoxin using a surgically-induced OA model; A propensity-score matched cohort study using The Health Improvement Network to examine the relationship between digoxin use and the risk of joint OA-associated replacement among patients with atrial fibrillation; identification and characterisation of the binding of digoxin to low-density lipoprotein receptor-related protein 4 (LRP4); various assays, including use of CRISPR-Cas9 genome editing to delete LRP4 in human chondrocytes, for examining the dependence on LRP4 of digoxin regulation of chondrocytes.

Results Serial screenings led to the identification of ouabain and digoxin as stimulators of chondrocyte differentiation and anabolism. Ouabain and digoxin protected against OA and relieved OA-associated pain. The cohort study of 56 794 patients revealed that digoxin use was associated with reduced risk of OA-associated joint replacement. LRP4 was isolated as a novel target of digoxin, and deletion of LRP4 abolished digoxin's regulations of chondrocytes.

Conclusions These findings not only provide new insights into the understanding of digoxin's chondroprotective action and underlying mechanisms, but also present new evidence for repurposing digoxin for OA.

Key messages

What is already known about this subject?

- Cardenolides, including digoxin, have been used in treating heart disease for centuries, and have been reported to enhance chondrocyte function in vitro, but how they regulate chondrocytes and whether they are therapeutic against osteoarthritis (OA) remains unknown.

What does this study add?

- This study identifies digoxin as a new chondroprotective factor that protects against OA and reduces OA-associated pain in a surgically-induced mouse OA model.
- Digoxin use is associated with reduced risk of knee or hip OA-associated joint replacement among patients with atrial fibrillation.
- This study isolates lipoprotein receptor-related protein 4 (LRP4) as a new target of digoxin, thus advancing our understanding of digoxin's action and underlying mechanisms and providing a solid foundation for future discoveries relating to the digoxin/LRP4 interaction in various conditions.

How might this impact on clinical practice or future developments?

- The chondroprotective effects of digoxin on OA support the concept that cardenolides, particularly digoxin, may be a new option to treat patients with OA in clinics.

INTRODUCTION

Osteoarthritis (OA) is the most common joint disease and the leading cause of disability in older adults.[1 2] OA incurs significant financial burden with the medical cost of the disease estimated to account for between 1% and 2.5% of the gross domestic product of various high-income countries.[3] Although the complex pathogenesis of OA is not fully understood, the progressive degeneration of articular cartilage is considered the major hallmark.[4 5] Articular cartilage is commonly known as a physiologically non-self-renewing avascular tissue comprised predominately of extracellular matrix (ECM) maintained through feedback from restricted population of chondrocytes, which is composed mainly of type II collagen and aggrecan.[6 7] It is increasingly understood that OA is an active dynamic alteration arising from an imbalance between the repair and destruction of joint tissues, and not a passive degeneration or so-called wear and tear disease as commonly described. Therefore, in addition to the well-known phenomena of increased cartilage-degrading metalloproteinases and cartilage erosion, attention should be also paid to synthesis of matrix molecules and ECM remodelling in OA.[8 9] Beyond that, articular cartilage has been shown to contain a population of stem cells or progenitor cells, similar to those found in many

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other adult tissues, that are thought to be involved in the maintenance of tissue homeostasis and in ECM remodelling in OA.[10 11]

To date, there is no safe treatment available that can halt OA progression. The main goals of the current disease management are pain control and functional improvement with avoidance of therapeutic toxicity.[12] Identifying disease-modifying treatment for OA remain an urgent unmet clinical need. Considering the huge costs in terms of time and money associated with drug development, identification of new uses for old drugs is desirable.[13–15]Taking that view, we screened a drug library composed of Food and Drug Administration (FDA)-approved drugs by use of mesenchymal stem cells and chondrocytes. After preliminary validation, we found that cardenolides, represented by ouabain and digoxin, may have potential therapeutic effects against OA.

Cardenolides, one of the two subgroups of cardiac glycosides, are a class of natural biologically active steroids derived from plants and have been used for the treatment of heart disease for centuries.[16] Ouabain and digoxin, two FDA-approved cardenolides, are used to increase the contractile force of the heart and decrease its rate of contraction by inhibiting the cellular Na^+/K^+-ATPase. Besides the well-known effect of ouabain and digoxin on the cardiovascular system, compelling evidence has indicated that they also participate in the regulation of inflammation. For instance, each has been found to inhibit the expressions of pro-inflammatory cytokines such as interleukin 6 (IL-6) and IL-17 under different pathological conditions.[17–20] Additionally, previous research reported that digoxin and ouabain enhanced the functional properties of tissue-engineered cartilage in vitro.[21] However, no studies have investigated the effects of cardenolides on OA. In the present study, we performed comprehensive assays with cell and animal models as well as a large population-based cohort study to demonstrate the potential clinical use of digoxin in treating OA. Additionally, we also identified low-density lipoprotein receptor-related protein 4 (LRP4) as a new binding target of digoxin and demonstrated that LRP4 was required for digoxin regulation of chondrocytes.

RESULTS

Isolation of digoxin as a potential chondroprotective drug

To isolate the small molecule drugs that may induce messenger RNA (mRNA) expression of type II collagen (*Col2a1*), the major component of cartilage ECM, a drug library containing 1046 FDA-approved drugs was first screened. Briefly, C3H10T1/2 mesenchymal stem cells were treated with drugs for 24 hours individually, followed by thousands of quantitative Real time PCR (qRT-PCR) assays for examining the expression of type II collagen in response to individual drug treatment. Twenty drugs that increased the expression of *Col2a1* potently were identified after the first round screening (online supplemental figure 1A). These 20 drug candidates were subjected to the second round screening by treating the C28I2 chondrocytes for 24 hours separately. There were only three candidates, particularly ouabain, that could induce the expression of *COL2A1* dramatically and dose-dependently (online supplemental figure 1B,C). To further evaluate their potential anabolic effects, we treated C28I2 cells with these three drugs then examined the expressions of various genes known to associated with chondrocyte differentiation and metabolism. Among three drugs analysed, only ouabain could robustly induce the expressions of anabolic marker genes, including *COL2A1, aggrecan* (*ACAN*) and cartilage oligomeric matrix protein (*COMP*). Intriguingly, it strongly inhibited the expression of RUNX Family Transcription Factor 2 (*RUNX2*), a marker gene of chondrocyte hypertrophy and OA (online supplemental figure 1D–F). As ouabain belongs to the family of cardenolides, we also treated cells with three other cardenolides: lanatoside C, cymarin and digoxin. All three cardenolides could induce the expression of *COL2A1*, but only digoxin could significantly increase the expressions of *ACAN* and *COMP*, two additional markers of anabolism (online supplemental figure 1G). Therefore, we ended up using ouabain and digoxin as representatives of cardenolides to conduct further experiments.

Digoxin induces chondrogenesis and regulates chondrocyte metabolism

Given the stimulatory effect of ouabain and digoxin on the expression of *Col2a1* in C3H10T1/2 mesenchymal stem cells, we first sought to determine whether these two drugs could induce chondrogenesis. We treated micromass cultured C3H10T1/2 mesenchymal stem cells with either drug for 7 or 14 days. As shown in figure 1A and B, alcian blue staining validated the enhanced chondrocyte differentiation in both ouabain and digoxin treated groups compared with the phosphate-buffered saline (PBS) group. Moreover, we also examined the transcriptional levels of chondrogenic marker genes. After treatment with either drug, the mRNA expressions of *Col2a1*, *Comp*, *Acan*, SRY-Box transcription factor 5 (*Sox5*), SRY-Box transcription factor 6 (*Sox6*) and SRY-Box transcription factor 9 (*Sox9*) were all significantly upregulated (figure 1C).

Next we tested their ability to promote anabolism in both human and mouse chondrocytes. We found that both ouabain and digoxin could induce the expressions of *COL2A1 and ACAN* in human C28I2 chondrocytes. In addition, the expression of *COMP*, which encodes a protein that is present in small amounts but plays a key role in matrix composition,[22 23] was also enhanced by the drugs (figure 1D and E). We next explored whether ouabain and digoxin could upregulate anabolic marker gene expressions in mouse and human primary chondrocytes. As shown in figure 1F and G, both ouabain and digoxin could stimulate chondrocyte anabolism in a dose dependent manner. In addition, human OA chondrocytes appeared to respond better than normal chondrocytes to drug treatment (figure 1G).

Given that these two drugs were previously reported to inhibit the expressions of pro-inflammatory cytokines under different pathological conditions,[17–20] we next determined whether ouabain and digoxin could also inhibit pro-inflammatory cytokine activated catabolism in chondrocytes.[24] C28I2 chondrocytes were incubated with pro-inflammatory cytokines (tumour necrosis factor α (TNFα) or IL-1β, known to play crucial roles in the pathogenesis of OA) for 24 hours, and both ouabain and digoxin significantly reduced the mRNA expressions of catabolic markers, a disintegrin and metalloproteinases with thrombospondin type 1 motif 4 (*ADAMTS4*) and matrix metallopeptidase 13 (*MMP13*)[25] (online supplemental figures 2A and 3A). Consistently, the protein levels of ADAMTS4, MMP13 and inducible nitric oxide synthase (iNOS) were also downregulated in ouabain or digoxin treated groups (online supplemental files 2D and 3D). Mouse primary chondrocytes were also treated as described above, as shown in online supplemental figures 2B,C and 3B,C, qRT-PCR revealed significant downregulation of the expressions of catabolic marker genes, mitochondrially encoded cytochrome c oxidase II, nitric oxide synthase 2 (*Nos2*), *Mmp13*, *Mmp3* and a disintegrin and metalloproteinases with thrombospondin type 1 motif 5 (*Adamts5)*, in the drug treatment groups. The decreased protein levels of iNOS, MMP13, ADAMST5 validated the occurrence of drugs-inhibited chondrocytes catabolism (online supplemental figures 2E and 3E).

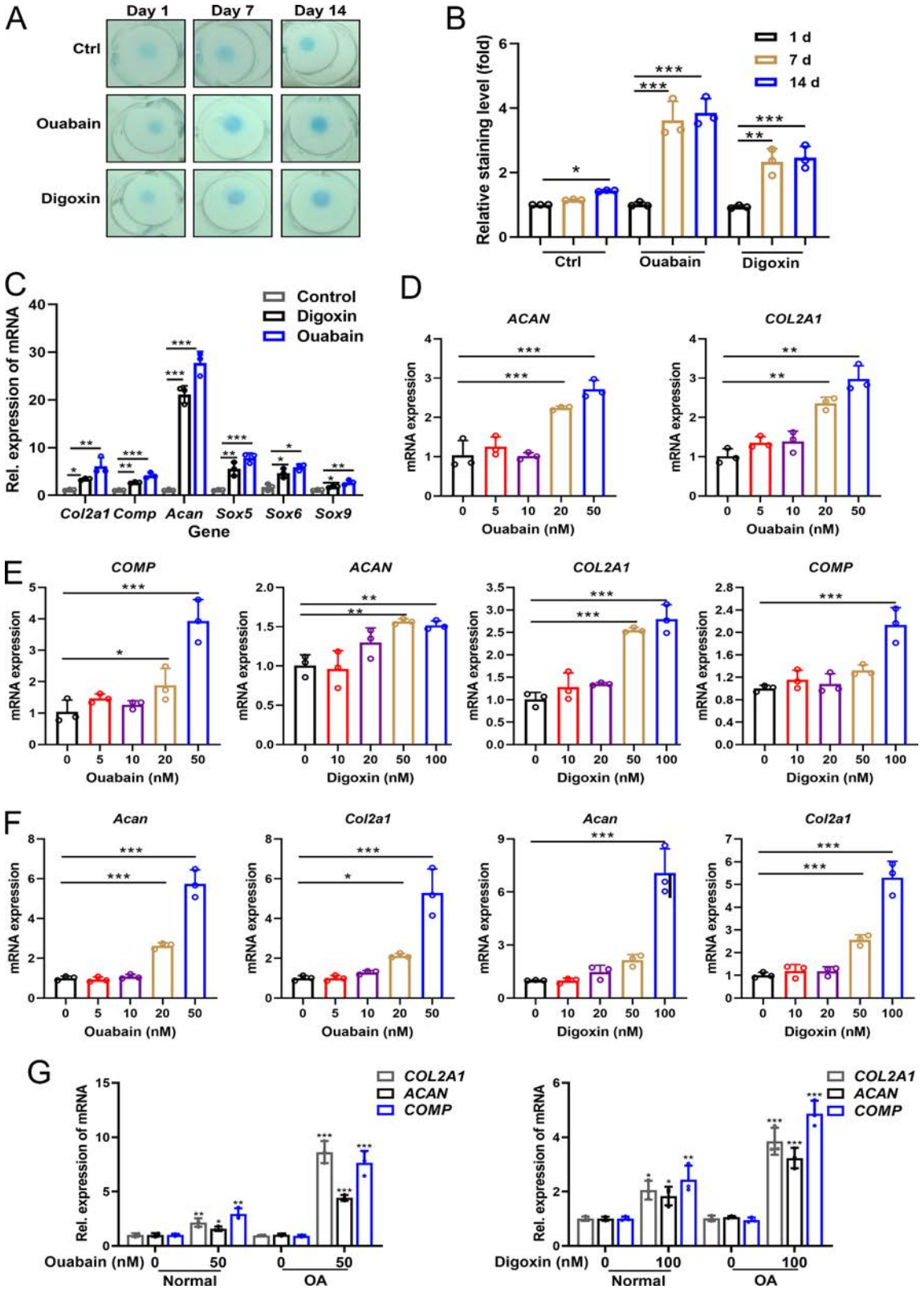


Figure 1 Ouabain and digoxin enhance chondrogenesis and stimulate chondrocyte anabolism. (A) C3H10T1/2 mesenchymal stem cells were incubated in the absence or presence of 50 nM ouabain or 100 nM digoxin for 1, 7, 14 days, followed by Alcian blue staining. (B) Quantification of (A). (C) C3H10T1/2 cells were incubated in the absence or presence of 50 nM ouabain or 100 nM digoxin for 2 or 7 days, qRT-PCR was performed to examine the expression of *Col2a1*, *Comp*, *Acan*, *Sox5*, *Sox6* and *Sox9*. (D, E) mRNA levels of *COL2A1*, *ACAN* and *COMP* in human C28I2 chondrocytes treated with a series of ouabain or digoxin for 24 hours, assayed by qRT-PCR analysis. (F) mRNA levels of *Col2a1 and Acan* in murine primary chondrocytes treated with various concentrations of ouabain or digoxin for 24 hours, assayed by qRT-PCR analysis. (G) mRNA levels of *COL2A1*, *ACAN* and *COMP* in human primary normal and OA chondrocytes treated with or without ouabain or digoxin for 24 hours, assayed by qRT-PCR analysis. The values are mean±SEM of at least three independent experiments; *p<0.05, **p<0.01, ***p<0.001 vs control group. ACAN, aggrecan; COL2A1, type II collagen; COMP, cartilage oligomeric matrix protein; mRNA, messenger RNA; OA, osteoarthritis; qRT-PCR, quantitative Real time PCR.

Digoxin protects against OA in a surgically induced model in vivo

To examine the effect of ouabain and digoxin on OA progression, we performed destabilisation of the medial meniscus (DMM) surgery[26] in 12-week-old male C57BL/6J mice with or without ouabain or digoxin administration commencing 3 days after surgery (figure 2A). We carried out histological analyses to assess knee joint damage 12 weeks after DMM surgery. As expected, cartilage degeneration was severe at 12 weeks after DMM surgery, evidenced by markedly increased Osteoarthritis Research Society International scores. The administration of both ouabain and digoxin (50 nM and 100 nM, respectively, for intra-articular injection) caused partial but significant reduction in cartilage degradation (figure 2B and C). By immunohistochemical staining, a significant decrease in type II collagen in cartilage was observed 12 weeks after DMM surgery, which was greatly inhibited by ouabain or digoxin use (figure 2D). However, the levels of the markers of chondrocytes catabolism (cleaved ACAN, MMP13 and ADAMTS5) was increased after DMM surgery, and ouabain or digoxin administration inhibited these changes (figure 2E). As illustrated in previous studies, during the evolution of OA, the subchondral bone undergoes marked changes in its composition and structural organisation.[27] Micro-computerised tomography (μCT) analysis performed in this study also showed significant difference in knee joint structure of mice in different treatment groups. Increased osteophyte formation was observed in mice 12 weeks after DMM surgery, and this effect was significantly inhibited by treatment with ouabain or digoxin (figure 2F–I). The volume of calcified meniscus and synovial tissue was also quantified and exhibited similar trends to those observed in osteophytes (figure 2F, J and K). We further analysed changes in subchondral bone mass and found that subchondral bone mass was significantly increased 12 weeks after DMM surgery and ouabain or digoxin had no significant effect on DMM-induced subchondral bone mass increase (online supplemental figures 4A-E). Collectively, these results suggest that ouabain and digoxin can limit OA development in the injury-induced OA mouse model.

We also performed a series of tests to determine if ouabain and digoxin could lower OA pain sensitivity. The results of von Frey tests showed significantly reduced paw withdrawal response thresholds after DMM surgery in mice. The administration of ouabain and digoxin could significantly increase paw withdrawal response threshold, reflecting reduced pain sensitivity in mice (figure 3A). We also tested spontaneous activity of the mice in response to DMM surgery and cardenolide treatment by performing an open field test. We found that travel distance, maximum walking speed, active time and absolute turn angle decreased over time after DMM surgery. Treatment with ouabain or digoxin could significantly reverse reduced spontaneous activity caused by DMM surgery (figure 3B–F). Taken together, ouabain and digoxin were associated with reduced mechanical pain sensitivity and enhanced spontaneous ambulatory activity relative to untreated controls.

Digoxin use is associated with reduced risk of knee or hip OA-associated joint replacement among patients with atrial fibrillation

To determine whether use of digoxin associates with OA in human patients, we performed a sequential propensity score matched cohort study using data from The Health Improvement Network (THIN) in the UK. The flow charts depict the selection process of individuals are shown in online supplemental file 5). The baseline characteristics of each propensity score matched cohort are shown in online supplemental table 2). After propensity score matching, 56 794 patients were included in the analysis (n=28 397 for each group). The mean age was 74.1 (SD: 9.8) years among the digoxin cohort and 75.1 (SD: 9.7) years among the non-user cohort; the female proportions were 49.1% and 51.5%, respectively. Overall, the characteristics among the propensity score matched cohorts were well-balanced, with all standardised differences <0.1. Digoxin initiators had a lower risk of knee or hip OA-associated joint replacement than non-users (figure 4). As shown in table 1, 739 cases of knee or hip OA-associated joint replacement occurred among 28 397 digoxin users (5.8 per 1000 person-years) and 854 cases occurred among 28 397 non-users (6.8 per 1000 person-years). The rate difference in knee or hip OA-associated joint replacement between digoxin initiators and non-users was −0.9 (95% CI: −1.5 to −0.3) per 1000 person-years and the HR was 0.85 (95% CI: 0.77 to 0.93). Results from the sensitivity analyses (ie, excluding participants who had knee or hip replacement within 3 months after the index date, excluding participants who had extreme propensity scores and missing data imputation analysis) did not change substantially (table 1)

Digoxin regulates chondrocyte metabolism through ERK1/2, AKT and NF-κB signalling pathways

It is widely accepted that the extracellular signalling-regulated kinases (ERK) and protein kinase B (PKB/AKT) signalling pathways play an essential role in chondrocyte anabolism.[28 29] To investigate whether ouabain and digoxin activated anabolism through ERK1/2 and AKT signalling pathways, we treated chondrocytes with ouabain or digoxin at different concentrations or different time points, and then performed Western blot analysis for total and phosphorylated ERK1/2 and AKT. As shown in online supplemental figure 6A,B, ouabain and digoxin could induce the phosphorylation of ERK1/2 and AKT in a dose and time dependent manner. Notably, ouabain and digoxin mediated activation of ERK1/2 and AKT signalling pathways and expressions of anabolic marker genes, including *COL2A1*, *ACAN* and *COMP*, were abolished by U0126 (ERK inhibitor) and wortmannin (AKT inhibitor), respectively, (online supplemental figure 6C-F). These results revealed that ouabain and digoxin activated chondrocyte anabolism through ERK1/2 and AKT signalling pathways.

Pro-inflammatory cytokines TNFα and IL-1β play key roles by activating p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) signalling and the transcription factor nuclear factor kappa B (NF-κB), in the pathogenesis of OA.[30] Ouabain and digoxin could inhibit both TNFα and IL-1β-induced chondrocytes catabolism promoted us to determine whether these two drugs could also affect these signalling pathways. Both ouabain and digoxin had no effect on the activation of p38 MAPK and JNK activated by TNFα, while dramatically inhibit NF-κB phosphorylation and transcriptional activity (online supplemental figure 7A-E). As illustrated in online supplemental figure 7F, p65 translocation from the cytoplasmic to the nuclear compartment on stimulation with TNFα was almost abolished in the presence of ouabain or digoxin.

LRP4 is a novel target of digoxin

Cardenolides bind to Na^+/K^+-ATPase and inhibit its activity. Accordingly, we sought to investigate whether ouabain and digoxin mediated chondrocyte metabolism through Na^+/K^+-ATPase. We thus suppressed the activity of Na^+/K^+-ATPase

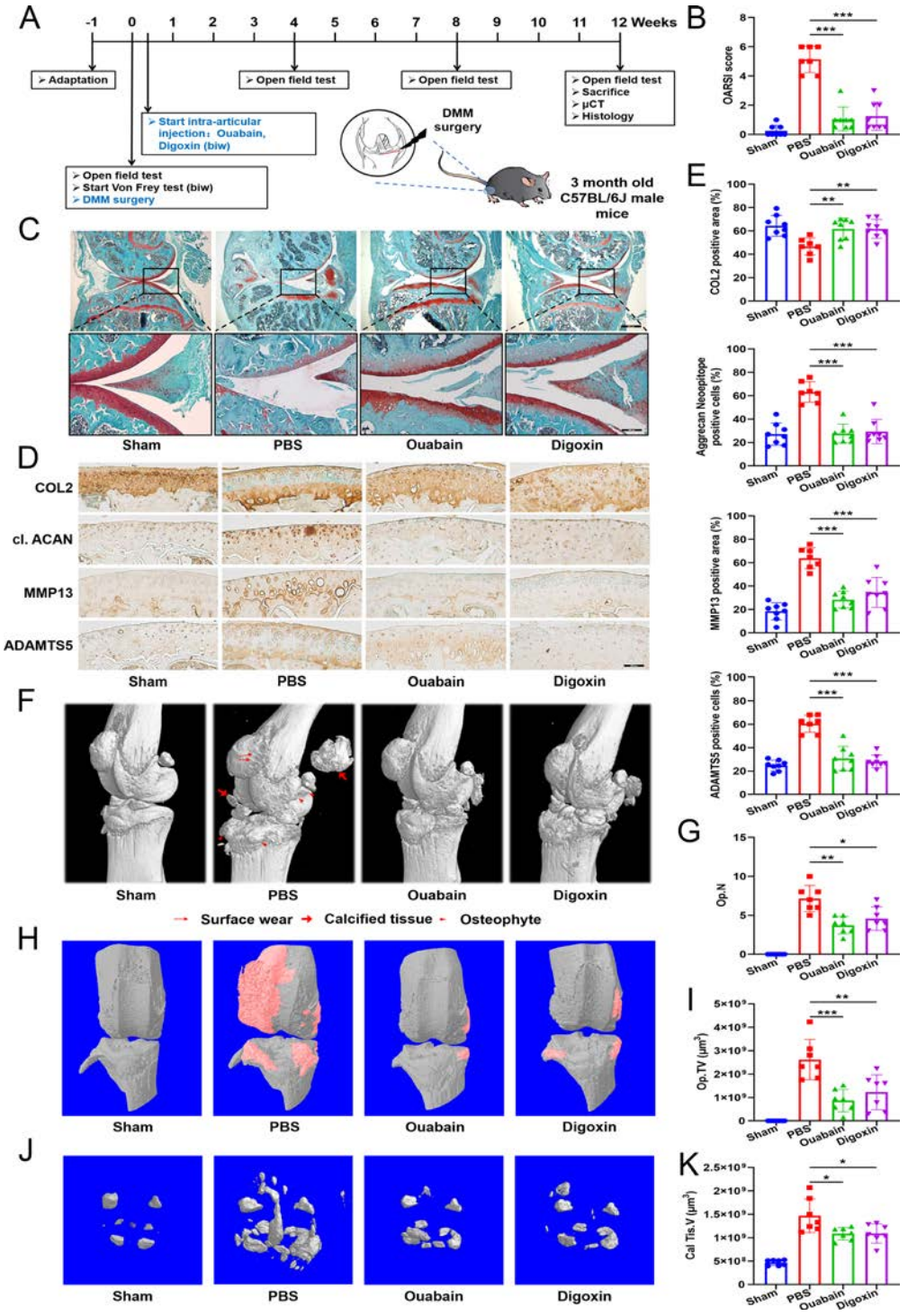


Figure 2 Ouabain and digoxin protect against OA in a surgically induced model in vivo. (A) Experimental flow chart. DMM surgery was performed in 12-week-old male C57BL/6 J mice. Ouabain or digoxin was administered 3 days after DMM surgery (n=8; a mouse in the PBS control group died of unknown causes 10 weeks after surgery). (B) The severity of OA-like phenotype 12 weeks after surgery was analysed by grading histological sections using the Osteoarthritis Research Society International score system. (C) Representative images of safranin O/Fast green stained sections of knee joints from mice treated with or without ouabain or digoxin for 12 weeks. Scale bar=800 μm (top panel) and 200 μm (bottom panel). (D, E) Representative images of immunohistochemical staining for type II collagen, aggrecan neoepitope, MMP13 and ADAMTS5 in knee joint sections of mice treated with or without ouabain or digoxin for 12 weeks. Scale bar=100 μm. Positive staining for type II collagen, aggrecan neoepitope, MMP13 and ADAMTS5 were quantified. (F) Three-dimensional mirco-CT images of pathological structural changes in the mouse knee 12 weeks after surgery. (G) Osteophyte number (Op.N) and (I) size (Op.TV) in the knee of mice after DMM surgery. (H) Three-dimensional mirco-CT images of osteophyte formation between the groups. The region marked in red shows osteophyte. (J) Three-dimensional mirco-CT images of calcified meniscus and synovial tissue between the groups. (K) The volume of calcified meniscus and synovial tissue (CAL Tis.V) was quantified. *p<0.05, **p<0.01, ***p<0.001 vs PBS control group. ADAMTS5, a disintegrin and metalloproteinases with thrombospondin type 1 motif 5; *COL2A1*, type II collagen; DMM, destabilisation of the medial meniscus; MMP13, matrix metallopeptidase 13; OA, osteoarthritis; PBS, phosphate-buffered saline; μCT, micro-computerised tomography.

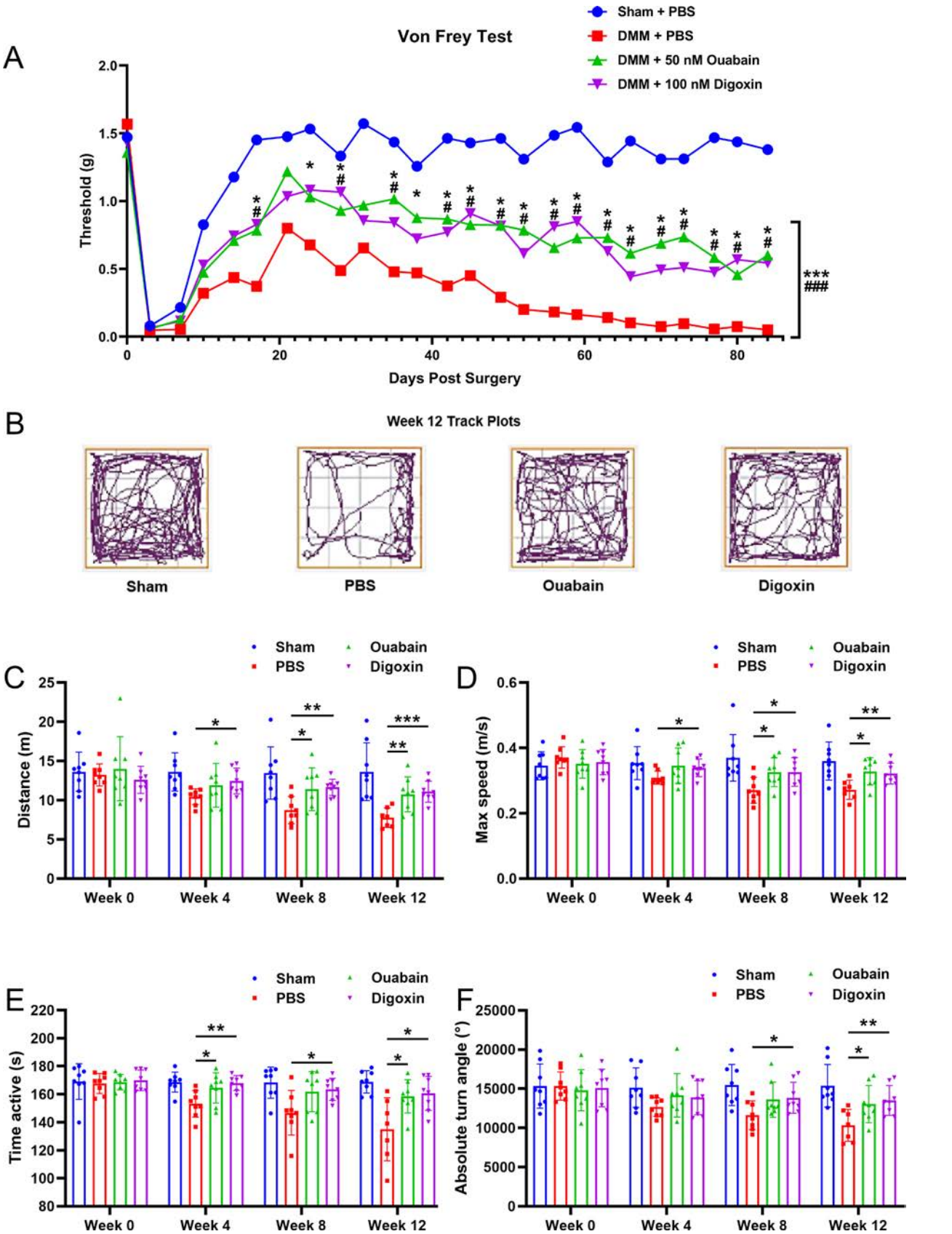
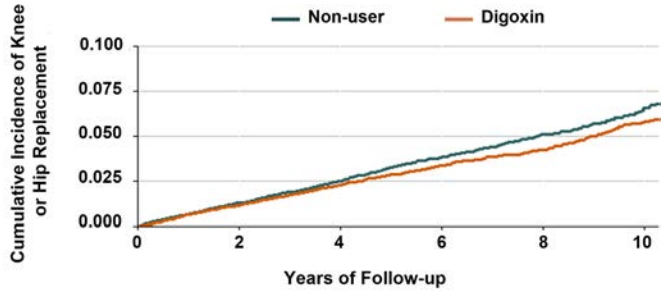


Figure 3 Effect of ouabain and digoxin on pain-related behaviours in the DMM-induced OA mice model. (A) Mechanical sensitivity was measured using von Frey filaments two times a week after DMM surgery. Statistical analysis was conducted using two-way analysis of variance and multiple T tests. P values were compared between ouabain (*) or digoxin (#) group and PBS control group. (B) Representative track plots show decreased spontaneous activity of mice in open field tests after DMM surgery. Changes in spontaneous activity, including (C) travel distance, (D) max speed, (E) active time and (F) absolute turn angle were evaluated 4, 8 and 12 weeks after DMM surgery. * or #p<0.05, ** or ##p<0.01, *** or ###p<0.001 vs PBS control group. DMM, destabilisation of the medial meniscus; OA, osteoarthritis; PBS, phosphate-buffered saline.



No. at risk						
Non-user	28397	19432	12789	8092	4891	2696
Digoxin	28397	19285	12840	8281	4974	2771

Figure 4 Cumulative incidence of knee or hip osteoarthritis-associated joint replacement in 28 397 digoxin users and 28 397 non-users, matched by propensity-score.

by treating C28I2 cells with Na^+/K^+-ATPase activity inhibitor istaroxime hydrochloride. To our surprise, the inhibition of Na^+/K^+-ATPase activity did not affect either drug enhancement of anabolism or inhibition of catabolism (online supplemental figure 8A,B). This finding indicates that ouabain and digoxin may have targets other than Na^+/K^+-ATPase in mediation of chondrocyte homeostasis.

To address this issue, we performed the drug affinity responsive target stability (DARTS) assay and separated proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A band with the molecular weight of around 200 kDa was shown to be clearly protected by ouabain (figure 5A). This band was excised for the unbiased protein identification by mass spectrometry (figure 5B), which identified LRP4 as a potential candidate. LRP4 is known to antagonise LRP5/6 signalling and mediate bone homeostasis.[31] To confirm whether LRP4 is the target of ouabain and digoxin, we employed DARTS assay using a series of concentrations of protease to digest cell lysate with or without ouabain or digoxin incubation, and found that both drugs could protect LRP4 against enzymatic digestion (figure 5C).

To further confirm the interaction between LRP4 and ouabain and digoxin, we performed the cellular thermal shift assay (CETSA),[32 33] which shows the change in thermal denaturation temperature for a target protein in the presence of various drug dosages. Both ouabain and digoxin prevented the denaturation of LRP4 at various temperatures, especially at 49°C, compared with control group (figure 5D). The melting curve showed a robust change of melting temperature (T_m) in the presence of ouabain or digoxin with T_m for control, ouabain and digoxin conditions 42.63, 46.86°C and 49.28°C, respectively (figure 5D). We also performed CETSA at 49°C with different concentrations of drugs and demonstrated that ouabain and digoxin prevented LRP4 denaturation in a dose-dependent manner with the EC50 of 5.98E-01 and 5.74E-02, respectively (figure 5E).

To further unravel the associations of LRP4 with ouabain and digoxin, we then employed molecular docking simulations. The structures of ouabain and digoxin were docked with three monomers of homology modelled human LRP4, respectively. Glide XP docking showed that both ouabain and digoxin had relatively lower binding free energy when they were docked into LRP4 monomer aa 146–737 compared with the other two monomers, which was reflected by the more negative values of docking scores (online supplemental figure 9), suggesting that ouabain and digoxin may have better affinity to the region from residue 146 to 737 of LRP4. Induced-fit docking (IFD) was then performed for ouabain and digoxin with LRP4 monomer aa146-737 to get optimal binding simulation. From IFD simulation, both ouabain and digoxin were predicted to majorly interact with LRP4 with docking scores of −12.496 kcal/mol and −10.149 kcal/mol (figure 5F). The binding pocket for ouabain and digoxin was lined by residues 232–239, 243–252 and 340–348 of LRP4. With abundant hydroxyl groups in the structure, ouabain and digoxin could form multiple hydrogen bonding interactions with LRP4, which may contribute to the good binding affinity. All the hydroxyl groups of ouabain are predicted to be involved in hydrogen bonding interactions with LRP4, which bonded with residues Arg232, Glu235, Phe236, Met237, Cys238, Arg249, Asn340 and Ser344, respectively. Several hydroxyl groups of digoxin served as hydrogen bond donors interacting with the side chains of Phe236, Asp239, Asp342, Glu343, and Asn348 of LRP4. Additionally, digoxin was exhibited to be involved in hydrogen bonds as acceptor of LRP4 at residues Asn340 and Asn348 (figure 5G–J).

We next mutated the sites of LRP4 mentioned above and performed DARTS to determine key site(s) for the binding between LRP4 and drugs (figure 5K). Results showed that ouabain largely lost, while digoxin totally lost, its protective effect on the Glu-343 mutant of LRP4 (figure 5L and M), demonstrating that Glu-343 is the critical amino acid for the drugs targeting to LRP4.

Table 1 Association between digoxin and risk of knee or hip replacement due to osteoarthritis among patients with atrial fibrillation

	Digoxin (n=28 397)	Non-user (n=28 397)
Event (n)	739	854
Mean follow-up (years)	4.46	4.45
Rate of event, /1000 person-years	5.83	6.75
Rate difference (95% CI), /1000 person-years	−0.9 (−1.5 to −0.3)	0.0 (reference)
HR (95% CI)	0.85 (0.77 to 0.93)	1.00 (reference)
Three-month lag, HR (95% CI)*	0.89 (0.80 to 0.98)	1.00 (reference)
PS trimming, HR (95% CI)†	0.86 (0.78 to 0.95)	1.00 (reference)
Missing data imputation, HR (95% CI)‡	0.85 (0.77 to 0.95)	1.00 (reference)

*This analysis introduced a 3 month exposure lag period to exclude patients with knee or hip replacement within 3 months after treat date.
†Asymmetric trimming was used to exclude participants whose propensity score was below the 2.5th percentile of the propensity score of the digoxin cohort and above the 97.5th percentile of the propensity score of the comparator cohort.
‡Imputation analysis was performed to deal with missing data. Specifically, missing values of the variables (ie, body mass index, smoking, drinking status or Townsend Deprivation Index) were imputed by a sequential regression method based on a set of covariates as predictors.
95% CI, 95% confidence interval; HR, hazard ratio; PS, propensity score; RD, rate difference.

LRP4 is downregulated in OA and its deficiency abolishes digoxin regulation of chondrocyte metabolism

To explore the relationship of LRP4 and OA, we examined the expression of LRP4 in human normal and patients with OA cartilage. LRP4 protein level was reduced in human OA cartilage compared with non-arthritic controls (figure 6A–C). Furthermore, in line with the results in human cartilage, the protein expression of LRP4 was decreased after murine DMM surgery, whereas intriguingly, the administration of ouabain and digoxin

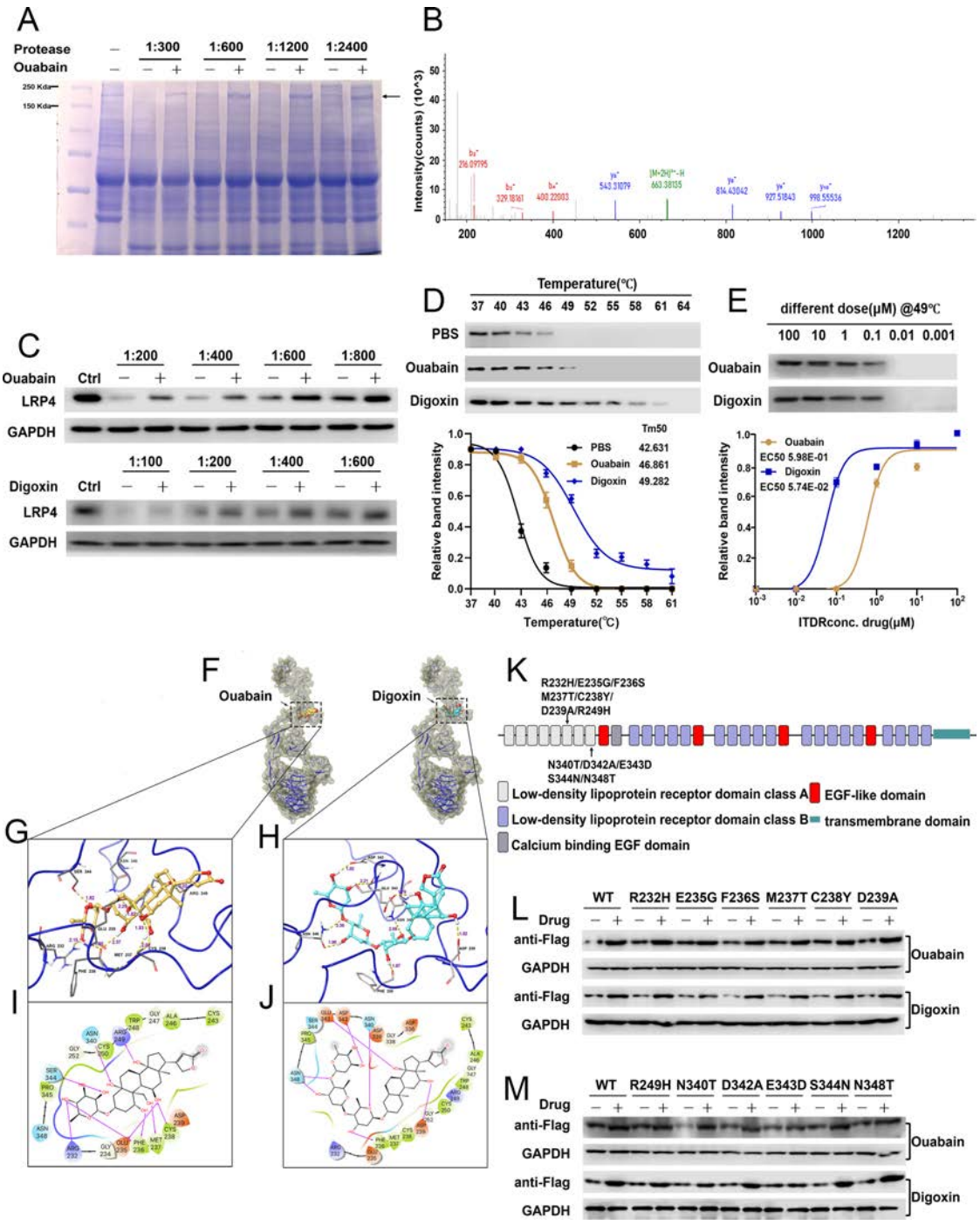


Figure 5 LRP4 is the target of ouabain and digoxin. (A) Coomassie blue staining of DARTS assay. The band with molecular weight around 200 kDa was protected by ouabain. (B) LRP4 adapted image from mass spectrometry. (C) C28I2 cells were digested with several dosages of protease with or without various concentrations of drugs, as indicated, then the level of LRP4 was assayed using Western blot. (D) C28I2 cell lysate was denatured under various temperatures and the protein level of LRP4 in control, ouabain-treated and digoxin-treated groups were assayed using Western blot and densitometry analysis curve. (E) Isothermal dose response with serial concentrations of drug. protein level of LRP4 was measured via Western blot with associated curve. (F) Overview of IFD-predicted binding positions of ouabain and digoxin in LRP4 AA 146–737 monomer. LRP4 is shown by ribbons along with yellow surface of 70% transparency. Ouabain and digoxin are shown by CPK representation with the following colour scheme: carbon-faded orange (ouabain) or teal (digoxin), oxygen-red, polar hydrogen-white. Non-polar hydrogen atoms are not shown. (G, H) IFD-predicted docked LRP4-Ouabain complex (G) and LRP4-digoxin complex (H) with the ligand depicted in ball and stick and the important interacting residues depicted as sticks. Hydrogen bonds are represented by dotted yellow lines and the distance of hydrogen bonds are measured in Å. (I, J) The two-dimensional interaction diagrams of ouabain (I) and digoxin (J) docked with LRP4 AA 146–737. The amino acids within 4 Å to the ligand are shown as coloured bubbles, where polar residues are cyan, hydrophobic residues are green, positively charged residues are purple, and negatively charged residues are red. Hydrogen bonds are shown by magenta arrows. (K) Schematic view of LRP4 receptor domain organisations and localisation of mutations tested. (L, M) DARTS assay for serial point mutants of LRP4. C28I2 cells were transfected with the plasmid expressing various Flag-tagged LRP4 mutants, as indicated. Mutants of LRP4 were detected by flag antibody. The values are mean±SEM of at least three independent experiments. CPK, Corey-Pauling-Koltun; DARTS, drug affinity responsive target stability; IFD, induced-fit docking; LRP4, lipoprotein receptor-related protein 4.

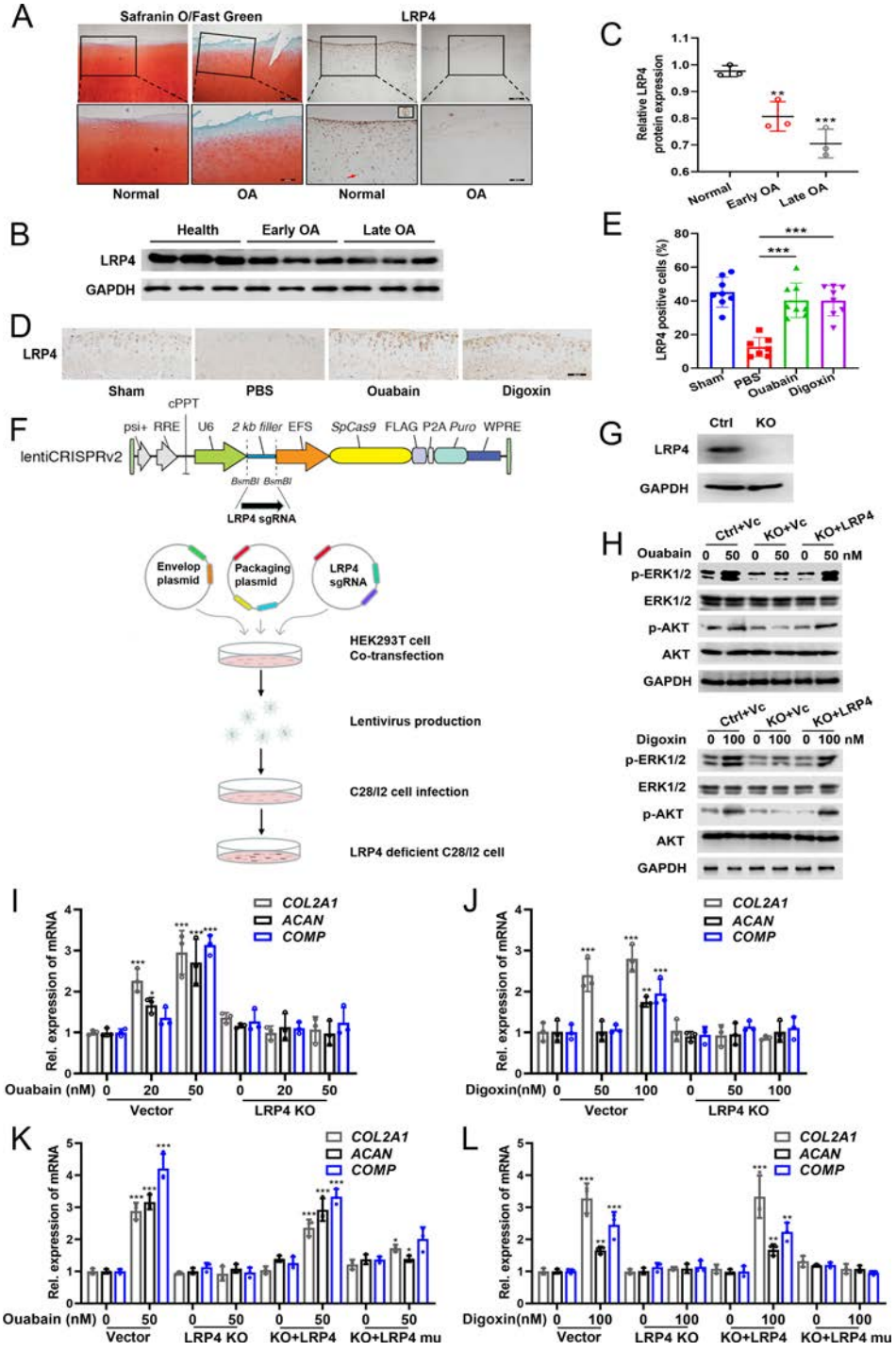


Figure 6 LRP4 is downregulated in OA cartilage and required for ouabain and digoxin regulation of chondrocyte anabolism. (A) Human non-arthritic and OA cartilage stained with safranin O/Fast green (left) and detected LRP4 by immunohistochemical staining (right). The red arrow and the inserted image indicate the expression of LRP4 in a single chondrocyte. Scale bar=400 µm (top panel) and 200 µm (bottom panel). (B) Expression of LRP4 was measured in healthy, early OA and late patients with OA via Western blot. (C) Densitometry analysis of immunoblotting results shown in (B). (D, E) Immunohistochemical staining of LRP4 and quantification of LRP4 positive cells in knee joint sections of C57BL/6 J mice treated with or without cardenolides for 12 weeks after DMM surgery. Scale bar=100 µm; n≥7. (F) Workflow of generating LRP4 knockout C28I2 cells using CRISPR/Cas9 technology. (G) Western blot confirmation of LRP4 knockout in C28I2 cells. (H) Immunoblotting of ERK1/2 and AKT signalling activation in control and LRP4 knockout C28I2 cells with or without re-expression of LRP4 treated with 50 nM ouabain or 100 nM digoxin for 30 min. (I, J) mRNA levels of *COL2A1*, *ACAN* and *COMP* in control or LRP4 knockout C28I2 cells treated with a series of ouabain or digoxin concentrations for 24 hours, assayed by qRT-PCR analysis. (K, L) Control and LRP4 knockout C28I2 cells with or without re-expression of wild type or Glu343 site mutated LRP4 were treated with 50 nM ouabain or 100 nM digoxin for 24 hours, mRNA levels of *COL2A1*, *ACAN* and *COMP* were detected by qRT-PCR. LRP4 mu: Glu343 site mutation of LRP4. The values are mean±SEM of at least three independent experiments; *p<0.05, **p<0.01, ***p<0.001 vs control group. ACAN, aggrecan; AKT, rotein kinase B/PKB; pCOL2A1, type II collagen; COMP, cartilage oligomeric matrix protein; DMM, destabilisation of the medial meniscus; ERK, extracellular signalling-regulated kinases; LRP4, lipoprotein receptor-related protein 4; mRNA, messenger RNA; OA, osteoarthritis; qRT-PCR, quantitative real time PCR.

prevented OA-associated LRP4 downregulation (figure 6D and E).

To define the role of LRP4 in mediating ouabain and digoxin's effects on chondrocyte metabolism, we efficiently deleted the *LRP4* gene using CRISPR-Cas9 genome editing strategy (figure 6F and G). Notably, the activation of ERK1/2, AKT signalling pathways induced by ouabain and digoxin were abolished in LRP4 knockout chondrocytes, leaving expression of the anabolic marker genes, such as *COL2A1, ACAN and COMP*, unchanged after drug treatment (figure 6H, I and J). Furthermore, ouabain and digoxin mediated inhibition of NF-κB activation was lost in LRP4 knockout cells, resulting in increased expression of catabolic genes, including *ADAMTS4* and *MMP13* (online supplemental figure 10A-D).

We next re-expressed wild type or Glu343 mutated LRP4 in LRP4 knockout C28I2 chondrocytes. Transfection of wild type LRP4 expression plasmid reinstated ouabain and digoxin induced activation of ERK1/2, AKT signalling pathways and chondrocyte anabolism (figure 6H, K and L) and also restored the anti-NF-κB effect of ouabain and digoxin and the inhibition of chondrocyte catabolism (online supplemental figure 10A,B,E,F). However, expression of LRP4 containing a Glu343 site mutation in LRP4 knockout C28I2 cells did not restore the regulatory effects of ouabain and digoxin on chondrocyte metabolism (figure 6K and L, online supplemental figure 10E,F). Taken together, these findings indicated that ouabain and digoxin facilitation of anabolism and inhibition of catabolism in chondrocytes is LRP4-dependent and Glu343 is critical for drug-mediated regulation of chondrocyte metabolism.

DISCUSSION

We have made several key observations in the studies presented here. (1) After performance of three rounds of qRT-PCR screening of an FDA-approved drug library, we identified ouabain and digoxin as candidates with potential to promote the anabolism of chondrocyte ECM. (2) We found that the administration of ouabain or digoxin limited OA development and relieved OA-associated pain sensitivity in mice, and our large population-based cohort study provides clinical evidence that digoxin use was associated with a reduced risk of knee or hip OA-associated joint replacement among patients with atrial fibrillation. (3) Through combined use of DARTS, proteomics, CETSA, IFD and generation of point mutations, we identified LRP4 as a previously unrecognised target of digoxin and found that digoxin's regulation of chondrocytes depends on the presence of LRP4.

No previous studies have directly investigated the effects of cardenolides use on OA. Though indirect evidence concerning the utility of these compounds in facilitating cartilage homeostasis has been provided through in vitro study which found that ouabain and digoxin could increase the functional properties of bovine articular chondrocytes. This promotion may be related to the amount of collagen cross-linking and increased Ca^{2+} oscillations.[21] In the current study, we have implemented a murine surgically–induced OA model to provide in vivo evidence that both ouabain and digoxin can limit OA development and relieve OA-associated pain. Consistently, the anabolism of ECM of chondrocytes is promoted, while the catabolism is inhibited, by ouabain and digoxin. To explore the mechanism of this dual protective effect, we studied multiple pathways closely related to OA by using specific inhibitors of each pathway. The results confirmed that activation of AKT and ERK pathways are involved in ouabain and digoxin-mediated promotion of ECM anabolism, while these cardenolides exert inhibitory effects on NF-κB pathway activation lending to reduced chondrocyte catabolism.

Digoxin is known as the only safe inotropic drug for oral use that improves hemodynamics.[34] This led us to hypothesise that records related to digoxin might be accessible in general practitioner based medical records databases. Herein, we performed a sequential propensity score matched cohort study via leveraging the data from THIN. THIN contains health information on approximately 17 million patients from 790 general practices in the UK. THIN data reflects a routine medical practice environment and have been shown to be valid for use in clinical and epidemiological research studies.[35–37] Excitingly, we found that digoxin use was associated with reduced risk of joint replacement surgery due to OA in a large population with atrial fibrillation. Despite the inevitable limitations of this database-based study (confounding factors, information authenticity and lack of knee or hip images), this study has significant implications for the clinical prospects of digoxin against OA.

Most of the biological activities of cardenolides are based on their ability to inhibit the membrane-bound Na^+/K^+-ATPase.[16] Inhibition of Na^+/K^+-ATPase has been shown to induce cell proliferation, autophagy and even apoptosis, not only in cardiac myocytes but also other several cell lines.[38 39] Under the intervention condition in this study, both ouabain and digoxin treatment did not show any toxicity (online supplemental figure 11). Neither the promoting effect of ouabain and digoxin on cartilage ECM anabolism nor the inhibitory effect on ECM catabolism was affected by inhibiting the activity of Na^+/K^+-ATPase with Istaroxime hydrochloride in chondrocytes. This suggested that ouabain and digoxin may regulate cartilage ECM homeostasis through a completely different target that has not yet been discovered. Therefore, through combined use of DARTS, proteomics, CETSA, IFD and point mutations, we identified LRP4 as the target and Glu-343 as the critical amino acid involved in the interaction with digoxin. By knockout and re-expression of LRP4 in chondrocytes, we demonstrated that LRP4 is indispensable in the process of both ouabain and digoxin promoting ECM synthesis by regulating ERK and AKT pathways and inhibiting ECM degradation by regulating NF-κB pathway (online supplemental figure 12).

LRP4 is a member of the low-density lipoprotein receptor family. At neuromuscular junction, neuronal agrin binds LRP4 in a complex with the muscle-specific kinase to form neuromuscular synapse.[40] In recent decades, increasing reports describing mutations of members of the LRP family and their relation to bone has reinforced the important role of LRP family members in the pathogenesis of devastating diseases such as osteoporosis, rheumatoid arthritis and OA.[31] LRP4 was reported to induce gene expressions of ECM proteins, as well as production of total proteoglycans in ATDC5 chondrocyte cells, whereas LRP4 knockdown had opposite effects and reduced mRNA expression of SOX9, a master regulator for chondrogenesis.[41] LRP4 was also reported to upregulate SOX9 expression in primary bovine chondrocytes, and LRP4 was indispensable for the promotion of chondrocyte differentiation and cartilage formation mediated by agrin.[42] These findings are consistent with our current report of reduced LRP4 expression in OA cartilage and that the dependence of the chondroprotective effect of ouabain and digoxin on LRP4. However, the specific mechanism by which LRP4 affects chondrocyte function warranties further investigations. Despite the lack of current knowledge regarding LRP4 in chondrocytes, the roles of LRP4 and other members of the LRP family in bone has long been studied. LRP4 and family-member

LRP5/6 have opposite effects on bone mass regulation[31 43] In OA specifically, in addition to the lack of reports on LRP4, the role of LRP5 and LRP6 is also controversial.[44–47] Therefore, although LRP4 has been found to regulate Wnt and bone morphogenetic protein (BMP) pathways in other physiological and pathological processes,[48–50] further studies are needed to determine whether LRP4 can affect the development of OA by regulating these pathways and thereby mediating the interaction with other LRPs. In our study, both ouabain and digoxin regulated the expression of the proteins related to AKT, ERK and NF-κB pathways in chondrocytes. This provides a new clue for the mechanism of LRP4 in chondrocyte function and the development of OA.

This study carries significance from several perspectives: This study identifies digoxin as a stimulator of chondrocyte differentiation and anabolism, and uncovers a new strategy for enhancing chondrocyte function and cartilage integrity. Thus, digoxin may be used for treating various chondrocyte-associated diseases and conditions, particularly OA. Due to the unique mechanism of action (eg, targeting LRP4 to enhance chondrocyte function), digoxin may be effective for patients with OA who fail to respond to current anti-inflammatory and anti-catabolic treatments. This study identifies LRP4 as a new target of digoxin, thus advancing our understanding of digoxin's action and underlying mechanisms, and also providing a solid foundation for future discoveries related to this digoxin/LRP4 interaction in various conditions.

Limitations of this study should be also acknowledged. Although previous studies have used joint replacement as a proxy for end-stage OA and joint replacement has been generally accepted as a clinically relevant 'hard' outcome in cohort studies of OA,[51–53] knee images and functional data were not available in THIN; thus, we were unable to examine the association between ddigoxin and the risk of progression of structural lesions and functional deterioration of OA. Although we conducted a 1:1 propensity score matched cohort study to control for many potential confounders, this may limit generalisability of our findings to patients with OA whose characteristics differ from patients with OA included in the current study. Although we have demonstrated that digoxin and ouabain can relieve OA-related pain in vivo, we failed to delve deeply into the mechanisms. The weak correlation between pain and cartilage loss, the complex classification of pain and the conduction involved,[54 55] give us reason to believe that further research, richer tools and extended time points are needed to uncover a full story. Further, additional in vivo characterisation and pharmaceutic kinetic assays of the drug in OA, including dose dependence, frequency and route of delivery, as well as treatment duration, warrant further investigations with preclinical animal models and human clinical trials.

In summary, functional studies, including various cell-based assays, in vivo animal model, and analysis of a large cohort of human patients, support the concept of using digoxin as novel treatment for patients with OA in clinic. Additionally, combined use of various approaches isolates LRP4 as the new target of digoxin responsible for its chondroprotective action. Thus, this study not only provides new insights into the understanding of digoxin's action and underlying mechanisms, but may also broaden the clinical application of digoxin.

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Contributors K-dW and XD designed and performed the experiments, collected and analysed the data and drafted the manuscript. NJ and X-yC participated in chondrogenesis-related experiments and identification of the drug target. CZ and JW performed the cohort study. AH assisted with human sample collection and language modification. AK participated in drug screening. Z-nL and Z-sC performed the induced-fit docking simulations. G-hL had full access to the database in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. C-jL had the concept, supervised all of the experiments and revised the manuscript. C-jL as the guarantor and responseible for the overall content. All authors have read, provided critical feedback on intellectual content and approved the final manuscript.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval All animal procedures were carried out in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee of New York University. Human subjects research was performed according to the Institutional Review Boards at New York University Medical Center (IRB Study Number i11-01488). This study was approved by the THIN Scientific Review Committee (20SRC003). THIN is a registered trademark of Cegedim SA in the UK and other countries. Reference made to the THIN database is intended to be descriptive of the data asset licensed by IQVIA. This work uses de-identified data provided by patients as part of their routine primary care.

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Data availability statement All data relevant to the study are included in the article. Not applicable.

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EPIDEMIOLOGICAL SCIENCE

Impact of adiposity on risk of female gout among those genetically predisposed: sex-specific prospective cohort study findings over >32 years

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ABSTRACT

Objectives To evaluate the joint (combined) association of excess adiposity and genetic predisposition with the risk of incident female gout, and compare to their male counterparts; and determine the proportion attributable to body mass index (BMI) only, genetic risk score (GRS) only, and to their interaction.

Methods We prospectively investigated potential gene-BMI interactions in 18 244 women from the Nurses' Health Study and compared with 10 888 men from the Health Professionals Follow-Up Study. GRS for hyperuricaemia was derived from 114 common urate-associated single nucleotide polymorphisms.

Results Multivariable relative risk (RR) for female gout was 1.49 (95% CI 1.42 to 1.56) per 5 kg/m^2 increment of BMI and 1.43 (1.35 to 1.52) per SD increment in the GRS. For their joint association of BMI and GRS, RR was 2.18 (2.03 to 2.36), more than the sum of each individual factor, indicating significant interaction on an additive scale (p for interaction <0.001). The attributable proportions of joint effect for female gout were 42% (37% to 46%) to adiposity, 37% (32% to 42%) to genetic predisposition and 22% (16% to 28%) to their interaction. Additive interaction among men was smaller although still significant (p interaction 0.002, p for heterogeneity 0.04 between women and men), and attributable proportion of joint effect was 14% (6% to 22%).

Conclusions While excess adiposity and genetic predisposition both are strongly associated with a higher risk of gout, the excess risk of both combined was higher than the sum of each, particularly among women.

Key messages

What is already known about this subject?

- The global gout burden is rising disproportionately in women, a historically overlooked population.
- Excess adiposity is a major risk factor for the development of gout; women with gout have had a higher prevalence of obesity than men with gout in prior cross-sectional studies.
- Gout is driven substantially by genetics with serum urate heritability estimates ranging from 25% to 60%.

What does this study add?

- The combination of higher genetic predisposition and overweight or obesity imposes an excess risk of incident gout, one larger than the sum of each exposure alone, particularly among women.

How might this impact on clinical practice or future developments?

- Keeping a healthy body weight would be especially important for prevention of female gout, particularly those genetically predisposed.

INTRODUCTION

Gout, the most common inflammatory arthritis, leads to excruciatingly painful flares and joint damage, and an excess burden of cardiometabolic-renal comorbidities.[1] Indeed, the global burden of gout and comorbidity has increased in recent decades,[2 3] disproportionately so among women.[2] Yet, traditionally considered a disease of men, data on female-specific gout are scarce, despite purported differences from males in risk factors[4–7] and clinical spectrums.[8] For example, obesity, the strongest modifiable risk factor for gout,[2 3 9–15] is more strongly associated with female gout than male gout in cross-sectional analyses,[4 6 7] as are the obesity-driven comorbidities such as myocardial infarction, hypertension and type 2 diabetes.[1 4 5] Obesity and associated insulin resistance lead to hyperuricaemia and gout primarily by decreasing renal excretion of urate[16–21]; the causality is supported by recent Mendelian randomisation studies.[22–27]

Furthermore, genetics substantially contribute to overall gout risk (serum urate heritability estimates range from 25% to 60%), with differential sex-specific effects from the prominent genes.[28–31] For example, of the two top genes, compared with male gout, the *SLC2A9* effect is stronger for female gout, whereas the *ABCG2* effect is weaker.[28 30] As such, the impact of excess adiposity on the risk of incident gout may vary according to one's genetic predisposition, particularly for female gout where obesity appears to play a larger role.[4 6 7] Correspondingly, excess adiposity may serve to exacerbate an individual's genetic susceptibility to gout. However, no relevant prospective data for the risk of incident female gout are available.

Our objective was to prospectively investigate the potential impact of adiposity on the risk of incident female gout, according to genetic predisposition, in a large prospective cohort of women (Nurses' Health Study (NHS) cohort) and to replicate in a

separate prospective cohort of younger women (NHS II). We also compared these results to men from the Health Professionals Follow-Up Study (HPFS). We addressed aetiological inferences as well as greater public health implications, using additive interactions,[32–38] and determined the proportion of excess risk attributable to body mass index (BMI) alone, to genetic predisposition alone and to their interaction.[35–38]

METHODS

Study population

The NHS is a prospective cohort study of 121 700 US female registered nurses who were 30–55 years of age on enrolment in 1976,[39] while the NHS II is a prospective cohort study of 116 430 US female registered nurses who were 25–42 years of age on enrolment in 1989. The HPFS is a prospective cohort of 51 529 US male health professionals who were 40–75 years of age on enrolment in 1986.[40] In all studies, participants were mailed validated food frequency questionnaires (FFQ)[41–45] every 4 years (starting from baseline for the HPFS and from the 1984 and 1991 follow-up questionnaires for the NHS and NHS II, respectively) and biennial questionnaires that asked about new medical diagnoses, medication use and lifestyle factors. Completion of the self-administered questionnaire was considered to imply informed consent. The current analysis, approved by the Massachusetts General Brigham Institutional Review Board, includes data from 26 490 women (N=18 244 from the NHS; N=8246 from the NHS II) and 10 899 men of European ancestry who did not report a history of gout at baseline, and for whom genotype data based on genome-wide association study data were available.[46] This study followed the Strengthening the Reporting of Observational Studies in Epidemiology reporting guideline.

Assessment of adiposity, covariates and incident gout

Adiposity was determined by BMI, weight in kilograms divided by height in metres squared (kg/m^2). For each cohort, information on weight and height was obtained on the baseline questionnaire, and weight was updated every 2 years. In validation studies, self-reported weight in the HPFS and NHS I was highly correlated with values obtained by technicians during home visits (r=0.97 for both).[47] Covariates of interest, which have also been validated and used extensively in these cohorts, included age, history of hypertension,[9] systolic and diastolic blood pressure, diuretic use,[9] and among women, menopausal status and the use of hormone replacement therapy[48]; these were ascertained from the biennial questionnaires. Alcohol consumption,[49] total energy intake and consumption of meat, seafood and dairy foods[40] were ascertained from the FFQ.[41–45] Incident gout was based on these nurses and health professionals' report of new-onset, physician-diagnosed gout on the biennial health questionnaires.[50] The present analysis uses data from questionnaires completed during the years 1984–2018 for the NHS, 1991–2017 for NHS II and 1986–2018 for the HPFS.

Genetic risk score and key individual genes

We constructed a genetic risk score (GRS) from 114 single nucleotide polymorphisms (SNPs) derived from European-ancestry meta-analysis of serum urate among 288 649 individuals.[29] GRS was computed as a weighted sum of risk alleles from the 114 SNPs, followed by standardisation to mean of 0 and SD of 1. Specifically, each SNP was weighted by its relative effect size for serum urate.[29] Higher scores indicate a greater genetic predisposition for hyperuricaemia and gout. We also assessed the individual SNPs mapped to the two most prominent serum urate genes driving gout risk, *SLC2A9* and *ABCG2*, both of which have exhibited sex-specific effects.[29 30]

Statistical analysis

We assessed the individual and joint (combined) associations between BMI, GRS and the risk of incident gout, using Cox proportional hazards models adjusting for previously identified risk factors for gout in a time-updated manner,[9 40 51–53] separately for each cohort: NHS (discovery), NHS II (replication) and HPFS (male comparators). Participants contributed person-time from the return of the first questionnaire (NHS, 1984; NHS II, 1991; HPFS, 1986) until gout diagnosis, death, loss to follow-up or end of the follow-up period (30 June 2018 for the NHS and HPFS and 30 June 2017 for NHS II), whichever came first.

To assess aetiological as well as greater public health implications,[32–34] we investigated the additive interaction[35–38] between continuous measures of adiposity and genetic predisposition on incident gout risk, considering BMI in 5 kg/m^2 increments and GRS in one SD increments. The relative excess risk due to interaction (RERI)[35–38 54 55] was assessed as an index of additive interaction[34 56]; 95% CIs were computed using the delta method described by Hosmer and Lemeshow.[57] Briefly, on the relative risk (RR) scale, we divided the RR from both exposures ($RR_{11}-1$) into the excess RR from BMI alone ($RR_{01}-1$), GRS alone ($RR_{10}-1$) and their additive interaction (RERI): $RR_{11}-1=(RR_{01}-1)+(RR_{10}-1)+RERI$. We used Cochrane's Q statistic and the I^2 statistic to examine heterogeneity in the associations for women and men. We subsequently calculated the proportion of the joint effect (the attributable proportion) due to BMI alone ($RR_{01}-1$)/($RR_{11}-1$); GRS alone ($RR_{10}-1$)/($RR_{11}-1$); and due to their additive interaction, RERI/($RR_{11}-1$), as done previously.[35–38] This represents the proportion of the excess incident gout cases (over-and-above the background risk) attributable either to elevated BMI alone, elevated genetic risk alone or the combination. We also evaluated for multiplicative interactions using the Wald test of a cross-product term of BMI and GRS.[58] We conducted the same analyses for the individual SNPs. Our secondary analyses assessed the exposures categorically, using obesity (BMI≥ vs <30) or overweight (BMI≥ vs < 25) cut-offs for adiposity and GRS above versus below the mean for genetic predisposition, and their joint effects.

Finally, to assess the contribution of overweight/obesity towards gout risk at the population level according to genetic predisposition, we calculated the population attributable risk (PAR).[59] This is an estimate of the percentage of incident gout cases in each cohort that would theoretically not have occurred if all individuals had been in the lowest-risk category (eg, BMI<25 kg/m^2) within each GRS stratum, assuming a causal relation between BMI and incident gout. All statistical analyses were performed with SAS V.9.4. All p values are two-sided.

Patient and public involvement

No patients or other members of the public were involved in setting the research question or the outcome measures, nor were they involved in the design and implementation of the study. The results of the research conducted in the three cohorts are regularly reported to study participants.

RESULTS

Baseline characteristics

We included 18 244 women from the NHS (mean age 47.1 years) in our primary (discovery) analysis and 8246 women from the NHS II (mean age 37.4) in our replication analysis, along

with 10 888 men from the HPFS in our comparison analysis (mean age 54.3 years at baseline). Total follow-up time among all participants exceeded 1 090 858 person-years. At baseline, the distribution of clinical gout risk factors was similar among those with GRS below and above the mean, in all three cohorts (table 1).

Risk of incident female gout according to BMI and GRS

There were 1360 cases of incident female gout in the NHS cohort (discovery analysis) and 188 cases in the NHS II (replication analysis). The frequency of obesity at the time of gout diagnosis in these two female cohorts was 41% and 57%, respectively. Individually, higher levels of adiposity and higher GRS were positively and significantly associated with the risk of incident gout in multivariable analysis (p for trend <0.01 in each cohort) (table 2, online supplemental tables 1 and 2). For example, the RR for NHS women in the highest BMI category (vs the lowest) was 4.86 (95% CI 3.96 to 5.95), while the RR for women in the highest GRS quintile (vs the lowest) was 2.89 (2.40 to 3.47) (table 2). These trends persisted on collapsing into three categories of BMI (corresponding to normal weight, overweight and obesity) and dichotomous GRS (above and below the mean) (online supplemental table S3). Moreover, joint effects analysis revealed that obese women with a higher genetic predisposition had a five-times higher risk of incident gout (RR 5.08 (4.15 to 6.22)) than women in the lowest-risk category (eg, normal weight and GRS below the mean), while that in men tended to be smaller (RR 3.46 (2.83 to 4.24)) (figure 1).

When examining the joint effects of continuous BMI and GRS on female gout risk, there was a significant additive interaction between these exposures among our discovery cohort (RERI=0.25 (95% CI 0.12 to 0.33), p<0.001) (table 3). Specifically, the RRs for incident gout in the NHS were 1.49 (1.42 to 1.56) per unit (5 kg/m^2) increase of BMI (adjusting for GRS), 1.43 (1.35 to 1.52) per SD increase of GRS (adjusting for BMI) and 2.18 (2.03 to 2.36) for their joint effect (table 3 and figure 2). Put another way, if there was no evidence of additive interaction (eg, RERI=0), the expected RR for the joint effect would be 1.92 (eg, 1.43+1.49–1). The attributable proportions of the joint effect were 42% for BMI alone, 37% for GRS alone and 22% for their interaction (figure 2). At the same time, there was no evidence of an interaction on the multiplicative scale (p≥0.15). These findings were replicated among younger women in the NHS II (p for interaction <0.001), where the attributable proportion due to interaction was 27% (16% to 38%) (table 3).

When examining these exposures categorically, there were additive interactions between obesity status and genetic risk among women. Obese women with a higher genetic predisposition had a 4.5-times higher risk of incident female gout (RR 4.49 (3.81 to 5.29)) than non-obese women with lower genetic predisposition (eg, BMI<30 kg/m^2 and GRS below the mean) (online supplemental table S4). The attributable proportion due to this interaction was 29%, while that for women in the NHS II was 53% (online supplemental table S4).

Comparison with incident gout among males

There were 1703 cases of incident gout in the HPFS (male comparison cohort) during the 32 years; obesity was much less frequent among incident male gout (21% were obese at the time of diagnosis) than incident female gout (41% and 57% were obese, respectively, in the NHS and NHS II). The RERI among males was 0.14 (0.05 to 0.22), significantly lower than that among females (p for heterogeneity between the male and pooled female cohorts=0.04) though still indicative of an additive BMI-GRS interaction (p=0.002) (table 3). Findings were

Table 1 Baseline gout risk factors in each cohort, by genetic predisposition

Characteristics	114 SNP genetic risk score	
	Below mean	Above mean
Nurses' Health Study, 1984: n=18 244 (women, discovery cohort)		
No. (%)	9162 (50.2)	9082 (49.8)
Age, years, mean (SD)	47.1 (6.9)	47.0 (6.9)
Hypertension, %	15	17
BMI, kg/m^2, mean (SD)	24.5 (4.5)	24.5 (4.4)
Physical activity, MET-hours/week, mean (SD)	14.6 (20.8)	14.0 (19.3)
Alcohol, g/day, mean (SD)	6.5 (10.2)	6.6 (10.5)
Sugar sweetened soft drink intake, servings/day, mean (SD)	0.3 (0.6)	0.3 (0.6)
Meat intake, servings/day, mean (SD)	1.1 (0.8)	1.1 (0.8)
Seafood intake, servings/day, mean (SD)	0.2 (0.2)	0.2 (0.2)
Low-fat dairy foods intake, servings/day, mean (SD)	0.9 (1.0)	0.9 (1.0)
High-fat dairy foods intake, servings/day, mean (SD)	1.4 (1.3)	1.4 (1.3)
Diuretic use, %	9.6	10.2
Nurses' Health Study II, 1991: n=8246 (women, replication cohort)		
No. (%)	4056 (49.2)	4190 (50.8)
Age, years, mean (SD)	37.5 (4.4)	37.3 (4.4)
Hypertension, %	3.21	3.61
BMI, kg/m^2, mean (SD)	24.3 (4.9)	24.5 (5.1)
Physical activity, MET-hours/week, mean (SD)	19.9 (24.9)	20.7 (28.3)
Alcohol, g/day, mean (SD)	3.2 (5.9)	3.2 (6.1)
Sugar sweetened soft drink intake, servings/day, mean (SD)	0.42 (0.8)	0.44 (0.8)
Meat intake, servings/day, mean (SD)	1.0 (0.7)	0.92 (0.6)
Seafood intake, servings/day, mean (SD)	0.5 (0.6)	0.51 (0.5)
Low-fat dairy foods intake, servings/day, mean (SD)	1.1 (1.9)	1.09 (1.1)
High-fat dairy foods intake, servings/day, mean (SD)	0.85 (0.9)	0.85 (0.9)
Diuretic use, %	2.6	2.7
Health Professionals Follow-Up Study, 1986: n=10 888 (men, comparison cohort)		
No. (%)	5463 (50.2)	5425 (49.8)
Age, years, mean (SD)	54.3 (8.6)	54.2 (8.7)
Hypertension, %	21	21
BMI, kg/m^2, mean (SD)	25.6 (3.2)	25.6 (3.1)
Physical activity, MET-hours/week, mean (SD)	20.2 (23.5)	19.9 (23.7)
Alcohol, g/day, mean (SD)	12.1 (15.6)	12.4 (16.3)
Sugar sweetened soft drink intake, servings/day, mean (SD)	0.3 (0.5)	0.3 (0.5)
Meat intake, servings/day, mean (SD)	1.6 (0.8)	1.6 (0.9)
Seafood intake, servings/day, mean (SD)	0.3 (0.3)	0.3 (0.3)
Low-fat dairy foods intake, servings/day, mean (SD)	1.0 (1.1)	1.0 (1.0)
High-fat dairy foods intake, servings/day, mean (SD)	1.3 (1.3)	1.3 (1.3)
Diuretic use, %	10.0	10.1

Values are age-adjusted (except for age).
BMI, body mass index; SNP, single nucleotide polymorphism.

Table 2 Relative risk (RR) of incident gout according to body mass index (BMI) and according to genetic risk score, Nurses' Health Study (women, discovery cohort)

Body mass index (kg/m^2)	<23	23–24.9	25–29.9	30–34.9	≥35	Per unit increase*	P for trend
N cases	201	172	433	316	238	–	–
Person-years	189 242	116 119	192 533	76 228	35 075	–	–
Incidence rate (per 1000 PY)	1.06	1.48	2.25	4.15	6.79	–	–
Age-adjusted RR (95% CI)	1.0 (ref)	1.41 (1.15 to 1.72)	2.09 (1.77 to 2.47)	3.97 (3.31 to 4.74)	7.00 (5.78 to 8.48)	1.63 (1.57 to 1.70)	<0.01
MV RR† (95% CI)	1.0 (ref)	1.32 (1.07 to 1.62)	1.78 (1.50 to 2.11)	2.95 (2.45 to 3.56)	4.66 (3.80 to 5.72)	1.47 (1.41 to 1.54)	<0.01
MV RR+GRS** (95% CI)	1.0 (ref)	1.33 (1.08 to 1.62)	1.80 (1.51 to 2.13)	2.98 (2.48 to 3.60)	4.86 (3.96 to 5.95)	1.49 (1.43 to 1.56)	<0.01
Genetic risk score	**Q1**	**Q2**	**Q3**	**Q4**	**Q5**	**Per unit increase‡**	**P for trend**
N cases	153	206	263	310	428	–	–
Person-years	121 847	121 819	121 887	121 867	121 777	–	–
Incidence rate (per 1000 PY)	1.26	1.69	2.16	2.54	3.51	–	–
Age-adjusted RR (95% CI)	1.0 (ref)	1.36 (1.10 to 1.68)	1.73 (1.42 to 2.11)	2.06 (1.70 to 2.50)	2.82 (2.35 to 3.40)	1.45 (1.37 to 1.53)	<0.01
MV RR† (95% CI)	1.0 (ref)	1.40 (1.14 to 1.73)	1.71 (1.40 to 2.08)	2.08 (1.71 to 2.52)	2.78 (2.31 to 3.35)	1.43 (1.36 to 1.51)	<0.01
MV RR +BMI*** (95% CI)	1.0 (ref)	1.45 (1.17 to 1.78)	1.72 (1.41 to 2.10)	2.14 (1.76 to 2.60)	2.89 (2.40 to 3.47)	1.45 (1.38 to 1.53)	<0.01

*Per 5 kg/m^2 increase.
†Multivariable (MV) relative risk adjusted for age, total energy intake, consumption of meat, seafood and dairy foods, history of hypertension, diuretic use, menopausal status, and use of oral contraceptive or postmenopausal hormone therapy.
‡Per SD increase.

consistent when assessing these exposures categorically (online supplemental tables S4 and S5); the RERI between obesity status and genetic predisposition among males tended to be lower than in females.

Interaction between BMI and key individual genes

Additive interactions were also observed between BMI and the individual SNPs mapped to the two most prominent serum urate genes, with RERI values of 0.21 (0.13 to 0.29) for *SLC2A9* and 0.23 (0.08 to 0.38) for *ABCG2* in the NHS (p for interaction <0.01). These findings were replicated within the NHS II (p for interaction <0.03). The additive interactions among men tended to be smaller, with RERI values of 0.14 (0.03 to 0.24) for *SLC2A9* and 0.03 (−0.19 to 0.25) for *ABCG2*.

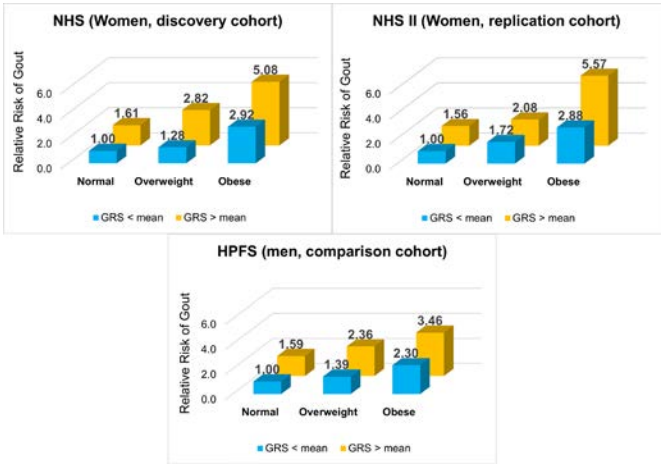

Figure 1 Joint association of body mass index (BMI) and genetic predisposition on the risk of incident gout. Normal weight=BMI<25 kg/m^2; overweight=30<BMI≥25 kg/m^2; obese=BMI≥30 kg/m^2. GRS, genetic risk score; HPFS, Health Professionals Follow-Up Study; NHS, Nurses' Health Study; NHS II, Nurses' Health Study II.

PAR of BMI according to genetic risk

Among women, the PAR of excess adiposity (BMI≥25 kg/m^2) was 42.0% (36.8 to 46.6) and 33.2% (25.0 to 39.9) among those with GRS above and below the mean, respectively. Excess adiposity tended to account for smaller proportions of incident gout cases among men, with PARs of 27.4% (20.9 to 33.1) and 24.7% (15.9 to 32.1) among those with GRS above and below the mean, respectively.

DISCUSSION

In our large-scale prospective cohort analysis of US women with 114-SNP GRS and serial BMI measures, higher adiposity and higher genetic predisposition (whether inferred from a polygenic score or key individual loci) were independently and jointly associated with the risk of incident female gout, adjusting for other pertinent health and lifestyle risk factors. Among women, 42% of the joint effect was attributable to higher BMI alone, 37% to higher genetic risk alone and 22% to an additive interaction between the two. These findings were replicated in a separate cohort of younger women among whom the attributable proportion due to interaction tended to be larger (27%), whereas that among men was only 14%. These findings underscore the importance of addressing excess adiposity for mitigating the risk of incident female gout and its cardiometabolic sequalae, especially for those with higher genetic predisposition, who are particularly prone to the deleterious effects of excess adiposity on gout.

Potential mechanisms

The mechanisms of this possible causal interaction,[60] particularly among women, remain to be clarified. The sex differences in the independent and joint effects of BMI on gout risk are

Table 3 Attributing effects to additive interaction between body mass index and genetic risk score on risk of incident gout

	NHS (women, discovery; N=1360 cases)	NHS II (women, replication; N=188 cases)	HPFS (men, comparison; N=1703 cases)
Main effects, relative risk (95% CI)			
Body mass index, kg/m^2*	1.49 (1.42 to 1.56)	1.33 (1.20 to 1.48)	1.42 (1.35 to 1.51)
Genetic risk score†	1.43 (1.35 to 1.52)	1.41 (1.19 to 1.68)	1.41 (1.34 to 1.48)
Joint effect	2.18 (2.03 to 2.36)	2.01 (1.69 to 2.39)	1.97 (1.83 to 2.12)
Relative excess risk (95% CI) due to interaction			
Relative excess risk due to interaction (RERI)‡	0.25 (0.12 to 0.33)	0.27 (0.14 to 0.40)	0.14 (0.05 to 0.22)
P value	<0.001	<0.001	0.002
Attributable proportion, % (95% CI)			
Body mass index, kg/m^2*	41.5 (37.3 to 45.8)	32.8 (22.4 to 43.1)	43.8 (37.8 to 49.8)
Genetic risk score†	36.8 (31.5 to 42.1)	40.4 (27.1 to 53.7)	42.1 (36.2 to 47.9)
Additive interaction	21.7 (16.2 to 27.7)	26.8 (15.9 to 37.7)	14.1 (6.1 to 22.2)

Multivariable relative risk adjusted for age, total energy intake, consumption of meat, seafood and dairy foods, history of hypertension, diuretic use, menopausal status (women only), and use of oral contraceptive or postmenopausal hormone therapy (women only).
*Per 5 kg/m^2 increase.
†Per SD increase.
‡RERI>0 is consistent with the presence of additive interaction.
HPFS, Health Professionals Follow-Up Study; NHS, Nurses' Health Study.

consistent with prior findings of higher prevalence of obesity (or mean level of BMI) in women with gout than in men, even after accounting for differences in age.[4 7] Whether sex hormones or related factors contribute to the interaction between genetic predisposition and excess adiposity warrants further investigation. Moreover, a recent analysis of four US-based prospective cohorts found that the risk of incident gout was 42% higher among men even after accounting for differences in serum urate levels,[61] suggesting the sex difference is explained by factors above and beyond hyperuricaemia. To that end, obesity is associated with increased inflammatory biomarkers,[62] adipokines, cytokines and reduced levels of adenosine monophosphate–activated protein kinase (AMPK) activity (as a master regulator of gouty inflammation),[63–66] which may promote the development of gout by affecting an inflammatory response to deposited monosodium urate crystals.[67] For example, leptin, an adipokine, was found to promote urate crystal induced inflammation in human and murine models[68]; reduced AMPK activity in obesity could contribute to higher mononuclear phagocyte responses to urate crystals, including NLRP3 inflammasome activation and interleukin 1β and chemokine release.[69 70]

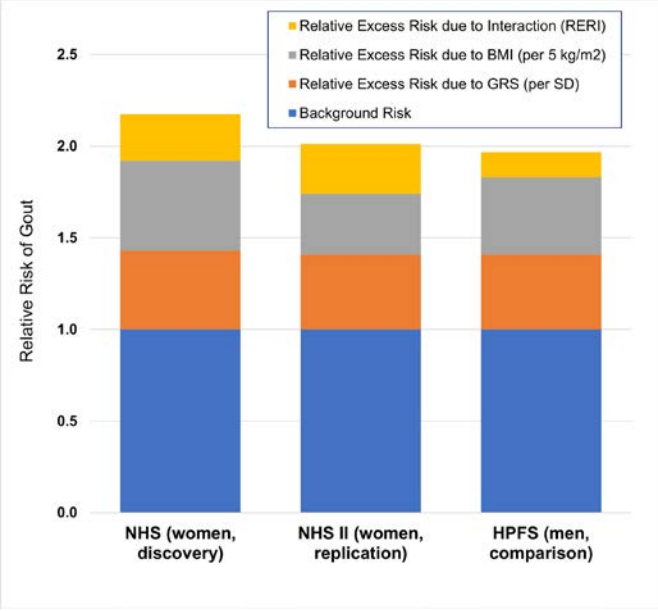

Figure 2 Joint association of body mass index (BMI) and genetic risk score (GRS) on the risk of incident gout. The area of each coloured bar represents the proportion of the excess risk of incident gout attributable to each individual exposure (BMI and GRS) and to their joint effects. HPFS, Health Professionals Follow-Up Study; NHS, Nurses' Health Study; NHS II, Nurses' Health Study II.

Public health implications

Our findings align with the recent Global Burden of Disease Study,[2 3] which reported a disproportionate rise in gout burden among women over 1990–2017.[2] Moreover, high BMI accounted for 31% of the burden of female gout globally in 2017, including 35% of the burden in Western Europe and 49% in high-income North America. From a public health standpoint, our findings reinforce adiposity as a major target for reducing the incidence and burden of female gout, with its higher frequencies of coronary heart disease,[1 5] type 2 diabetes[1 4 5] and other cardiometabolic-renal comorbidities than male gout, particularly for women born with a greater genetic predisposition to developing this disease. For example, while our data indicated the combination of obesity and genetic predisposition resulted in an additional 29% of cases of incident female gout (above-and-beyond the background rate), this 29% would not occur if one of these factors (namely obesity, the modifiable one) were absent. Successful interventions for achieving and maintaining a healthy weight could be tailored towards an individual's comorbidity profile and personal preferences.[14 15] For example, in our recent ancillary analysis of the Dietary Intervention Randomized Controlled Trial (DIRECT),[71] three established healthy weight loss diets (Mediterranean, low-fat and low-carbohydrate) each resulted in considerable reductions in serum urate levels, particularly among those at risk with baseline hyperuricaemia (by 1.9–2.4 mg/dL by 6 months and 1.1–1.4 mg/dL by 24 months), which were mediated by significant reductions in body weight and plasma insulin levels.[72] The addition of physical activity to these healthy diets may further mitigate gout risk through the direct effects on BMI[73] or indirect effects on insulin resistance.[15 74]

Strengths and limitations of the study

Strengths of our study included the sex-specific analysis of large cohorts of females, and replication of our findings in another female cohort, the NHS II, and comparison to male counterparts (HPFS). Another important focus of our study is the absolute risk (additive) scale, which has greater implications for population health[32–34 75] as applied to related endpoints.[35–38 55 76] In fact, the absence of a significant multiplicative interaction between exposures (eg, RR ratio not significantly different between exposure groups) can mislead about the impact of joint exposures; a significant additive interaction still implies that a larger number of cases is expected when the group with one deleterious risk factor is exposed to a second deleterious factor. The FFQs and other instruments for collecting covariate data have been well validated in these cohorts,[41–45 47 48 77] and participants' height and weight data were found to be highly reliable.[47] We collected biennial measures of BMI and a comprehensive set of other health and lifestyle factors repeatedly, thus minimising measurement error. Moreover, our prospective collection of exposure data, before gout diagnosis, eliminated the potential for recall bias and gave the rare opportunity to prospectively assess the interactions between gout-risk genes and BMI measured before diagnosis, unlike previous cross-sectional studies with unclear temporal relations with BMI and relevant lifestyle covariates.[67 78–80] However, despite our comprehensive adjustment for covariates, these findings are subject to potential residual and unmeasured confounding, like with any observational study. Although the absolute rates of gout and the distribution of adiposity of our cohorts may not be representative of a random sample of Americans, all risk factors identified from our study cohorts have been consistently replicated as those for hyperuricaemia in the US general population based on the National Health and Nutrition Examination Survey.[9 40 49 51 53 81–90] Furthermore, similar associations, including those for BMI and the risk of gout,[91] have been reported in somewhat older-aged, sociodemographically diverse cohorts such as the Atherosclerosis Risk in Communities study in the USA (eg, age 45–64 years at baseline, 25% African American, 40% with annual household income ≤$25,000.[92]

In these prospective female cohorts, both excess adiposity and genetic predisposition were strongly associated with a higher risk of gout, and the excess risk of both combined was higher than the sum of each, whereas a weaker interaction was present in male counterparts. These findings suggest addressing excess adiposity could prevent a large proportion of female gout cases in particular, as well as its cardiometabolic comorbidities, and the benefit could be greater in genetically predisposed women.

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EPIDEMIOLOGICAL SCIENCE

Two-dose COVID-19 vaccination and possible arthritis flare among patients with rheumatoid arthritis in Hong Kong

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ABSTRACT

Objectives To investigate the relationship between COVID-19 full vaccination (two completed doses) and possible arthritis flare.

Methods Patients with rheumatoid arthritis (RA) were identified from population-based electronic medical records with vaccination linkage and categorised into BNT162b2 (mRNA vaccine), CoronaVac (inactive virus vaccine) and non-vaccinated groups. The risk of possible arthritis flare after vaccination was compared using a propensity-weighted cohort study design. We defined possible arthritis flare as hospitalisation and outpatient consultation related to RA or reactive arthritis, based on diagnosis records during the episode. Weekly prescriptions of rheumatic drugs since the launch of COVID-19 vaccination programme were compared to complement the findings from a diagnosis-based analysis.

Results Among 5493 patients with RA (BNT162b2: 653; CoronaVac: 671; non-vaccinated: 4169), propensity-scored weighted Poisson regression showed no significant association between arthritis flare and COVID-19 vaccination ((BNT162b2: adjusted incidence rate ratio 0.86, 95% Confidence Interval 0.73 to 1.01); CoronaVac: 0.87 (0.74 to 1.02)). The distribution of weekly rheumatic drug prescriptions showed no significant differences among the three groups since the launch of the mass vaccination programme (all p values >0.1 from Kruskal-Wallis test).

Conclusions Current evidence does not support that full vaccination of mRNA or inactivated virus COVID-19 vaccines is associated with possible arthritis flare.

Key message

What is already known about this subject?
⇒ Fear of arthritis flare after vaccination could introduce vaccine hesitancy.
⇒ To date, there are no analytical studies on COVID-19 vaccination and arthritis flare among patients with rheumatoid arthritis (RA).

What does this study add?
⇒ Current cohort study showed no evidence of increased risk of possible arthritis flare among patients with RA who were fully vaccinated with mRNA or inactivated virus COVID-19 vaccines.

How might this impact on clinical practice or future developments?
⇒ Individuals with RA should be encouraged to receive the vaccine against COVID-19.
⇒ Real-world COVID-19 vaccine safety surveillance should continue to provide more robust evidence on the association between arthritis flare and COVID-19 vaccines with direct disease activity tests and consideration of immunomodulated medications.

INTRODUCTION

Vaccine is an effective public health measurement to control the global COVID-19 pandemic. Patients with rheumatoid arthritis (RA) are twofold more vulnerable to infections that result in hospitalisation and impaired quality of life.[1] With consideration to the benefits of vaccination outweighing the risks, the European Alliance of Associations for Rheumatology (EULAR)[2] recommends that patients with RA should receive COVID-19 vaccines without needing major adjustment to their ongoing treatment regimens. However, one of the major barriers to vaccine uptake among patients with RA is the fear of arthritis flare despite non-relevant evidence from landmark trials and few case reports in the post marketing.[3]

Understanding the association between arthritis flare and vaccination is important to overcome vaccine hesitancy. Currently, the Hong Kong (HK) Government Vaccination Programme provides two authorised COVID-19 vaccines: CoronaVac (inactivated virus vaccine; recommended vaccination interval 28 days) and BNT162b2 (mRNA vaccine; recommended vaccination interval 21 days). Since the launch of the vaccination programme on 23 February 2021, more than 8 million doses have been administered with close safety monitoring. In this study, we analysed the territory-wide electronic medical records (EMRs) database and aimed to investigate the population-level risk of possible

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arthritis flare following full vaccination based on two technology platforms.

METHOD

Data sources

We analysed population-based EMRs from the Hospital Authority (HA) with linked vaccination records from the Department of Health (DH) of the HK Government.[4] HA provides publicly funded health services to around 7 million HK residents. The EMRs database managed by the HA holds centralised medical records from 42 public hospitals with high population coverage, representativeness and coding accuracy.[5 6] This study linked the EMRs with the vaccination records of all HK residents ≥16 years old who ever used the HA service. We used de-identified and non-reversible series numbers for the record linkage to protect patient privacy.

Study design and population

This was a retrospective cohort study among patients with RA. Risk of possible arthritis flare was compared among vaccine recipients and non-vaccinated individuals. Based on the International Classification of Diseases Ninth version, Clinical Modification (ICD-9-CM) diagnosis (online supplemental table 1), we identified the RA cohort from the EMRs, excluding patients who had cancer or other autoimmune diseases to avoid cohort contamination. We matched each vaccine recipient with non-vaccinated individuals by age and sex using maximum ratio matching and assigned the vaccination date as the pseudo index date for non-vaccinated individuals (controls). Individuals with completed two-dose vaccination and their matched controls were followed up from the date of second dose vaccination or the age-sex matched pseudo index date until the occurrence of interested outcome, death or the end date of data availability (31 July 2021), whichever was earlier. The record linkage, matching procedure and cohort identification is illustrated in online supplemental figure 1.

Outcome measurements

After vaccination, any specialist outpatient clinic (SOPC) consultation or hospitalisation related to RA or reactive arthritis was considered a proxy of arthritis flare. Primary outcome is a recorded diagnosis of RA or reactive arthritis from inpatient or SOPC settings. Secondary outcome is a relevant diagnosis at inpatient setting as the proxy of severe arthritis flare.

Statistical analysis

To balance the patient characteristics among groups (CoronaVac, BNT162b2 and non-vaccinated), we used multi-group Inverse Probability Treatment Weighting method and weighted variables including age, sex, medical history and health service utilisation since 2018 and the recent 90 days of medication use. We applied Poisson regression to estimate the adjusted incidence rate ratio (IRR) with 95% Confidence Interval (CI) using the non-vaccination group as reference. Fisher's exact test was used to examine the association between delayed second dose (defined as interdose interval more than 42 days,which is the maximum dose interval used in BNT162b2 clinical trials)[7] and the occurrence of flare.

In addition, we analysed the weekly prescription pattern of rheumatoid drugs (online supplemental table 2) between 23 February (the start date of mass vaccination programme) and 31 July 2021, hypothesising that the prescription volume of non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids would increase sharply if there was a significant arthritis flare in the study cohort. Number of prescriptions (per-patient) and proportion of each drug category (NSAIDs, corticosteroids, conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) and biological/target synthetic disease-modifying antirheumatic drugs (b/tsDMARDs)) among CoronaVac, BNT162b2 and non-vaccinated groups were compared using Kruskal-Wallis test.

Patient and public involvement

This study used de-identified electronic medical records and was conducted without patient and public involvement.

RESULTS

We obtained 3 983 529 records of HA active patients with affirmed vaccination status. Following the cohort selection procedure, 5493 patients with RA (BNT162b2: 653; CoronaVac: 671 and non-vaccinated individuals: 4169) were included. Compared with non-vaccinated individuals, vaccine recipients were younger and less likely to have pre-existing chronic diseases. After weighting, all variables were well balanced with a standardised mean difference smaller than 0.2 (table 1).[8 9] Median interdose interval was 21 days (IQR 21–23) for BNT162b2 and 28 days (IQR 28–29) for CoronaVac recipients. Delaying the second dose was very uncommon for both vaccine groups (BNT162b2: 0.5%; CoronaVac: 0.8%).

During a median follow-up of 32 days (IQR 14–72), 35 BNT162b2 recipients (crude incidence 0.45 (95% CI 0.32 to 0.62) per person-year) had RA or reactive arthritis-related hospitalisation or SOPC attendance. The number of CoronaVac recipients is 41 (crude incidence 0.45 (0.33 to 0.61) per person-year) with a median follow-up of 30 days (IQR 15–95). Receiving two doses of BNT162b2 (adjusted IRR 0.86 (95% CI 0.73 to 1.01)) or CoronaVac (adjusted IRR 0.87 (95% CI 0.74 to 1.02)) showed no significant association with arthritis flare as defined. Similarly, no significant association was detected when focusing on events identified from inpatient setting only (table 2). Delayed second dose was not associated with the occurrence of possible flare (p=0.3042 for BNT162b2; p=0.5422 for CoronaVac and p=0.1454 for overall from Fisher's exact test).

Weekly prescription of four major rheumatoid drugs were presented in figure 1. Since the launch of the COVID-19 vaccination programme in HK, weekly arthritis-related prescriptions ranged between 0.09 and 0.14 per patient. NSAIDs and corticosteroids accounted for 23%–27% of overall prescriptions. The per-patient prescription and distribution of four rheumatoid drug categories showed no significant differences among the BNT162b2 and CoronaVac recipients, and the non-vaccinated individuals (all p values >0.1 from Kruskal-Wallis test).

DISCUSSION

Using territory-wide EMRs in HK, we found that after full vaccination with BNT162b2 or CoronaVac, patients with RA did not show an increased risk of possible arthritis flare. The weekly prescription trends of major rheumatoid drugs also presented no significant differences among patients with or without vaccination. Currently, safety evidence on COVID-19 vaccine among patients with rheumatic diseases are from case reports,[3 10 11] self-report surveys[12] or trials among RA patients with controlled disease activities.[13] Since the launch of vaccination in HK, uptake of the vaccine (approximate 24% (95% CI 22.99% to 25.25%) with full vaccination based on our study cohort) among patients with RA is gradually increasing (online

Table 1 Baseline characteristics before and after multi-group inverse probability treatment weighting

	Before weighting				After weighting			
	BNT162b2	CoronaVac	None	SMD	BNT162b2	CoronaVac	None	SMD
N	653	671	4169		3893.56	4051.97	4169	
Male (N (%))	136 (20.8)	194 (28.9)	850 (20.4)	0.132	681.6 (17.5)	865.8 (21.4)	850.0 (20.4)	0.065
Age (mean (SD))	55.83 (11.89)	59.52 (11.04)	63.97 (14.73)	0.424	61.98 (12.38)	61.60 (10.85)	63.97 (14.73)	0.12
Comorbidities (N (%))								
Asthma	9 (1.4)	9 (1.3)	72 (1.7)	0.021	53.6 (1.4)	55.7 (1.4)	72.0 (1.7)	0.019
Cerebrovascular disease	6 (0.9)	18 (2.7)	230 (5.5)	0.18	163.9 (4.2)	166.0 (4.1)	230.0 (5.5)	0.044
Chronic obstructive pulmonary disease	12 (1.8)	16 (2.4)	235 (5.6)	0.135	218.7 (5.6)	264.4 (6.5)	235.0 (5.6)	0.025
Congestive heart failure	1 (0.2)	2 (0.3)	118 (2.8)	0.153	120.5 (3.1)	50.7 (1.3)	118.0 (2.8)	0.085
Chronic renal failure	0 (0.0)	5 (0.7)	76 (1.8)	0.137	0.0 (0.0)	72.9 (1.8)	76.0 (1.8)	0.129
Dementia	0 (0.0)	0 (0.0)	17 (0.4)	0.06	0.0 (0.0)	0.0 (0.0)	17.0 (0.4)	0.06
Diabetes	29 (4.4)	45 (6.7)	488 (11.7)	0.18	503.8 (12.9)	384.3 (9.5)	488.0 (11.7)	0.073
Mild liver disease	0 (0.0)	1 (0.1)	13 (0.3)	0.056	0.0 (0.0)	3.8 (0.1)	13.0 (0.3)	0.057
Moderate-severe liver disease	1 (0.2)	0 (0.0)	1 (0.0)	0.04	0.0 (0.0)	0.0 (0.0)	1.0 (0.0)	0.015
Myocardial infarction	4 (0.6)	1 (0.1)	48 (1.2)	0.086	25.8 (0.7)	82.8 (2.0)	48.0 (1.2)	0.081
Peripheral vascular disease	0 (0.0)	1 (0.1)	39 (0.9)	0.1	0.0 (0.0)	27.7 (0.7)	39.0 (0.9)	0.094
Paralysis	0 (0.0)	1 (0.1)	17 (0.4)	0.065	0.0 (0.0)	7.6 (0.2)	17.0 (0.4)	0.064
Respiratory infections	20 (3.1)	23 (3.4)	390 (9.4)	0.176	278.5 (7.2)	354.8 (8.8)	390.0 (9.4)	0.053
Stroke or systemic embolism	2 (0.3)	7 (1.0)	95 (2.3)	0.121	73.5 (1.9)	56.4 (1.4)	95.0 (2.3)	0.044
Ulcers	3 (0.5)	14 (2.1)	106 (2.5)	0.116	80.1 (2.1)	97.7 (2.4)	106.0 (2.5)	0.022
Viral infections	0 (0.0)	2 (0.3)	43 (1.0)	0.104	0.0 (0.0)	36.9 (0.9)	43.0 (1.0)	0.097
Health service utilisation (N (%))								
Emergency or hospital admission	471 (72.1)	508 (75.7)	3464 (83.1)	0.177	3185.5 (81.8)	3327.5 (82.1)	3464.0 (83.1)	0.022
Outpatient visits	641 (98.2)	665 (99.1)	4122 (98.9)	0.054	3826.2 (98.3)	4011.8 (99.0)	4122.0 (98.9)	0.043
Medication usage within 90 days (N (%))								
Immunosuppressants	11 (1.7)	7 (1.0)	134 (3.2)	0.102	82.0 (2.1)	115.4 (2.8)	134.0 (3.2)	0.046
NSAIDs	284 (43.5)	295 (44.0)	1617 (38.8)	0.07	1529.5 (39.3)	1671.0 (41.2)	1617.0 (38.8)	0.033
Corticosteroids	0 (0.0)	0 (0.0)	1 (0.0)	0.015	0.0 (0.0)	0.0 (0.0)	1.0 (0.0)	0.015
b/tsDMARDs	191 (29.2)	187 (27.9)	1287 (30.9)	0.044	1230.7 (31.6)	1418.6 (35.0)	1287.0 (30.9)	0.059
csDMARDs	486 (74.4)	508 (75.7)	3041 (72.9)	0.042	2767.6 (71.1)	2988.2 (73.7)	3041.0 (72.9)	0.04
Drugs for gout	9 (1.4)	26 (3.9)	120 (2.9)	0.105	79.7 (2.0)	93.1 (2.3)	120.0 (2.9)	0.036

bDMARDs, biological disease-modifying antirheumatic drugs; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; NSAIDs, Non-steroidal anti-inflammatory drugs; SMD, standardised mean difference; tsDMARDs, target synthetic disease-modifying antirheumatic drugs.

supplemental figure 2), although remaining suboptimal. Findings from this study provide real-world evidence of COVID-19 vaccine safety and could potentially overcome vaccine hesitancy among patients with RA.

We acknowledge that if individuals who experienced flare after the first dose, then they would be less likely to take the second dose, which could theoretically introduce biased estimation for the current two-dose analysis. To clarify this issue, we conducted post hoc analysis to estimate the number of patients received single-dose only. We included patients who received the first-dose vaccine on or before 19 June 2021 and had no record of second dose until the study end date (31 July 2021). It would ensure at least 42-day observation period after the first dose and exclude the possibility that the second dose was scheduled beyond the study period. Although the recommended dosing interval is 21 and 28 days for BNT162b2 and CoronaVac, respectively, the HK Government allows flexibility of interval between doses for logistic or clinical reasons. Analysis of the phase III efficacy data of BNT162b2 showed it was feasible to administer the second dose from 19 to 42 days.[6] Therefore, we defined

Table 2 Risk of flare among two-dose vaccine recipients vs unvaccinated individuals, after propensity score weighting

	N	Follow-up time (person-year)	Crude incidence (per person-year, 95% CI)	Adjusted IRR* (95% CI)	P-value
Primary outcome					
BNT162b2	35	78.23	0.45 (0.32 to 0.62)	0.86 (0.73 to 1.01)	0.0702
CoronaVac	41	91.02	0.45 (0.33 to 0.61)	0.87 (0.74 to 1.02)	0.0962
None	330	612.63	0.54 (0.48 to 0.60)	Ref	–
Secondary outcome					
BNT162b2	33	78.65	0.42 (0.29 to 0.58)	0.96 (0.81 to 1.14)	0.6486
CoronaVac	38	91.58	0.41 (0.30 to 0.56)	1.03 (0.87 to 1.22)	0.7373
None	275	620.26	0.44 (0.39 to 0.50)	Ref	–

*Adjusted variables with standard mean difference >0.1; IRR estimated using non-vaccinated group as reference
IRR, incidence rate ratio.

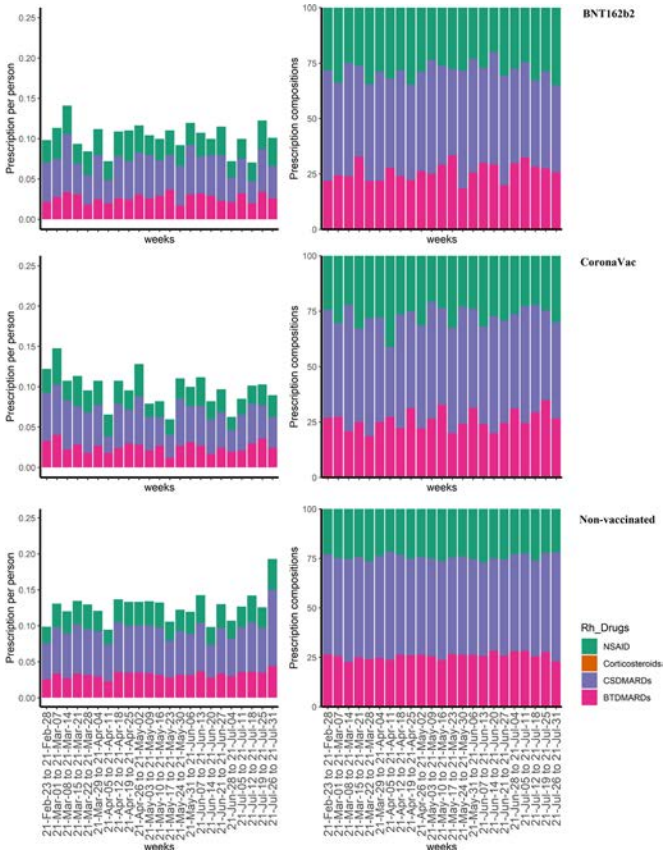

Figure 1 Weekly arthritis-related prescriptions among vaccine recipients and non-vaccinated individuals, between 1 February and 31 July 2021. BTDMARDs, biological or target synthetic disease-modifying antirheumatic drugs; CSDMARDs, conventional synthetic disease-modifying antirheumatic drugs; NSAID, non-steroidal anti-inflammatory drug. Kruskal-Wallis test showed all p values >0.1 for each week comparison, indicating the distribution of arthritis-related prescriptions showed no differences among BNT162b2 recipients, CoronaVac recipients and non-vaccinated individuals.

an interdose interval within 42 days is acceptable. Based on the above definition, the number of subjects who received single-dose only is very small for both vaccine groups (BNT162b2: 4; CoronaVac: 7). Therefore, we anticipate the theoretical bias is neglectable and will not affect the interpretation of our current results. We also conducted a post hoc analysis to evaluate the potential effect of delayed second dose, that is, more than 42 days. Our RA cohort showed only less than 1% of the subjects received the second dose more than42 days after the first dose. Fisher's exact test also showed no association between delayed second dose and the occurrence of flares. In summary, non-taken or delayed second dose is very uncommon in our study cohort with minimum impact to the results interpretation of current study.

Nevertheless, multiple factors could trigger arthritis flare, such as infection, stress and poor medication adherence.[14] Flare is preventable, manageable and reversible if an appropriate regimen and dosing adjustment of DMARDs is followed. For possible flare resulting in hospitalisation, our data showed that the maximum length of stay was 6 days with no recorded registered death, indicating a satisfactory prognosis. Vaccine hesitancy is also related to the uncertainty of immunogenicity in patients with inflammatory diseases because of their immunocompromised conditions.[15 16] Individuals with inflammatory disease were observed to have a higher risk of severe conditions after COVID-19 infection compared with those without inflammatory diseases.[17 18] It was established that the immunogenicity of COVID-19 vaccine could achieve an acceptable threshold for protection.[13 19] Combining the current evidence of safety and effectiveness, vaccination with two doses is highly recommended to achieve adequate self-protection in patients with RA.[20]

To the best of our knowledge, this is the first population-based analytical study with valid vaccination record linkage for COVID-19 vaccine safety monitoring among patients with RA. The study assessed the safety of two different vaccine technology platforms with relatively larger sample sizes and a longer follow-up period. Our cohort identification was based on ICD-9-CM diagnosis codes (714.xx) recorded in either inpatient or SOPC settings with clinical diagnoses made by rheumatology specialists. Furthermore, prescription data analysis showed, in our study cohort, 96% of the patients diagnosed with RA had arthritis-related prescription records (cs/b/tsDMARD, NSAIDs or corticosteroid) between 1 January 2018 and 31 July 2021 (the period of data availability), which supports the high validity of RA cohort we identified.

However, as a common drawback with EMR-based studies, information on the clinically relevant definition of flares, such as disease activity assessment (eg, Disease Activity Score-28 for Rheumatoid Arthritis) and patient-reported symptoms (eg, pain, stiffness and fever), is not available. Using arthritis-related hospital admission and SOPC consultation as a proxy of flare may underestimate the accurate occurrence. The supplementary analysis using arthritis-related prescription as a surrogate outcome of flare enables the validation of diagnosis-based outcome definition. This consistent finding further supports the non-significant association between COVID-19 vaccination and arthritis flare. Of note, almost no patients were recorded as using corticosteroids at cohort entry, indicating that those who received the vaccine were at the maintenance stage of RA with stable disease activity or in remission. The study conclusion is not entirely generalisable to patients with active RA. Our database is also restricted to patients who use the HA service. HA is the statutory body responsible for managing all the public hospitals in HK and provides a highly subsidised health service to all eligible HK residents. It is anticipated that the majority of possible flare is captured in this study, particularly severe cases resulting in hospitalisation, although we possibly missed patients consulting private rheumatologists for flare management. However, there is no evidence to show differential use of private consultants between vaccinated and unvaccinated subjects; hence, it is unlikely to affect our conclusion.

In conclusion, among patients with RA, there is no increased risk of possible flare following two doses of COVID-19 vaccination. Real-world vaccine safety surveillance with direct disease activity testing related to arthritis flare should continue to provide more robust evidence on the association.

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EPIDEMIOLOGICAL SCIENCE

Survival after COVID-19-associated organ failure among inpatients with systemic lupus erythematosus in France: a nationwide study

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ABSTRACT

Objective We analysed the incidence of, the specific outcomes and factors associated with COVID-19-associated organ failure (AOF) in patients with systemic lupus erythematosus (SLE) in France.

Methods We performed a cohort study using the French national medical/administrative hospital database for the January 2011–November 2020 period. Each patient with SLE diagnosed in a French hospital with a COVID-19-AOF until November 2020 was randomly matched with five non-SLE patients with COVID-19-AOF. We performed an exact matching procedure taking age ±2 years, gender and comorbidities as matching variables. COVID-19-AOF was defined as the combination of at least one code of COVID-19 diagnosis with one code referring to an organ failure diagnosis.

Results From March to November 2020, 127 380 hospital stays in France matched the definition of COVID-19-AOF, out of which 196 corresponded with patients diagnosed with SLE. Based on the presence of comorbidities, we matched 908 non-SLE patients with COVID-19-AOF with 190 SLE patients with COVID-19-AOF. On day 30, 43 in-hospital deaths (22.6%) occurred in SLE patients with COVID-19-AOF vs 198 (21.8%) in matched non-SLE patients with COVID-19-AOF: HR 0.98 (0.71–1.34). Seventy-five patients in the SLE COVID-19-AOF group and 299 in the matched control group were followed up from day 30 to day 90. During this period, 19 in-hospital deaths occurred in the SLE group (25.3%) vs 46 (15.4%) in the matched control group; the HR associated with death occurring after COVID-19-AOF among patients with SLE was 1.83 (1.05–3.20).

Conclusions COVID-19-AOF is associated with a poor late-onset prognosis among patients with SLE.

Key messages

What is already known about this subject?
- The interplay between COVID-19 and systemic lupus erythematosus (SLE) has yet to be defined.
- Currently available data regarding the impact of COVID-19 in SLE sound reassuring, but most studies are based on a small number of patients.

What does this study add?
- In this nationwide cohort study, we found that patients with SLE had a poor late-onset prognosis after COVID-19-associated organ failure compared with a matched control population.
- The HR associated with SLE for risk of death between day 30 and day 90 after the first day in the hospital for COVID-19-associated organ failure was 1.83 (1.05–3.20; p=0.03).

How might this impact on clinical practice or future developments?
- Anti-SARS-CoV-2 vaccination among the SLE population appears to be a priority.

INTRODUCTION

The interplay between COVID-19 and systemic lupus erythematosus (SLE) has yet to be defined. Indeed, many patients with SLE are exposed to immunosuppressive drugs, are more susceptible to viral infections and often suffer from chronic kidney or cardiovascular diseases, which are additional risk factors for severe COVID-19.[1] On the other hand, glucocorticoids and hydroxychloroquine, the drugs most widely used in SLE treatment, have also been investigated to treat COVID-19.[2 3] Moreover, type 1 interferon (IFN) such as IFNα and the antibodies anti-IFNα are involved both in SLE and severe COVID-19.[4–7] Although currently available data regarding the impact of COVID-19 in SLE sound reassuring,[8–11] most studies are based on a small number of patients. Finally, comparison between SLE, which mainly affects women of childbearing age, and the general population with regard to severe COVID-19 may be challenging.

We used a French nationwide medical and administrative database to analyse the incidence, the specific outcomes and the characteristics associated with COVID-19-associated organ failure (COVID-19-AOF) in patients with SLE.

METHODS

Study population and data source

Data of all patients admitted to French hospitals from January 2011 to November 2020 with at least one diagnosis of infection associated with an organ failure and/or SLE were collected from the national medical administrative database, the PMSI (Programme de Médicalisation des Systèmes d'Informations, Information System Medicalization Program). The PMSI database provides a summary with diagnosis and individual medical conditions at discharge of any public or private French healthcare

facilities. Information covers both medical and administrative data. Each facility produces its own anonymous standardised set of data, which are then compiled at the national level. Even though these data are anonymous, the system allows to follow all hospital stays for each individual patient. Routinely collected medical data include, among other data, main diagnosis, secondary diagnoses and the procedures performed. Administrative data include, among other data, age, gender, year, duration of hospital stay and location of the hospital. In-hospital death is also reported. Diagnoses identified during the hospital stay are coded according to the International Classification of Diseases, 10th Revision (ICD-10). Procedures performed during the hospital stay are coded according to the 'Classification Commune des Actes Médicaux' (French Common Classification of Medical Procedures). Since 2004, each hospital's budget depends on the medical activity described in this specific programme. Regular checks are made by the social insurance authority to ensure that data are correctly imputed. To select the SLE population, we first extracted from the PMSI database all records of patients for whom at least one ICD-10 M32 diagnosis was reported. We excluded patients younger than 15 years old and patients admitted to hospital only for scheduled sessions (chronic haemodialysis, radiotherapy, chemotherapy).

Definitions

We defined COVID-19-AOF as the combination of at least one of the diagnosis codes of COVID-19 (ICD-10 codes 'U071', 'U0710', 'U0711', 'U0712', 'U0714', 'U0715'), with one code referring to an organ failure diagnosis (listed in online supplemental materials). This definition, which matches the definition for sepsis, has been previously used and validated in medical administrative database studies.[12–14] To be allocated in the SLE group, COVID-19-AOF had to follow or be concomitant with an SLE 'M32' code. For an exhaustive description of diagnosis and procedure codes used, see online supplemental materials. To determine patients' phenotype, we used all the specific diagnostic codes reported during or before the COVID-19-AOF stay.

Matching procedure

Each patient with SLE who experienced COVID-19-AOF during the period of study was randomly matched with five non-SLE control patients with COVID-19-AOF and one patient with SLE without evidence of COVID-19 infection. We used a random exact matching procedure (without replacement) using the following matching variables: age ±2 years, gender, chronic kidney disease, arterial hypertension, cardiovascular history, diabetes mellitus, chronic pulmonary disease and obesity. We verified matching accuracy and efficacy by calculating the standardised differences for the matching variables between the various matched populations.

Survival analysis

Kaplan-Meier method was used to present 90-day survival, taking day 0 as the first hospital admission for COVID-19-AOF. For the control SLE non-COVID-19 population, we used the first day of a randomly selected stay before 2020 (2011–2020 period) as day 0.

In order to calculate the HR of death after COVID-19-AOF according to SLE status, and because we cannot assume the proportional hazard hypothesis for the whole period between day 0 (D0) and day 90 (D90), we split this period into two parts: D0–D30 and D30–D90. For the second time period, we considered only the subgroups of patients who survived after 30 days of follow-up, taking D30 as the new day 0. We used standard univariable Cox proportional hazard model for the unmatched analysis and univariable marginal Cox proportional hazard model[15] for the postmatching analysis.

Statistical statement

Categorical variables are presented as number (percentage). Quantitative variables are presented as median (first quartile–third quartile). HRs are presented with their 95% CI. We used Student's t-test and χ^2 test for univariable comparisons, as appropriate. All analyses were performed using SAS V.9.4 software. Kaplan-Meier curves were built with R V.4.0.3 software.

RESULTS

Characteristics of patients with SLE experiencing COVID-19-AOF

From March to November 2020, 127 380 hospital stays in France matched the definition of COVID-19-AOF. Among them, there were 196 unique patients with SLE and 113 567 unique patients without SLE. A flow chart of these selected populations is presented in online supplemental figure S1. A comparison of SLE patients with COVID-19-AOF versus non-SLE patients with COVID-19-AOF is presented in table 1. The characteristics of patients with SLE admitted to hospital within the study period but without any evidence of COVID-19 are also presented for information. Briefly, SLE patients with COVID-19-AOF were younger (65 (52–76) years vs 76 (64–86) years; p<0.0001), less frequently male (n=50 (25.5%) vs n=56 601 (57.8%); p<0.0001) and had more comorbidities than the general population with COVID-19-AOF. Patients with SLE were also more frequently admitted to the intensive care unit (ICU) (n=83 (42.4%) vs n=40 304 (35.6%); p=0.04) and underwent more often renal replacement therapy for acute kidney injury (AKI; n=14 (7.1%) vs n=3744 (3.3%); p=0.003).

Crude analysis of 30-day and 90-day survival of patients with SLE experiencing COVID-19-AOF

At D30, 43 (21.9%) in-hospital deaths occurred among SLE patients with COVID-19-AOF vs 31 274 (27.6 %) in the unmatched non-SLE patients with COVID-19-AOF. In the D0–D30 period, the HR of death associated with presence of SLE was 0.69 (0.51–0.93). At D90, there was no perceptible difference regarding in-hospital mortality between both groups: 59 deaths (30.1%) in the SLE group vs 35 130 (30.9%) in the control group. In the D30–D90 period, the HR of death associated with presence of SLE was 1.52 (0.93–2.47). A sensitivity analysis using Cox model adjusted for age and sex is presented in online supplemental figure S6.

The Kaplan-Meier curve of the 90-day survival of these populations is displayed in figure 1. The survival of an unmatched SLE population without any evidence of COVID-19 is also displayed for information.

Postmatching analysis of 30-day and 90-day survival of patients with SLE experiencing COVID-19-AOF

Based on the presence of comorbidities, we were able to match 908 non-SLE patients with COVID-19-AOF and 170 SLE patients without COVID-19 with 190 SLE patients with COVID-19-AOF.

The characteristics of these matched populations as well as the standardised differences for the matching variables are displayed in table 2. The rate of ICU admission was similar

Table 1 Characteristics of patients with and without SLE experiencing COVID-19-AOF from March to November 2020 in France

	COVID-19-AOF			SLE non-COVID-19
	SLE	Non-SLE		
	n=196	n=113 371	P value*	n=7139
Age, years, median (Q1–Q3)	65 (52–76)	76 (64–86)	<0.001	45 (33–59)
Male sex, n (%)	50 (25.5)	56 601 (57.8)	<0.0001	992 (13.9)
Arterial hypertension, n (%)	139 (70.9)	72 701 (64.1)	0.05	2384 (33.4)
Cardiovascular history, n (%)	85 (43.4)	41 675 (36.8)	0.05	1699 (23.8)
Chronic kidney disease, n (%)	81 (41.3)	21 750 (19.2)	<0.001	1100 (15.4)
History of solid organ transplantation, n (%)	8 (4.6)	579 (0.5)	<0.001	157 (2.2)
Obesity, n (%)	69 (35.2)	31 210 (27.5)	0.2	888 (12.4)
Chronic pulmonary disease, n (%)	55 (28.1)	22 182 (19.6)	0.003	831 (11.6)
ICU admission, n (%)	83 (42.4)	40 304 (35.6)	0.04	
SAPS II at ICU admission, median (Q1–Q3)†	35 (26–52)	36 (27–47)	0.41	
Invasive mechanical ventilation, n (%)	36 (18.4)	17 513 (15.5)	0.26	
Renal replacement therapy for AKI, n (%)	14 (7.1)	3774 (3.3)	0.0003	
Use of pressor amines, n (%)	31 (15.8)	14 683 (13.0)	0.23	

Characteristics of the SLE population admitted to French hospitals without any evidence of COVID-19 during the same period are also presented for information.
*P values are given for significance of the difference between the first two groups.
†SAPS II is only available for ICU-admitted patients.
AKI, acute kidney injury; AOF, associated organ failure; ICU, intensive care unit; Q1, first quartile; Q3, third quartile; SAPS II, Simplified Acute Physiology Score; SLE, systemic lupus erythematosus.

between patients with SLE and matched patients without SLE experiencing COVID-19-AOF: n=82 (43.2%) in the SLE group vs n=242 (43.3%) in the non-SLE matched control group.

The Kaplan-Meier curve of the 90-day survival of these matched populations is displayed in figure 2. More details about the type of discharge after hospital stay following COVID-19-AOF are provided in online supplemental figure S3.

At D30, 43 deaths (22.6%) were observed within SLE patients with COVID-19-AOF vs 198 (21.8%) within the matched non-SLE patients with COVID-19-AOF. The HR of death associated with presence of SLE for this period was 0.98 (0.71–1.34).

A follow-up from D30 to D90 was possible for 75 SLE patients with COVID-19-AOF and 299 non-SLE patients with COVID-19-AOF. A comparison of their baseline features as well as detailed data of their follow-up is available in online

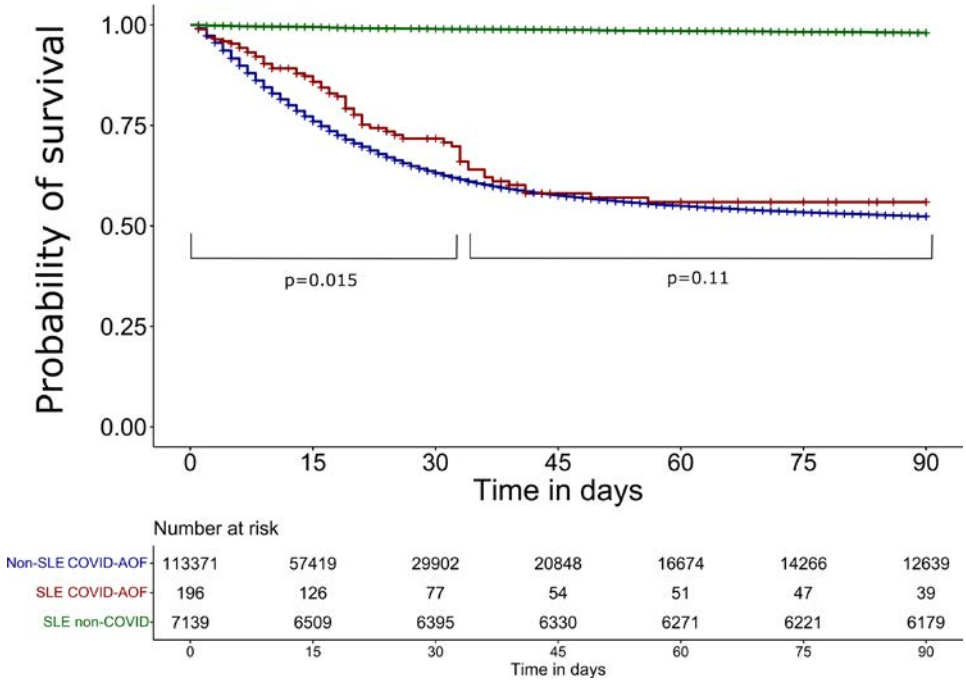

Figure 1 Survival at D90 of patients with SLE experiencing COVID-19-AOF in France (in red) from March 2020 to November 2020 compared with an unmatched control population without SLE (in blue) with COVID-19-AOF during the same period. For information, the survival of an unmatched SLE population admitted in France during the same period without any evidence of COVID-19 is shown in green. P value is given for the time periods D0–D30 and D30–D90 for comparison between SLE patients with COVID-19-AOF and non-SLE patients with COVID-19-AOF. AOF, associated organ failure; D, day; SLE, systemic lupus erythematosus.

Table 2 Characteristics of matched patients with and without SLE experiencing COVID-19-SLE from March to November 2020 in France

	COVID-19-AOF		SLE non-COVID-19	
	SLE	Non-SLE		Standardised differences†
	n=190	n=908	n=170	
Age, years, median (Q1–Q3)‡	65 (54–76)	66 (55–77)	63 (52–75)	−0.0630
Male sex, n (%)‡	48 (25.3)	235 (25.8)	37 (21.8)	0.0142
Arterial hypertension, n (%)‡	137 (72.1)	656 (72.3)	124 (72.9)	−0.0032
Cardiovascular history, n (%)‡	81 (42.6)	380 (41.9)	69 (40.6)	0.0158
Chronic kidney disease, n (%)‡	75 (39.5)	341 (37.6)	66 (38.8)	0.0394
Obesity, n (%)‡	68 (35.8)	318 (35.0)	56 (32.9)	0.0160
Chronic pulmonary disease, n (%)‡	53 (27.9)	253 (27.9)	40 (23.5)	0.0007
Diabetes mellitus, n (%)‡	51 (26.8)	254 (27.9)	42 (24.7)	−0.0254
ICU admission, n (%)	82 (43.2)	391 (43.1)		
SAPS II at ICU admission, median (Q1–Q3)*	36 (27–53)	37 (27–50)		0.0028
Invasive mechanical ventilation, n (%)	36 (18.9)	169 (18.6)		0.0086
Renal replacement therapy for AKI, n (%)	13 (6.8)	61 (6.7)		0.0049
Use of pressor amines, n (%)	31 (16.3)	147 (16.2)		0.0034

Characteristics of a matched SLE population admitted to French hospitals without any evidence of COVID-19 during the same period are also presented for information.
*SAPS II is only available for ICU-admitted patients.
†Standardised differences are given for significance of the difference between the first two groups.
‡Matching variables.
AKI, acute kidney injury; AOF, associated organ failure; ICU, intensive care unit; Q1, first quartile; Q3, third quartile; SAPS II, Simplified Acute Physiology Score; SLE, systemic lupus erythematosus.

supplemental figures S3–S4. The Kaplan-Meier curve of their D30–D90 survival is displayed in figure 3. During this period, we observed 19 deaths in the SLE group (25.3%) and 46 (15.4%) in the matched control group. The HR for death occurring from day 30 to day 90 after COVID-19-AOF among patients with SLE was 1.83 (1.05–3.20). A sensitivity analysis using a rematch procedure for patients still alive at D30 is presented in online supplemental figure S7–S8.

All HRs calculated for the crude and the matched analyses are summarised in table 3.

Healthcare use after COVID-19-AOF

Analysis of healthcare use post COVID-19-AOF for the matched population still alive at D30 is presented in online supplemental figure S5. We observed that patients with SLE had more reports of coinfection diagnoses than the matched control patients (mean (±SD): 9.09 (±8.5) in the SLE group vs 7.6 (±6.1) in the control group). Otherwise, we found no difference in the number of SLE-related outcomes such as renal biopsy or dialysis for AKI. Similarly, we found no difference

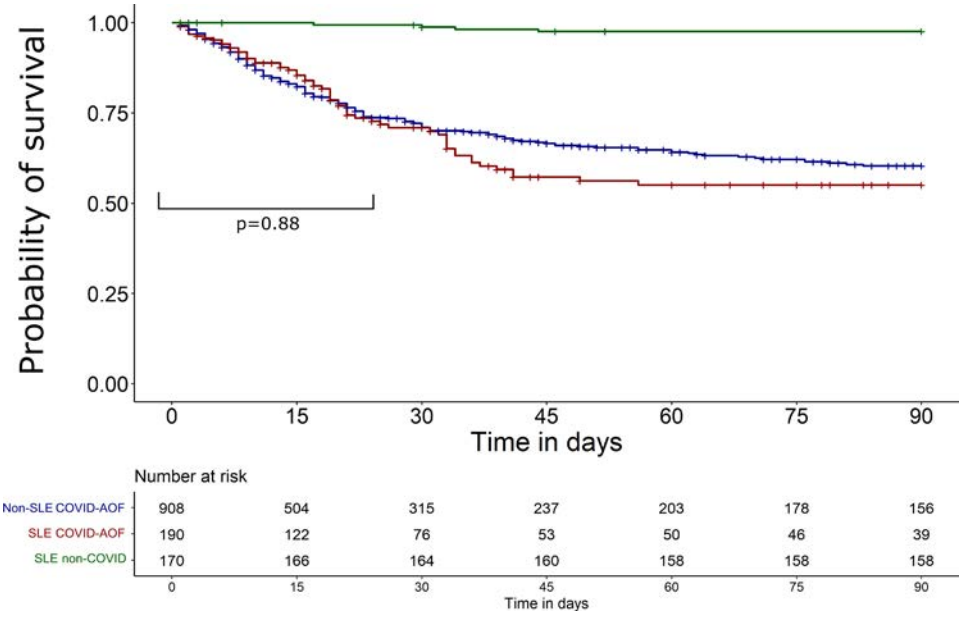

Figure 2 Survival at D90 of patients with SLE experiencing COVID-19-AOF in France (in red) from March 2020 to November 2020 compared with a matched control population (in blue) with COVID-19-AOF but without SLE admitted during the same period. For information, survival of a matched SLE population admitted in France during the same period without any evidence of COVID-19 is shown in green. P value is given for the time period D0–D30 for comparison between SLE patients with COVID-19-AOF and non-SLE patients with COVID-19-AOF. AOF, associated organ failure; D, day; SLE, systemic lupus erythematosus.

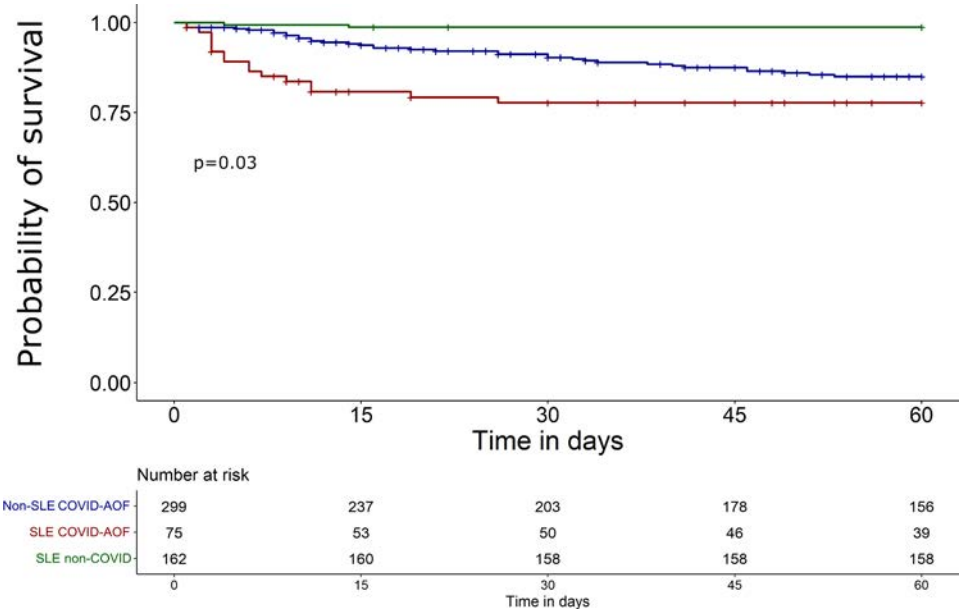

Figure 3 D30–D90 survival of patients still alive at D30. Patients with SLE experiencing COVID-19-AOF in France from March 2020 to November 2020 (in red) compared with a matched control population without SLE (in blue) with COVID-19-AOF during the same period. For information, survival of a matched SLE population admitted in France during the same period without any evidence of COVID-19 is shown in green. P value is given for comparison between SLE patients with COVID-19-AOF and non-SLE patients with COVID-19-AOF. AOF, associated organ failure; D, day; SLE, systemic lupus erythematosus.

in unspecific outcomes such as number of hospital stays, diagnosis of pulmonary embolism or coronary angiography for myocardial infarction.

DISCUSSION

Our analysis of the French national medical and administrative database showed that SLE is associated with a worsened prognosis during COVID-19-AOF requiring hospitalisation. Importantly, we observed an increased late-onset mortality, between D30 and D90, for hospitalised patients with SLE still alive 30 days after the first day of admission, independently of age, gender and various comorbidities such as chronic kidney disease.

Conversely, in our selected unmatched population, the crude analysis of the COVID-19-AOF outcome showed that hospitalised patients with SLE have an unchanged prognosis as compared with the general population. Such observation may be biased because patients with SLE are younger and more frequently female. On the other hand, patients with SLE have more comorbidities.[16] Using a matching strategy that was devised to limit such biases, a specific delayed risk was unravelled in hospitalised SLE patients with COVID-19-AOF. Such increased risk might be related to the high coinfection rate during or after COVID-19 in these patients. Patients with SLE could be more susceptible to coinfection due to their treatment. They may also be at risk of COVID-19-induced immune paralysis. Of note, SLE flares or cardiovascular events rates did not seem to be increased after COVID-19-AOF. Since we did not have access to patients' detailed files and treatments, we were not able to confirm that SLE disease went uneventful.

Several studies have assessed the specific prognosis of patients with SLE during COVID-19 and displayed heterogenous results.[8 9 17–20] Most studies analysed a mixed subset with various rheumatic diseases and included a very limited number of patients with SLE. Moreover, no matching strategy was used to limit the bias related to age, sex and comorbidities among the SLE population.

We found a relatively low number of patients with SLE among the French population with COVID-19-AOF (196 of 113 371, 0.2%) between March and November 2020, whereas Cordtz *et al*[21] recently reported an increased risk of hospitalisation for SLE patients with COVID-19 in Denmark compared with the general population. It might be due to a lack of precision in coding SLE procedures; however, our results fit well with the prevalence of SLE in France, estimated at 5 for 10 000.[22] Moreover, the characteristics of our patients with SLE are consistent with previously published large epidemiological studies on patients with SLE conducted in France.[22]

Because it is a hospital database, we only had access to in-hospital mortality. Since patients with SLE are more likely to be admitted to hospital, the proportion of in-hospital mortality for patients with SLE is expected to be higher than for the general population. However, we found that most of the patients discharged before D90 went home; therefore, we can assume that a very limited number of them died after discharge. Follow-up was limited to 90 days after COVID-19, with data gathered before November 2020; thus, the investigated population encountered almost exclusively the original 'Wuhan' SARS-CoV-2 strain.

Table 3 Summary of the main results of the study

Analysis	Period	HR of SLE	95% CI
Crude	D0–D30	0.69	0.51 to 0.93
	D30–D90	1.52	0.93 to 2.47
Matched	D0–D30	0.98	0.71 to 1.34
	D30–D90	1.83	1.05 to 3.20

HR is given for risk of death associated with SLE diagnosis after a COVID-19-AOF.
For crude analysis HR was calculated using a standard univariable Cox proportional hazard model.
HR for the matched analysis was calculated using a univariable marginal Cox proportional hazard model.
AOF, associated organ failure; SLE, systemic lupus erythematosus.

Our work has several strengths. First, in accordance with the French Health Insurance System, PMSI gather exhaustive data of all French hospitals, meaning that our data included every patient with at least one diagnosis of SLE reported from 2011 to 2020. Thanks to linking between the successive hospitalisation episodes, we were able to examine all hospital records of each individual patient and to assess 30-day and 90-day outcomes. Our matched study allowed us to take into account several confounding factors that usually blur the comparison between patients with SLE and the general population. Although the severity of COVID-19-AOF was not included in the matching procedure, we observed a very similar rate of ICU admission, Simplified Acute Physiology Score II and healthcare use between patients with SLE and the matched control population, confirming the validity of the matching process.

COVID-19-AOF has a late-onset poor prognosis in patients with SLE. Further studies are warranted to delineate the clinical course of patients with SLE who survived severe COVID-19.

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Contributors AM designed and conducted the analysis and wrote the manuscript. TP, SR, DvG, AS and KS were involved in the project development and edited the manuscript. J-FT directed the project and wrote the manuscript. AM is the guarantor of this study.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval In accordance with the French regulatory system regarding personal and medical data and after agreement of the institutional review board (IRB), our institution was allowed to access the PMSI database. We only had access to patients diagnosed with infection-associated organ failure and/or SLE according to our definition. All data were anonymised. This study does not involve human participants.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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CLINICAL SCIENCE

BNT162b2 vaccine-induced humoral and cellular responses against SARS-CoV-2 variants in systemic lupus erythematosus

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ABSTRACT

Objectives Our aim was to evaluate systemic lupus erythematosus (SLE) disease activity and SARS-CoV-2-specific immune responses after BNT162b2 vaccination.

Methods In this prospective study, disease activity and clinical assessments were recorded from the first dose of vaccine until day 15 after the second dose in 126 patients with SLE. SARS-CoV-2 antibody responses were measured against wild-type spike antigen, while serum-neutralising activity was assessed against the SARS-CoV-2 historical strain and variants of concerns (VOCs). Vaccine-specific T cell responses were quantified by interferon-γ release assay after the second dose.

Results BNT162b2 was well tolerated and no statistically significant variations of BILAG (British Isles Lupus Assessment Group) and SLEDAI (SLE Disease Activity Index) scores were observed throughout the study in patients with SLE with active and inactive disease at baseline. Mycophenolate mofetil (MMF) and methotrexate (MTX) treatments were associated with drastically reduced BNT162b2 antibody response ($\beta=-78$, $p=0.007$; $\beta=-122$, $p<0.001$, respectively). Anti-spike antibody response was positively associated with baseline total immunoglobulin G serum levels, naïve B cell frequencies ($\beta=2$, $p=0.018$; $\beta=2.5$, $p=0.003$) and SARS-CoV-2-specific T cell response ($r=0.462$, $p=0.003$). In responders, serum neutralisation activity decreased against VOCs bearing the E484K mutation but remained detectable in a majority of patients.

Conclusion MMF, MTX and poor baseline humoral immune status, particularly low naïve B cell frequencies, are independently associated with impaired BNT162b2 mRNA antibody response, delineating patients with SLE who might need adapted vaccine regimens and follow-up.

Key messages

What is already known about this subject?

- BNT162b2 efficacy and safety has been described in studies mixing different rheumatic and musculoskeletal diseases (RMDs).

What does this study add?

- No serious adverse effects nor systemic lupus erythematosus (SLE) flares have been documented after BNT162b2 in patients with SLE.
- Not only mycophenolate mofetil and methotrexate, but also a poor humoral immune status at baseline, impair vaccine antibody response.
- Although decreased, serum neutralising activity against variants of concerns is conferred to vaccine responders.

How might this impact on clinical practice or future developments?

- These parameters could be helpful for physicians to delineate which patients should have antibody measurement after full BNT162b2 vaccination and should be proposed a third injection of BNT162b2 vaccine.

INTRODUCTION

Because of the tremendous paucity of data on the impact of rheumatic and musculoskeletal diseases (RMDs) and associated immune-modulatory treatments on SARS-CoV-2 vaccination efficacy, most of the recommendations are currently based on expert opinions. Messenger RNA (mRNA) vaccination is a novel practice, and its tolerance, immunogenicity and efficacy are poorly documented in RMD. Consequently, rules for vaccine against SARS-CoV-2 vary according to country and over time.[1 2] Factors affecting the anti-SARS-CoV-2 antibody response have been explored, but only after a first dose or in studies mixing RMD.[3 4] Furthermore, the impact of treatments on the vaccine response is often studied mixing different RMDs.[5] Importantly, Simon *et al* recently showed that interindividual variations to vaccination were more related to the disease itself rather than to concomitant treatments.[3] Additionally, most of these studies focused on RMD treatments and not on the immunological status, which may also affect the antibody response. Among RMDs, systemic lupus erythematosus (SLE) could represent a peculiar challenge to vaccination against SARS-CoV-2.[6] The deregulation of type I interferon (IFN) pathways associated with this condition[7] might impact on vaccine antibody response.[8] SLE-associated impaired lymphocyte functions might

also impair vaccine efficacy.[9 10] Altogether, the risk of flares induced by vaccines is highly dependent on the disease studied and the specific scores used to measure this activity. It is therefore important to focus vaccine evaluation on homogeneous groups of patients.

Compared with the general population, patients with SLE do not seem to be at higher risk of SARS-CoV-2 infections or severe COVID-19,[11–14] but this finding remains controversial as other studies found that patients with SLE may be at higher risk of hospitalisation during their COVID-19 course.[15 16] Increase of SLE disease activity has been previously reported during COVID-19[17 18] but the risk of SLE flares following vaccination does not appear to be increased,[18] although this point still requires confirmation through follow-up of patients with SLE evaluated at identical pre-vaccination and post-vaccination timepoints in a prospective study. Finally, it remains unclear whether failures to induce antibody responses in patients under immunomodulatory regimens such as abatacept, mycophenolate mofetil (MMF), CD-20 inhibitors, calcineurin inhibitors[5 19] are also associated, or not, with an absence of vaccine-induced SARS-CoV-2-specific T cell responses. Here, we report post-vaccination disease activity data in 126 patients with SLE, prospectively followed during the completion of a two-dose mRNA Pfizer/BioNTech (BNT162b2) vaccination regimen. SARS-CoV-2-specific humoral and cellular responses were monitored against not only the SARS-CoV-2 historical strain but also against SARS-CoV-2 variants of concern (VOCs).

METHODS

Patients

The clinical study was conducted in the Internal Medicine Department 2, French National Reference Center for SLE, Pitié-Salpêtrière Hospital, Paris, France. Eligible patients were 18 years or older, with a diagnosis of SLE according to the revised American College of Rheumatology (ACR) classification criteria.[20] Active lupus was defined with two scores: (1) at least 1 British Isles Lupus Assessment Group (BILAG) B in any organ, (2) SLE Disease Activity Index (SLEDAI) 2K score >4. Patients were vaccinated according to the French recommendations for COVID-19 vaccination.[2]

Outcomes and follow-up

Patients were vaccinated at baseline (first dose) against SARS-CoV-2 with Pfizer/BioNTech (BNT162b2) vaccine and received the second dose at day (D) 21–28, unless contraindicated. Patients were evaluated at baseline and at D7–14, D21–D28, D42. Patients were asked to contact their physician if they developed any symptoms in order to be promptly examined.

At each visit, the following endpoints were assessed:

- Adverse events.[21]
- SLE activity measured with SLEDAI 2K score[22 23] and BILAG score.[24]
- SLE flares defined with the SELENA-SLEDAI Flare Index (SFI)[22 23] and BILAG 2004 score.[24–26]
- SARS-CoV-2 infection measured with anti-nucleocapsid antibodies.
- Changes in serological activity (anti-dsDNA antibodies and C3), IFN-α, anti-phospholipid antibodies.[27]
- Anti-spike antibodies.
- B, T and natural killer cell quantification.
- B lymphocyte subsets.

Patient and public involvement

Patients were not involved in the design, or conduct, or reporting or dissemination plans of this research.

Serological analysis

SARS-CoV-2-specific immunoglobulin G (IgG) antibodies were measured as previously described.[28] Serum samples were tested with the Maverick SARS-CoV-2 Multi-Antigen Serology Panel (Genalyte, USA) according to the manufacturer's instructions. The panel is designed to detect antibodies to five SARS-CoV-2 antigens: nucleocapsid, spike S1 receptor binding domain (RBD), spike S1S2, spike S2 and spike S1, within a multiplex format based on photonic ring resonance technology. Briefly, 10 µL of each serum sample was added to a sample well plate array containing required diluents and buffers, and the plate and chip were loaded in the instrument for chip equilibration with the diluent buffer to measure baseline resonance. The serum sample was then charged over the chip to bind specific antibodies to antigens present on the chip. The chip was then washed to remove low-affinity binders, and specific antibodies were detected with anti-IgG secondary antibodies.

Pseudoneutralisation assay

Lentiviral particles carrying the luciferase gene and pseudotyped with spikes of SARS-CoV-2 historical strain or VOCs were produced by triple transfection of 293T cells as previously described.[28] Serum dilutions were mixed and co-incubated with 300 transducing units of pseudotyped lentiviral particles at room temperature for 30 min and then diluted in culture medium (Dulbecco's modified Eagle's medium–GlutaMAX (Gibco) +10% fetal calf serum (Gibco) +1% penicillin/streptomycin (Gibco)). This mixture was then plated on tissue culture–treated black 96-well plates (Costar) with 20 000 HEK 293T-hACE2 cells per well in suspension. To prepare the suspension, cell flasks were washed with Dulbecco's phosphate buffer saline (DPBS) twice (Gibco), and a single-cell suspension was made in DPBS +0.1% EDTA (Promega) to preserve integrity of hACE2 protein. After 48 hours, the medium was removed from each well and bioluminescence was measured using a luciferase assay system (Promega) on an EnSpire plate reader (PerkinElmer).

B cell phenotyping

B cell phenotyping was assessed on fresh whole blood. Briefly, 400 µL of blood was washed in PBS1X-RPMI 5% (Gibco) then transferred in tubes containing anti-CD45 V500, anti-CD19 APC, anti-IgD FITC, anti-CD38 PerCPCy5.5, CD27 PE-Cy7, CD24 APC-H7, CD86 PE, CD3 BV421, CD14 BV421, CD21 BV421 lyophilised antibodies (BD Horizon Lyo technology). This lyophilised version of the multicolour panel increases the reagent stability and the assay performance. Cell staining was performed at room temperature for 15 min, then cells were washed and fixed (BD Cell Fix). Events were acquired on a BD FACS Canto II flow cytometer (BD Biosciences) and analysed with FlowJo V.10 software (FlowJo, LLC) according to the gating strategy presented in online supplemental figure S1.

SARS-CoV-2-specific T cell responses

SARS-CoV-2-specific T cell responses were assessed in the clinical immunology laboratory of Pitié-Salpêtrière Hospital by a whole blood Interferon-Gamma Release Assay (IGRA) following manufacturer's instructions (Quantiferon SARS-CoV-2, Qiagen). This test uses two Qiagen proprietary mixes of SARS-CoV-2

spike protein (Ag.1 and Ag.2) selected to activate both CD4 and CD8 T cells. Briefly, venous blood samples were transferred into the Quantiferon tubes containing spike peptides as well as positive and negative controls. Whole blood was incubated at 37°C for 16–24 hours and centrifuged to separate plasma. IFN-γ (IU/mL) was measured in these plasma samples using QuantiFERON Human IFN-γ SARS-CoV-2 ELISA kit (Qiagen) on Dynex DS2 analyser (Qiagen).

Statistical analysis

Baseline characteristics are reported with descriptive statistics. Linear regression models were used to assess the association between clinical and biological characteristics and the titre of IgG anti-RBD at day 42 in unadjusted and multivariable analysis. We considered potential confounders known or suspected to be associated with vaccine response such as demographic features (age, sex), activity of SLE, concomitant immune modulatory treatments and data from T, B and NK cells phenotyping. The beta coefficient is the degree of change in the outcome variable for every 1 unit of change in the predictor variable. If the beta coefficient is not statistically significant (ie, the p value is not significant), the variable does not significantly predict the outcome. If the beta coefficient is significant, examine the sign of the beta. If the beta coefficient is positive, the interpretation is that for every 1-unit increase in the predictor variable, the outcome variable will increase by the beta coefficient value. If the beta coefficient is negative, the interpretation is that for every 1-unit increase in the predictor variable, the outcome variable will decrease by the beta coefficient value. For example, if the beta coefficient is 0.80 and statistically significant, then for each 1-unit increase in the predictor variable, the outcome variable will increase by 0.80 unit. Paired t-tests were used to detect differences in activity scores and biological data over time. As we excluded the 10 patients for whom follow-up was incomplete, we did not have to perform any imputation for missing data. Non-parametric test were used as Mann-Whitney U test to compare two independent groups, Wilcoxon test to compare paired values and Pearson coefficient to calculate correlation. Significant p values are indicated as follows: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Statistical analysis was performed using R software (V.4.1.0) and GraphPad Prism software, V.6 (GraphPad, San Diego, California, USA).

RESULTS

Demographic and disease characteristics

Vaccination against SARS-CoV-2 with Pfizer/BioNTech vaccine was proposed by their SLE referring physician to 180 patients with SLE; 127 (70.5 %) immediately accepted, 35 (19.4%) patients refused and 18 (10.0%) eventually accepted on reflection, 9 of them were vaccinated in another centre (figure 1). A total of 136 patients with SLE were enrolled and received one first dose; 3 patients received only one dose: either because they developed COVID-19 within 10 days after the first dose (n=2) or because COVID-19 had been contracted 3 months before the first dose (n=1). Among the 133 patients with SLE who received two doses, 126 (92.6%) completed all the visits and were included in the final analysis. Baseline clinical characteristics of these 126 patients are summarised (table 1). Treatments received from D1 to D42 were distributed as follows: hydroxychloroquine (n=106; 84.1%; median daily dose: 400 mg), prednisone (n=70; 55.5%) with 57 patients (45.2%) receiving less than 10 mg daily (median daily dose: 5 mg) and 13 (10.3%) more than 10 mg daily (median daily dose: 19 mg), methotrexate

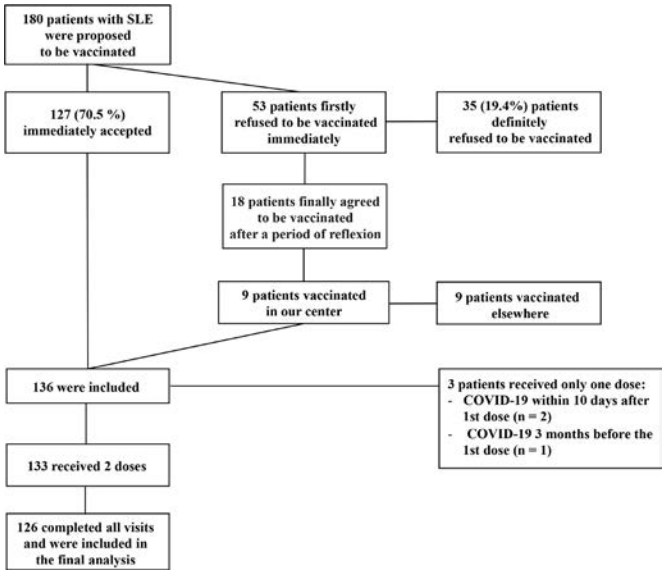

Figure 1 Study population and enrolment process. Patients with systemic lupus erythematosus (SLE) were offered BNT162b2 vaccine through 15 January 2021.

(n=20; 15.9%; median weekly dose 15 mg); mycophenolate mofetil (n=24; 19.0%; median daily dose=2000 mg), azathioprine (n=5; 4.0%; median daily dose: 100 mg) and belimumab (n=15; 11.9%), of whom 7 had intravenous and 8 subcutaneous injections, respectively.

Adverse BNT162b2 vaccine-associated events in patients with SLE

No related serious adverse events (AEs), no grade 4 reactions and no withdrawals due to related AEs were observed (online

Table 1 Demographics and clinico-biological features of patients with SLE

	N=126
Female sex	114 (90.5%)
Age, years	46.6 (33.9, 58.7)
Time from SLE onset, months	14.1 (7.2, 23.1)
Time from last flare, months	2.4 (0.5, 6.2)
SLEDAI 2K	2.0 (0.0, 4.0)
SLEDAI 2K>4	24 (19.0%)
At least one BILAG score ≥B	20 (16.7%)
Hydroxychloroquine blood dosage, µg/L	855.5 (641.0, 1,123.0)
Low complement C3 (<0.7 g/L)	22 (17.5%)
Increased dsDNA binding (>30 IU/mL)	63 (50.0%)
Detectable interferon alpha (>2 IU/mL)	17 (14.8%)
Hydroxychloroquine	106 (84.1%)
No corticosteroids	56 (44.4%)
Corticosteroids≤10 mg/day	57 (45.2%)
Corticosteroids>10 mg/day	13 (10.3%)
Belimumab (intravenous, n=7, subcutaneous, n=8)	15 (11.9%)
Mycophenolate mofetil	24 (19.0%)
Azathioprine	5 (4.0%)
Methotrexate	20 (15.9%)

Qualitative variables are presented as n (%). Quantitative variables are presented as median (IQR).
.BILAG, British Isles Lupus Assessment Group; dsDNA, double-stranded DNA; SLE, systemic lupus erythematosus; SLEDAI, SLE Disease Activity Score.

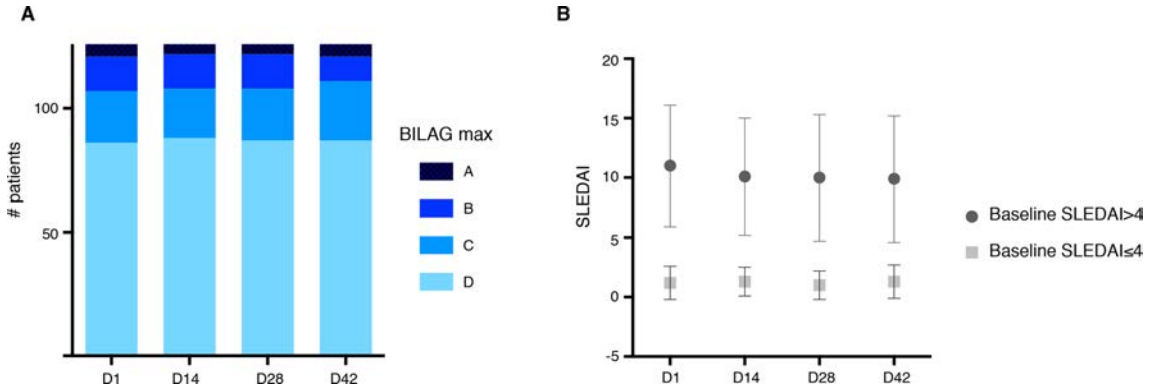

Figure 2 Evolution of systemic lupus erythematosus (SLE) activity following vaccination. (A) Repartition of maximal British Isles Lupus Assessment Group (BILAG) score at baseline and following vaccination. (B) Evolution of mean SLE Disease Activity Index (SLEDAI) 2K score following vaccination.

supplemental figure S2 and online supplemental table S1). Local reactions, predominantly pain at the injection site, were mild to moderate (grade 1 and 2).

BNT162b2 vaccine effect on SLE disease activity

At baseline, 29 (23.0%) and 20 (16.7%) patients had active SLE according to SLEDAI (SLEDAI 2K>4) and to BILAG (≥1 BILAG B), respectively. Within 42 days following vaccination (figure 2A), mild disease flares were observed in three patients following vaccination, with a mucocutaneous BILAG score going from C to A in one individual, a musculoskeletal BILAG going from C to B in one individual and from D to C for one another vaccinated patient. In return, nine patients (five active and four inactive) clinically improved following vaccination with a musculoskeletal BILAG going from B to C for four patients and from C to D for three patients, a mucocutaneous BILAG going from A to C for one patient and a renal BILAG going from A to B for one patient. No statistically significant variation of SLEDAI score was observed throughout the study for patients with active and inactive SLE according to initial SLEDAI score (SLEDAI 2K score ≤4 at day 1: mean (SD); 1.2 (1.4) day 1; 1.3 (1.2) day 14; 1.0 (1.2) day 28; 1.3 (1.4) day 42, ns; SLEDAI score >4 at day 1: 11 (5.1) day 1; 10.1 (4.9) day 14; 10.0 (5.3) day 28; 9.9 (5.3) day 42, ns; figure 2B). Altogether, vaccination is not preferentially associated with exacerbation of SLE symptoms than with clinical improvement. When observed, variations of BILAG and SLEDAI scores were not preferentially observed in patients with either active or inactive SLE at baseline.

Effect of treatments and baseline immune status on the immunogenicity of the BNT162b2 vaccine in SLE

Higher total serum IgG levels measured at baseline were associated with better seropositivity rates (β=2.0; 95% CI 0.34 to 3.6; p=0.018), while MMF and MTX uses were associated with lower anti-spike antibody production(β = −78; 95% CI −133 to −22; p=0.007 and β=−122; 95% CI −184 to −61; p<0.001, respectively) measured 14.7 days on average after the second injection (SD 1.9 days). Total lymphocyte counts and IFN-α levels at baseline were not significantly associated with seropositivity rates (table 2). Hydroxychloroquine, steroids (either high or low dose) or belimumab use during the 42 days following vaccination did not impact anti-spike antibody production. Of note, SLE activity was not correlated with anti-spike antibody response, regardless of the score used to measure disease activity (see table 2 and online supplemental table S2 with BILAG and SLEDAI, respectively).

Since IgG levels but not total lymphocyte counts were significantly associated with the antibody response, we next studied the effect of lymphocyte subpopulation counts at baseline (table 3).

We found that B lymphocyte counts were the sole lymphocyte population associated with anti-spike antibody response (β=0.38; 95% CI 0.13 to 0.62; p=0.003). We further characterised the effect of B lymphocyte subsets at baseline. Treatments modifying B cell subpopulations were adjusted in this analysis (table 4). Strikingly, naïve B lymphocyte frequency at baseline was positively associated with anti-spike antibody response at D42 (β=2.5; 95% CI 0.87 to 4.0; p=0.003; table 4).

Effect of treatments on BNT162b2-induced neutralisation responses

We next analysed whether vaccine-induced antibody responses may be protective by evaluating serum-neutralising activity. As expected, we confirm a strong correlation between anti-RBD antibody levels and neutralisation titres (SARS-CoV-2 D614G

Table 2 Baseline predictors of day 42 anti-SARS-CoV-2 RBD IgG titres according to linear regression model

	β	95% CI	P value
Age, years	−0.61	−2.0 to 0.75	0.4
Male sex	−62	−127 to 3.7	0.064
At least one BILAG score ≥B	−45	−106 to 17	0.2
C3, g/L	35	−77 to 147	0.5
dsDNA antibodies, IU/mL	0.04	−0.06 to 0.13	0.4
Detectable IFN-α	−3.4	−7.4 to 0.58	0.093
Total serum IgA, g/L	1.8	−12 to 16	0.8
Total serum IgG, g/L	2.0	0.34 to 3.6	0.018
Total serum IgM, g/L	12	−1.0 to 24	0.071
Lymphocytes count, G/L	6.6	−31 to 44	0.7
Corticosteroids low	−20	−66 to 26	0.4
Corticosteroids high	−50	−127 to 28	0.2
Hydroxychloroquine	−27	−85 to 31	0.4
Azathioprine	−118	−242 to 6.5	0.063
Belimumab	−18	−90 to 54	0.6
Mycophenolate mofetil	−78	−133 to 22	0.007
Methotrexate	−122	−184 to 61	<0.001
Other immunosuppressor	62	−32 to 156	0.2

SLE activity is measured with BILAG score.
*See the Methods section.
BILAG, British Isles Lupus Assessment Group; dsDNA, double-stranded DNA; IFN, interferon; IgG, immunoglobulin G; RBD, receptor binding domain.

Table 3 Baseline B, T and NK cell count predictors of day 42 anti-SARS-CoV-2 RBD IgG titres according to linear regression model

	β*	95% CI	P value
B lymphocyte count, G/L	0.38	0.13 to 0.62	0.003
NK lymphocyte count, G/L	0.21	−0.37 to 0.80	0.5
CD4 + T lymphocyte count, G/L	0.01	−0.09 to 0.11	0.9
CD8 + T lymphocytes, G/L	−0.01	−0.16 to 0.13	0.8

*See the Methods section
IgG, immunoglobulin G; RBD, receptor binding domain.

r=0.82, p<0.0001; figure 3A). Consequently, parameters listed above influencing seroconversion also influenced neutralisation activity (online supplemental table S3). MMF and MTX in particular have a negative impact on induction of neutralising activity (β=−1.1; 95% CI −1.9 to −0.34; p=0.005 and β=−1.9; 95% CI −2.7 to −1.1; p<0.001, respectively, online supplemental table S3). While a majority of MMF/MTX-treated patients still harboured detectable neutralising activity (65% (15/23) MMF-treated patients, 68% (13/19) MTX-treated patients vs 96% (81/84) patients without MMF or MTX), their serum neutralising activity drastically dropped compared with patients receiving other treatments (inhibitory dilution 50 (ID50) D614G median(min–max); 111.2 (30–18 910) in MMF-treated patients vs 90.4 (30–5527) in MTX-treated patients and 684.6 (30–12061) in other patients; p<0.05; figure 3B).

Effect of baseline immune status on BNT162b2-induced neutralisation responses

Consistent with serological studies, naïve B cell decrease at baseline was negatively associated with serum D42 neutralising activity (β=0.04; 95% CI 0.01 to 0.07; p=0.006; online supplemental table S4). As shown in figure 3C, patients with a low naïve B cell compartment (<42% of B cells) developed a lower neutralising activity than patients with normal or high naïve B cell subset frequencies (229.2 (30–2510) in low naïve B cell patients vs 468.3 (30–5421); p<0.05; figure 3C). To more accurately evaluate the effect of naïve B cells on neutralising antibody response, we divided patients with SLE into four groups according to their naïve baseline B cell counts (median(min–max) naïve B cell counts/μL: 9 (0.01–23.2); 41 (27.2–50.9); 68.1 (57.2–98.7); 133.8 (110.1–160.2) in quartiles 1, 2, 3 and 4, respectively; figure 3D). We confirm that naïve B cell counts are positively associated with vaccine-induced neutralising antibody responses (ID50 D614G 93.4 (30–246.5) vs 340.1 (30–1632) in quartiles 1 and 2, respectively; p<0.05 vs 315.2 (30–721.1) in quartile 3; p<0.05; vs 679.9 (60.4–2510) in quartile 4; p<0.001; figure 3D).

These data therefore underline the importance of interrogating initial B cell status as well as immunosuppressive treatments to predict vaccine response.

Table 4 Baseline B cell predictors of day 42 anti-SARS-CoV-2 RBD IgG titres according to linear regression model

	β*	95% CI	P value
Corticosteroids≤10 mg/day	−42	−93 to 9.0	0.10
Corticosteroids>10 mg/day	−135	−230 to -39	0.007
Hydroxychloroquine	−34	−110 to 43	0.4
Azathioprine	−71	−233 to 92	0.4
Belimumab	9.6	−105 to 125	0.9
Mycophenolate mofetil	−146	−224 to -68	<0.001
Methotrexate	−121	−202 to -41	0.004
Other immunosuppressor	203	5.6 to 401	0.044
Marginal zone B lymphocytes, day 1 (%)	−0.22	−2.7 to 2.3	0.9
Autoreactive B lymphocytes, day 1 (%)	−1.5	−4.3 to 1.3	0.3
Naïve B lymphocytes, day 1 (%)	2.5	0.87 to 4.0	0.003
Double negative B lymphocytes, day 1 (%)	3.8	−2.2 to 9.9	0.2
Memory B lymphocytes, day 1 (%)	−0.57	−2.3 to 1.2	0.5

*See the Methods section. Autoreactive B cells (CD21lowCD38low); double negative B cells (CD27-IgD-); marginal zone B cells (CD27 +IgD+); memory B cells (CD27 +IgD-); naïve B cells (CD27-IgD+). B cell subset frequencies are measured in total B cells.

Broad neutralising activity against VOCs in BNT162b2 vaccine responders

The Pfizer/BioNTech vaccine was designed to target the Wuhan isolate described by the end of 2019. However, emerging variants, with enhanced infectivity and the ability to escape immune control, rapidly became dominant. Concerns have been raised as to whether Pfizer/BioNTech vaccine will be effective against these emerging variants, particularly in vaccinated individuals receiving immunosuppressive drugs. We therefore measured neutralising activities in the last 46 serum samples longitudinally collected against four major SARS-CoV-2 lineages: B.1.1.7 (originating in the UK), B.1.351 (described in South Africa), B.1.1.28 (reported in Brazil) and B.1.617 (emerged in India). Consistent with previous studies,[29 30] we found that vaccine-induced IgG antibodies efficiently cross-neutralise variants B.1.1.7 (ID50 median (min–max); D614G 1453 (30–18 910) and B.1.1.7 514.5 (30–12 625), ns; figure 3E). It is noteworthy that serum neutralisation activity decreased with lineages bearing the E484K mutation in the RBD (ID50 B1.617.1 341.1 (30–3996), p<0.001; B.1.617.2 379.3 (30–4982), p<0.001; B.1.617.3 317.9 (30–3604), p<0.01; B.1.1.28 302.3 (30–5757) and B.1.351 88.1 (30–2389); p<0.0001; figure 3E), but remained detectable in a majority of patients (82% for B.1.1.7; 73% for B.1.617.1; 76% for B.1.617.2; 71% for B.1.617.3; 73% for B.1.1.28; 60% for B.1.351; figure 3F). Among patients with neutralising antibody activity against D614G strain, 100% (37/37) of patients also efficiently neutralised B.1.1.7 strain, 89% (33/37) B.1.617.1 variant, 92% (34/37) B.1.617.2 variant, 87% (32/37) B.1.1.28 variant, 89% (33/37) B.1.1.28 variant and 60% of patients (27/37) had detectable neutralising activity against B.1.351.

Altogether, these results demonstrated that vaccinated-SLE harboured decreased neutralising activity against VOCs, as previously described in vaccinated healthy donors.[31 32]

SARS-CoV-2-specific T cell responses induced by the BNT162b2 vaccine in SLE

Beyond antibodies, T cell immunity is required to confer optimal immune protection. In order to gain insight into the specific SARS-CoV-2 T cell response after vaccination in patients with SLE, we evaluated IFN-γ secretion levels after specific T cell stimulation at day 15 after vaccination. While SARS-CoV-2-specific T cell responses were detected in 57% (17/30) of patients who had neutralising antibody titres, cellular responses were only detected in 10% (1/10) of patients who had non-neutralising antibody titres (p<0.05; figure 4A). Interestingly, SARS-CoV-2-specific T cell responses were nevertheless detected in two out of six patients with very low levels of neutralising activity in their serum (ID50 below 100 for D614G strain). Overall, the strength of neutralising antibody response correlates with IFN-γ production by SARS-CoV-2-specific T cells (antigen 1, r=0.462, p=0.003; antigen 2 r=0.424, p=0.007, figure 4B).

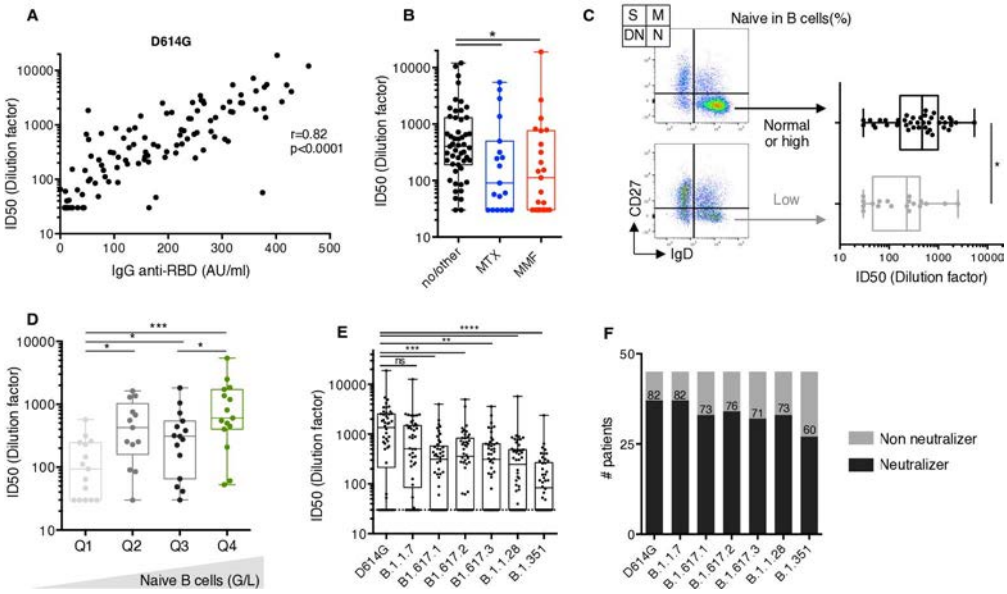

Figure 3 Vaccine-induced neutralising potency. (A) Comparison of serum anti-RBD IgG levels measured by photonic ring immunoassay with neutralising capacity against D614G SARS-CoV-2 (n=126). Spearman coefficient (r) and p value (p) are indicated. (B) Serum neutralising activities against D614G SARS-CoV-2 measured as inhibitory dilution 50 (ID50) in 126 serum samples at D42. Methotrexate (MTX)-treated and mycophenolate mofetil (MMF)-treated patients are colour coded (blue and red, respectively). Patients receiving other treatments are indicated in black. The boxplots show medians (middle line) and first and third quartiles, while the whiskers indicate minimal and maximal values. P value was calculated using Kruskal-Wallis test (*p<0.05). (C) Comparison of serum neutralising activities measured as ID50 against D614G SARS-CoV-2 in patients with systemic lupus erythematosus (SLE) with baseline low (grey, n=19) or high (black, n=40) naïve B cell frequency (arbitrary cut-off=42% of total B cells). Naïve B cells (N) are defined as CD27-Ig+ B cells, switched memory B cells (S) as CD27 +IgD-, marginal zone B cells (M) as CD27 +IgD+ and double negative B cells (DN) as CD27-IgD-. The boxplots show medians (middle line) and first and third quartiles, while the whiskers indicate minimal and maximal values. P value was calculated using Mann-Whitney test (*p<0.05). (D) Serum neutralising activities against D614G SARS-CoV-2 measured as ID50 in 59 patients with SLE classified according to their naïve B cell counts. Q1, Q2, Q3 and Q4 defined the naïve B cell count quartiles. P value was calculated using Kruskal-Wallis test (*p<0.05; ***p<0.001). (E) Serum neutralising activities against indicated SARS-CoV-2 variants B.1.1.7 (Alpha), B.1.617.1 (Kappa), B.1.617.2 (Delta), B.1.617.3, B.1.28 (Gamma) and B.1.351 (Beta) measured as ID50 in 46 serum samples at D42. The boxplots show medians (middle line) and first and third quartiles, while the whiskers indicate minimal and maximal values. P value was calculated using Kruskal-Wallis test (**p<0.01; ***p<0.001; ****p<0.0001). (F) Positive rates of serum neutralising activity against SARS-CoV-2 variants in 46 SLE samples at day 42. Patients were defined as 'neutralisers' (black) or 'non-neutralisers' (grey) according to the presence of neutralising activity at first serum dilution (1/30), or not. IgG, immunoglobulin G; RBD, receptor binding domain.

DISCUSSION

Here, we report BNT162b2 antibody response measured both with anti-RBD antibody levels and neutralisation activity in a cohort of 126 French patients with SLE, with both active and inactive disease. To our knowledge, this is the first evaluation of BNT162b2-induced T cell and neutralisation responses against VOCs in a cohort of patients with SLE.

Global acceptance of BNT162b2 vaccine was 80.5%, in line with previous studies.[33] Most patients with SLE were followed up for a long time before vaccination in our centre and vaccine was proposed by their treating physician. Interestingly, 18 (10%) patients who first refused vaccination finally agreed to be vaccinated after a reflection time, a finding that is often lacking in COVID-19 vaccine acceptance studies. Tolerance of BNT162b2 vaccine was also good with a majority of local reactions and few systemic reactions.

SLE activity at time of vaccination, assessed either with the BILAG or the SLEDAI scores, neither reduced vaccine efficacy nor increased the risk of subsequent SLE flares or vaccine side effects. Consistent with this finding, previous meta-analysis of seasonal influenza and pneumococcal vaccinations in SLE demonstrated that immunisation had no significant effect on the SLE activity measured with SLEDAI score.[34] Our results support the recommendation not to defer mRNA vaccination in patients with active SLE.[1]

One should note, however, that patients with active SLE would subsequently receive treatments that could blunt BNT162b2 antibody response. Indeed, MMF profoundly lowers BNT162b2 antibody response as previously reported in transplant recipients[35] and patients with RMDs.[5] MTX, a drug that is widely used for SLE, decreases Covid-vaccine antibody response in a similar extent to MMF. Our results confirm recent studies[5 36] showing that MTX hampers immunogenicity to BNT162b2 mRNA COVID-19 vaccine in immune-mediated inflammatory diseases. However, since these two studies mixed different RMDs, the impact of these two drugs on BNT162b2 mRNA antibody response was assessed without adjusting with specific SLE parameters that could also affect BNT162b2 antibody response (disease activity, IFN-α levels). Reduced humoral responses to both seasonal influenza and pneumococcal vaccines with MTX in patients with rheumatoid arthritis (RA) have been previously reported[37 38] while transitory MTX discontinuation improves the immunogenicity of seasonal influenza vaccination in patients with RA.[39–42] Based on these trials, the ACR recommended that vaccination should be performed at least seven days after MTX treatment,[1] but the evidence supporting this recommendation is unclear and was counterbalanced by the potential for RA flare associated with withholding MTX for a too long period, a recommendation that could not be extrapolated to SLE.

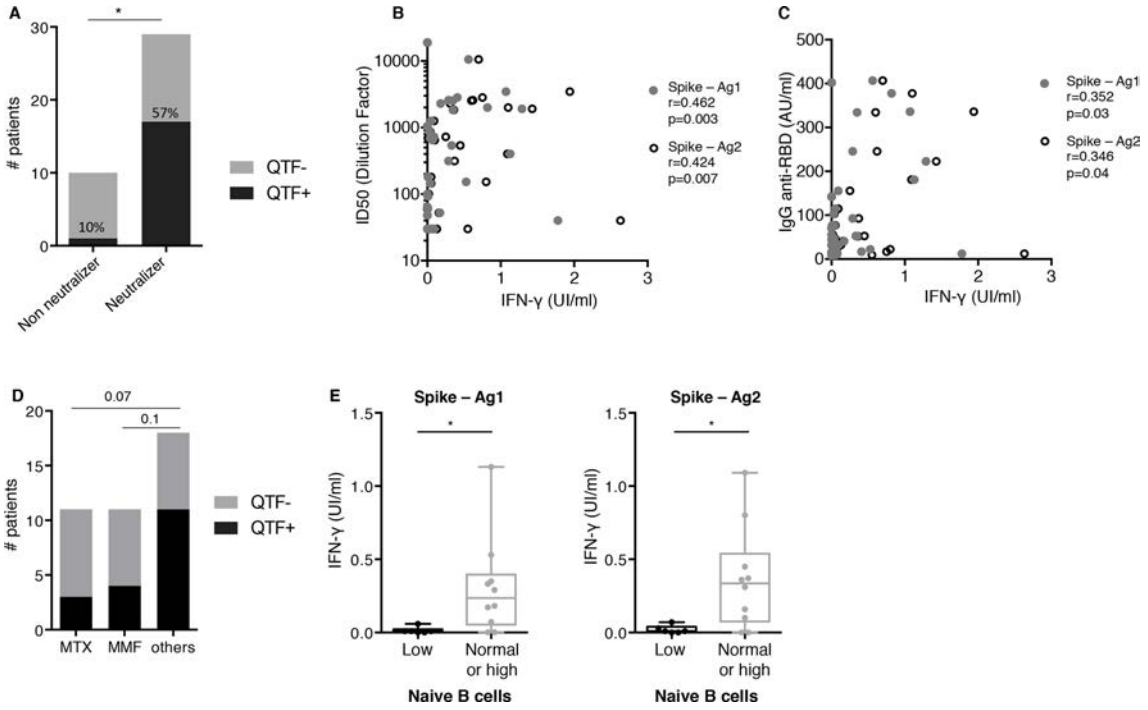

Figure 4 T cell responses correlate with anti-SARS-CoV-2 humoral responses. (A) Positive rates of Quantiferon SARS-CoV-2 testing in 40 patients with systemic lupus erythematosus (SLE) at day (D) 42, grouped according to serum neutraliser and non-neutraliser status, as defined in figure 3D. Numbers indicate the percentage of patients with a detectable T cell response. (B) Comparison of nterferon-γ (IFN-γ) levels (UI/mL) after specific T cell stimulation using Quantiferon SARS-CoV-2 test and serum neutralising activity reported with inhibitory dilution (ID50) in 40 patients with SLE at D45. Spearman coefficient (r) and p value (p) are indicated. MMF, mycophenolate mofetil; MTX, methotrexate.

By contrast, neither hydroxychloroquine nor anti-BAFF belimumab did affect vaccine antibody response.

High-dose steroids were not associated either with a lower vaccine-induced antibody response. The median prednisone daily high dose was 19 mg in our study, a threshold that is lower than the one used for transplant recipient patients.[43] There is still controversy regarding the effect of steroids on SARS-CoV-2 vaccine efficacy, in particular whether a daily dose prednisone threshold above which antibody response might be blunted could be defined.[1] As a consequence, there is currently no expert panel recommendation to delay or not COVID-19 vaccination in patients with RMD receiving glucocorticoids at a prednisone-equivalent dose of ≥20 mg/day.[1] Optimal antibody responses seem to be elicited in RMDs patients on glucocorticoid monotherapy,[44] although the daily prednisone dose was not reported in the latter study. Our data suggest that patients with SLE with a daily dose of prednisone close to 20 mg should properly respond to BNT162b2 vaccine.

Elevated IFN-α serum levels were not associated with impaired BNT162b2 antibody response, an observation in line with the lack of influence of SLE activity on vaccine efficacy. By contrast, elevated baseline total serum IgG levels were associated with a better antibody response. This association remains significant (p=0.018) when the analysis is adjusted for immunosuppressive drugs that could decrease IgG levels. For patients with chronic lymphocytic leukaemia, higher serum immunoglobulin levels at time of BNT162b2 mRNA vaccination were independently associated with a better response rate (IgG levels ≥550 mg/dL (OR 3.70, 95% CI 1.08 to 12.66)).[45] IgM levels were also an independently associated with serologic response (IgM ≥40 mg/dL (OR 2.92, 95% CI 1.21 to 7.02)) in these patients. The influence of baseline IgG and IgM levels on COVID-19 vaccine antibody response has never been reported before in RMD and might be considered in future studies.

Our data also underline the importance of interrogating initial B cell compartments as correlates of predicted vaccine response. A marked decrease of naïve B cells is known to be characteristic of SLE and not only the result of immunosuppressive drugs.[46 47] Here, we observed a strong correlation of naïve B cell loss with poor vaccine antibody response, which likely points the role of naïve B cells as a source of spike reactive B cells. In recent studies, extensive screening of pre-pandemic naïve B cell repertoires revealed the presence of SARS-CoV-2-neutralising antibody precursors. This subset of germline antibodies bound SARS-CoV-2 ACE2 RBD, although weakly, and may be engaged on vaccine exposure to generate germinal centres and then follow affinity maturation process.[48 49] Indeed, Rincon-Arevalo *et al* observed a significant difference in the frequency of SARS-CoV-2 RBD-specific naïve B cells between BNT162b2 responders and non-responders.[50] Reduced naïve B cell pool in SLE would thus readily impact precursor frequency available to encounter the antigen, therefore impairing vaccine efficiency. Future vaccination strategies in SLE should consider naïve B cells as an essential biomarker to define individuals at high risk of suboptimal response that might benefit from reinforced vaccine regimens.

It will remain to define in future studies whether patients eventually seroconverting after a third dose would have had readily detectable T cell responses after the second dose. Finally, much larger studies will be necessary to determine whether BNT162b2-induced T cell responses are solely sufficient to prevent at least from severe forms of the COVID-19 in patients.

Our study has some limitations. It is surprising to note that SARS-CoV-2-specific T cell responses were detected in only 57% of patients who had neutralising antibody titres. This

observation questions the sensitivity of the quantiferon (QTF) assay used in our study and another.[51] Future studies should include other assays such as T cell ELISPOT[52] to confirm this observation and whether low T cell responses would be more likely associated with SLE, compared with other RMDs and to healthy controls. Moreover, QTF assay was performed 15 days after the second dose, a timing that may be too short to optimally detect SARS-CoV-2-specific T cell response. Longitudinal studies are thus required to determine whether patients with SLE develop a delayed cellular immune response.

Unlike previous authors,[3–5] we did not use antibody response positivity thresholds. There are, however, no studies showing that these thresholds give patients with RMD real protection against the risk of subsequent infection with SARS-CoV-2. It is not yet clear as to what immunogenicity parameter is predictive of vaccine-induced protection. Additionally, these thresholds vary according to the assays used and the variants studied, their clinical relevance is therefore questionable. To address this issue, Khoury *et al*[53] recently analysed the relationship between in vitro neutralisation levels and the observed protection from SARS-CoV-2 infection using data from seven current vaccines and from convalescent cohorts. These authors found that despite expected inconsistencies between studies, comparison of normalised neutralisation levels and vaccine efficacy demonstrates a remarkably strong non-linear relationship between mean neutralisation level and the reported protection across different vaccines (Spearman r=0.905; p=0.0046). In this setting, the strong correlation we observed between RBD antibody levels and the neutralising activity is reassuring about the usefulness of serology in clinical practice. In our survey, only 1 out of 126 patients presented high IgG anti-RBD levels and low neutralising activity (figure 3A). Antibody response was assessed 14 days after the second injection. We cannot rule out the hypothesis that a higher antibody response would have been observed later.[44] Of note, Polack *et al* measured antibody responses as soon as 7 days after second injection[21] and were able to link BNT162b2 efficacy to prevention of SARS-CoV-2 infections in healthy individuals. Lastly, this SLE cohort did not comprise rituximab-treated patients, in whom antibody responses are abrogated.[54] Rituximab is not approved for SLE, although it is being used in clinical practice.

Despite its limitations, this study provides evidence that in SLE, use of MMF or MTX is associated with reduced vaccine efficacy. We also show that low baseline IgG levels and a reduced pool of naïve B cells are predictive of impaired vaccination-induced neutralising activity against SARS-CoV-2. These parameters could be helpful for physicians to delineate which patients should have antibody measurement after full BNT162b2 vaccination and should be proposed a third injection of BNT162b2 vaccine.

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Contributors QM, AM, RL, FA, JH, MP, LB and ZA recruited patients. QM, PB, HC, PMF and PG-D collected demographic and clinico-biological data. MM, DS and PG-D prepared the specimens. MM, QM, FA and DS designed and performed experiments. QM, DS, FA, MM, SdA and SM analysed data. QM and DS prepared the figures. QM, DS, GG and ZA wrote the manuscript. QM, DS, FA, MM, GG and ZA designed the study and reviewed the manuscript. QM, DS, MM and FA contributed equally to this work.GG and ZA jointly directed this work.

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CLINICAL SCIENCE

Average corticosteroid dose and risk for HBV reactivation and hepatitis flare in patients with resolved hepatitis B infection

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ABSTRACT

Objectives Corticosteroids remain the mainstay of treatment for rheumatic diseases but can cause hepatitis B virus (HBV) reactivation in patients with resolved HBV infection. Risk assessment and stratification are needed to guide the management of these patients before corticosteroid therapy.

Methods We prospectively enrolled patients with negative hepatitis B surface antigen positive Anti-hepatitis B core status with or without corticosteroid use and determined corticosteroid exposure by calculating cumulative dose and time-weighted average daily dose of prednisone. The primary outcome was the time to a composite of HBV reactivation, hepatitis flare or severe hepatitis.

Results Among 1303 participants, the median of cumulative dose and time-weighted average dose of prednisone used in this cohort was 3000 mg (IQR: 300–6750 mg) and 15 mg/day (IQR: 10–20 mg/day), respectively. In multivariable analyses, cumulative dose showed inverted V-shaped relationship with primary events, which peaked at a cumulative dose of 1506 mg (HR: 3.72; 95% CI, 1.96 to 7.08). Quartiles of time-weighted average dose were independently associated with a monotonic increase in event risk (HR per quartile increase: 2.15; 95% CI, 1.56 to 2.98), reaching an HR of 49.48 (95% CI, 6.24 to 392.48) in the top quartile. The incidence of primary outcome was 16.67 per 100 person-years in the top quartile of time-weighted average dose (Q4>20 mg/day). Other quartiles all had an incidence of primary outcome less than 10 per 100 person-years.

Conclusion Patients with time-weighted average prednisone dose greater than 20 mg/day would be classified as the high risk for HBV reactivation or hepatitis flare. Prophylactic Anti-HBV therapy may be needed for these high-risk patients.

Trial registration number ChiCTR1900023955.

Key messages

What is already known about this subject?

- Corticosteroids remain the mainstay of treatment of musculoskeletal conditions, arthritic disease and connective tissue disorders.
- Guidelines are currently in consensus on recommending the use of anti-hepatitis B virus (HBV) prophylaxis in patients with positive hepatitis B surface antigen (HBsAg) before corticosteroid therapy, but such a consensus has not been reached in the management of patients with resolved HBV infection with an HBsAg-negative Anti-hepatitis B core positive status.
- Due to the lack of systemically collected data, reliable risk assessment and stratification of HBV reactivation in corticosteroid users with resolved HBV infection are lacking.

What does this study add?

- This study proposed a time-weighted average dose of prednisone to quantify corticosteroid exposure, and this indicator, instead of cumulative dose, positively predicted the risk of HBV reactivation or hepatitis flare in patients with resolved HBV infection.
- Use of prednisone with a time-weighted average dose greater than 20 mg/day resulted in an incidence of HBV reactivation or hepatitis flare more than 10 per 100 person-years in patients with resolved HBV infection, and prophylactic Anti-HBV therapy may therefore be needed for these high-risk patients.

How might this impact on clinical practice or future developments?

- Assessment of time-weighted average prednisone dose, instead of the peak dose, treatment duration and cumulative dose, would allow for the risk stratification for HBV reactivation in patients with resolved HBV infection treated with corticosteroids for a variety of rheumatic diseases.

INTRODUCTION

Corticosteroids currently remain the mainstay of treatment of musculoskeletal conditions, arthritic disease and connective tissue disorders. However, given the fact that hepatitis B is a common comorbidity among rheumatic patients, improper use of corticosteroids may cause hepatitis B virus (HBV) reactivation or hepatitis flare. HBV reactivation and hepatitis flare are a potentially serious disorder, which may lead to fulminant hepatic failure and death.[1] A prevailing opinion on the prevention of HBV reactivation is to initiate prophylactic antiviral therapy according to risk stratification instead of universal prophylaxis before corticosteroid therapy.[2 3] HBV reactivation more frequently occurs in patients with positive hepatitis B surface

antigen (HBsAg), and guidelines are currently in consensus on recommending the use of Anti-HBV prophylaxis in HBsAg-positive patients.[4] HBV reactivation may also occur in patients with resolved HBV infection with an HBsAg-negative and antibody against hepatitis B core (Anti-HBc) positive status; however, guidelines are not always in agreement on the initiation of Anti-HBV prophylaxis in these patients.[4] Reliable risk assessment and stratification are, therefore, needed to guide the management of these patients.

Most of existing studies concerning HBV reactivation focused on novel biological drugs, such as rituximab, abatacept and anti-TNF agents.[5 6] Due to the lack of systemically collected data, there is difficulty in estimating the precise risk of HBV reactivation in corticosteroid users with resolved HBV infection. Currently, these patients are usually categorised by prednisone or equivalent dose (low dose: <10 mg/day; moderate dose: 10–20 mg/day; high dose: >20 mg/day) and treatment duration (<4 weeks vs ≥4 weeks).[7] Nevertheless, according to the dose and duration of corticosteroids used, the estimated risk levels are not always consistent among several guidelines.[7–9] Furthermore, corticosteroids are often used in a tapering or pulse manner, which may have an impact on the course of HBV reactivation,[7] but these situations have not been considered in the current risk assessment. As medication patterns may vary markedly in different rheumatic diseases, the reported incidence of HBV reactivation was largely distinct,[10–12] and a more generalised tool may be needed to quantify the risk of HBV reactivation in various rheumatic diseases when given a corticosteroid therapy.

In the prospective study, we perform a continuous monitoring of corticosteroid medication and HBV reactivation in patients with resolved HBV infection with uveitis. This condition is in a wide association with a variety of acute or chronic autoimmune and autoinflammatory diseases, such as ankylosing spondylitis, Behçet's disease, inflammatory bowel disease, psoriasis and sarcoidosis,[13 14] and corticosteroids currently remain the mainstay of treatment.[15] We assess the association of the cumulative dose and the time-weighted average dose of prednisone use with the risk of HBV reactivation in the analysis of different corticosteroid medication patterns for these immune-related diseases. We hypothesise that such indicators would be most likely to characterise the extent of corticosteroid exposure and to quantify the risk of HBV reactivation.

METHODS

Study design and participants

This was a prospective observational study conducted at Uveitis Centre of the First Affiliated Hospital of Chongqing Medical University, Chongqing, China. We enrolled consecutive uveitis patients who had an HBsAg-negative Anti-HBc-positive status and would receive systemic corticosteroids or not according to their ocular and systemic conditions to observe whether these patients would have a composite endpoint event of HBV reactivation, hepatitis flare or severe hepatitis. Those patients reaching the endpoints received immediate best medical judgement and proper antiviral therapy, and observation for the study purpose would be terminated. Evidence suggested that the deferred preemptive use of antiviral agents would be feasible to control HBV reactivation,[16 17] and, therefore, entecavir (0.5 mg/day) was initiated in patients when HBV reactivation was encountered. In this study, eligible participants were aged 18 years or older, seropositive for Anti-HBc and negative for HBsAg. Key exclusion criteria included past or concurrent infection with hepatitis C or hepatitis D virus; being receiving antiviral therapy; serum alanine aminotransferase (ALT) concentrations above normal; high HBV DNA level ($\geq 1\times10^7$ IU/mL); evidence of liver cirrhosis; concomitant other chronic liver diseases or other severe health problems. Online supplemental table S1 shows a complete list of inclusion and exclusion criteria. This cohort study is prospectively registered with Chinese Clinical Trial Register.

Study procedures and clinic visits

Treatment was generally implemented based on the recommendations of current guidelines.[15 18] In this study, according to the severity of ocular and systemic condition, patients received either different doses of oral prednisone or not, in combination with or without cyclosporine (2–5 mg/kg/day) as a corticosteroid-sparing immunosuppressive agent. In addition to systemic therapies, patients were also allowed to use any forms of topical treatments as a complementary therapy. Those patients in no need for systemic corticosteroids and cyclosporine only received topical therapies or even observation. To preclude other drug effects, additional systemic immunosuppressive agents, non-steroidal anti-inflammatory drugs and antiviral therapy were prohibited, otherwise observation would be terminated with data censored. Clinic visits were scheduled at baseline, at the end of 2 weeks, 4 weeks and 8 weeks, and approximately every 2 months thereafter. For each visit, we performed HBV DNA quantification and biochemical measures (serum ALT, aspartate aminotransferase (AST) and bilirubin) for all participants. HBV serology makers, including HBsAg, antibody against HBsAg (Anti-HBs), hepatitis B e antigen (HBeAg), antibody against HBeAg (Anti-HBe) and Anti-HBc, were measured every 6 months since baseline (see laboratory tests in online supplemental table S2). If suppression of disease was achieved and maintained, an attempt would be made to taper oral prednisone according to the patient's ocular and systemic conditions. Patients' conditions were evaluated until the occurrence of study endpoints, initiation of other treatments (eg, adding another immunosuppressant), withdrawal or loss to follow-up, or the completion of study. Corticosteroid tapering to discontinuation was not part of reasons for termination of evaluation, and the observation would continue.

Definitions, measurements and outcomes

The primary exposure factor was corticosteroid use, characterised by two continuous variables, cumulative dose and time-weighted average daily dose of prednisone, calculated as follows. We first determined the prednisone duration–dose curve for each participant based on the daily dose of prednisone over time from baseline to prednisone discontinuation if occurred during the study period, or to the termination of evaluation as mentioned before (online supplemental figure S1). During the period from baseline to the last evaluation, we performed interpolation over the days of missing data on daily dose, if any, by using the last observation carried forward. The cumulative dose was, therefore, defined as the total dose of prednisone accumulated over the duration interval measured as area under the curve. The time-weighted average dose was calculated by dividing the cumulative dose by the drug duration (days). In this study, the primary outcome was the time to a composite of HBV reactivation, hepatitis flare or severe hepatitis. Endpoint events were adjudicated by two independent clinicians who were unknown of the treatment assignment according to the AASLD recommended criteria (online supplemental table S3).[19]

Covariates

Covariates prospectively measured in this study included the dose of cyclosporine used, age, sex, body mass index, residence (rural vs urban), educational level (primary school and less, middle school, high school, and college and higher), smoking (none, past and current), drinking (none, past and current), presence of hypertension, diabetes, coronary heart disease or malignancy, uveitis affected eye (one eye vs both eyes), best corrected visual acuity in the worse-seeing eye (logarithm of the minimum angle of resolution (logMAR) transformed; a higher logMAR score indicates a worse visual acuity), Anti-HBs status, ALT level, AST level, total bilirubin level and creatinine level.

Statistical analyses

Data are expressed as numbers and percentages for categorised variables, as means and SD for normally distributed data, and as medians and IQRs for skewed data. Normality was evaluated with the Shapiro-Wilk test. Data on cumulative dose and time-weighted average dose were categorised into each quartile. Incidence rates of study endpoints for each quartile were calculated as events per 100 person-years. To account for potential confounders, we used the propensity score-based inverse probability weighting to obtain incidence estimates, in which each observation was weighted by the inverse of the probability of a patient being in each quartile. The propensity scores based on the probability of being in each quartile were generated using the multinomial logistic regression with the full set of covariates as independent variables. This approach produced a pseudopopulation, where incidence rates were estimated to represent the population-average treatment effects of each quartile independent of measured covariates.[20] We used the Cox proportional hazards regression model to estimate adjusted HRs with 95% CIs for endpoint events. Participants without an endpoint event had their data censored on their last evaluation. Schoenfeld residuals indicated no violations of the proportional hazards assumption. All multivariable models adjusted for the full set of covariates measured at baseline unless stated otherwise. A linear trend was estimated by modelling the factor as a continuous variable. Non-linear relationship was explored by remodelling the Cox regression equation with restricted cubic splines. To examine the subgroup effects, we additionally used a multivariable Cox regression model, including the interaction between each subgroup variable and time-weighted average prednisone dose quartiles as a factor. A p value of <0.05 on the Wald χ^2 test was considered to indicate statistical significance for the interaction term. In a sensitivity analysis, HRs for the primary composite outcome were estimated with a multivariable Cox model adjusted for suitable minimally sufficient adjustment sets that were identified by a directed acyclic graph (DAG). The DAG is composed of nodes representing variables and arrows showing associations between these variables, and produces the minimum number of covariates required to account for confounding.[21] The DAG was generated with the use of DAGitty V.3.0.[22] All tests were two-sided. No adjustments were made for multiple comparisons. Statistical analyses were performed with SPSS Statistics V.25 or R V.3.5.0.

Patient and public involvement

No patients were involved in setting the research question or the outcome measures, nor were they involved in developing plans for recruitment, design or implementation of the study. No patients were asked to advise on interpretation or writing up of results. There are no plans to disseminate the results of the research to study participants or the relevant patient community.

RESULTS

Study population and events

From July 2019 through March 2021, a total of 2996 consecutive patients underwent eligibility assessment, and 1303 HBsAg-negative Anti-HBc-positive patients were enrolled (online supplemental figure S2). The median age of our cohort was 47.5 (IQR: 38–56) years, and 47.5% were female. Participants had a median ALT level of 18 (IQR: 14–24) U/L, 842 (64.6%) patients were Anti-HBs-positive and all had an undetectable HBV DNA level ($<1\times10^2$ IU/mL) at enrolment. Other baseline demographic and clinical characteristics are summarised according to the cumulative dose and time-weighted average dose quartiles in tables 1 and 2. Oral prednisone was initiated in 77.8% of participants at a median dose of 20 mg/day (IQR: 15–20 mg/day), and cyclosporine in 56.3% at 100 mg/day (IQR: 100–125 mg/day). The median of cumulative prednisone dose and time-weighted average prednisone dose used in this cohort was 3000 mg (IQR: 300–6750 mg) and 15 mg/day (IQR: 10–20 mg/day), respectively. This study was completed on 31 March 2021, which provided a median follow-up of 10 months (IQR: 4–16 months) for participants. There were 70 participants initiating other systemic therapies than prednisone and cyclosporine, for whom the evaluation was terminated and data were censored. During the study period, a total of 51 participants had the incident HBV reactivation or hepatitis flare, reaching the primary composite endpoint. The mean detectable HBV DNA level was 1.81×10^3 IU/mL at the first sign of HBV reactivation. No severe hepatitis occurred in the cohort.

Cumulative dose

A higher incidence of primary outcome was not seen in patients with a higher cumulative prednisone dose (table 3). After inverse probability weighting, patients in the top quartile of cumulative dose had the lowest incidence rate of primary endpoint events (0.17 per 100 person-years), while those in the second quartile had the highest incidence (48 per 100 person-years). In Cox regression analysis with the fully adjusted model, as compared with the bottom quartile, the highest risk of primary endpoint events was detected in the second quartile of cumulative dose (HR: 6.03; 95% CI, 2.60 to 14.01) and the lowest risk in the top quartile (HR: 0.06; 95% CI, 0.01 to 0.49) (table 4). In linear remodelling, there seemed to be an inverse association between cumulative dose and event risk (HR per quartile increase: 0.46; 95% CI, 0.33 to 0.65). In cubic spline analyses, cumulative dose showed an inverted V-shaped relationship with the event risk, which peaked at a cumulative dose value of 1506 mg (HR: 3.72; 95% CI, 1.96 to 7.08) (figure 1 and online supplemental table S4).

Time-weighted average dose

Participants using a higher time-weighted average prednisone dose had a higher incidence of HBV reactivation or hepatitis flare, and the relationship between the time-weighted average dose and event risk appeared to be dose dependent (table 3). After inverse probability weighting, the incidence rate of primary endpoint events was 16.67 per 100 person-years in the top quartile of time-weighted average dose (Q4: >20 mg/day). All other quartiles (Q1: ≤10 mg/day; Q2: >10 mg/day but ≤15 mg/day; Q3: >15 mg/day but ≤20 mg/day) had a lower incidence of primary endpoint

Table 1 Baseline demographic and clinical characteristics according to cumulative prednisone dose categories

Characteristics	Cumulative prednisone dose*			
	Q1	Q2	Q3	Q4
No. of participants	383	269	328	323
Age, median (IQR), years	47.5 (40–56)	48 (38–57)	47.5 (37–56)	47.5 (36–56)
Female, no. (%)	182 (47.4)	121 (45.1)	173 (52.7)	170 (52.6)
BMI, median (IQR), kg/m^2	23.2 (22.1–24.2)	23.2 (21.5–25.4)	23.2 (22.0–24.6)	23.2 (22.2–24.4)
Rural residents, no. (%)	120 (31.3)	113 (42.2)	146 (44.5)	197 (61.0)
Education level, no. (%)				
Primary school and less	93 (24.2)	69 (25.7)	94 (24.6)	84 (26.0)
Middle school	91 (23.7)	77 (28.7)	107 (32.6)	119 (36.8)
High school	68 (17.7)	61 (22.8)	72 (22.0)	71 (22.0)
College and higher	132 (34.4)	61 (22.8)	55 (16.8)	49 (15.2)
Smoking status, no. (%)				
None	275 (71.6)	180 (67.2)	211 (64.3)	173 (53.6)
Past	44 (11.5)	35 (13.1)	50 (15.2)	74 (22.9)
Current	65 (16.9)	53 (19.8)	67 (20.4)	76 (23.5)
Drinking alcohol, no. (%)				
None	251 (65.4)	171 (63.8)	196 (59.8)	178 (55.1)
Past	68 (17.7)	50 (18.7)	68 (20.7)	80 (24.8)
Current	65 (16.9)	47 (17.5)	64 (19.5)	65 (20.1)
Hypertension, no. (%)	29 (7.6)	34 (12.7)	32 (9.8)	37 (11.5)
Diabetes, no. (%)	21 (5.5)	11 (4.1)	11 (3.4)	14 (4.3)
Coronary heart disease, no. (%)	3 (0.8)	9 (3.4)	6 (1.8)	3 (0.9)
Malignancy, no. (%)	4 (1.0)	4 (1.5)	3 (0.9)	0 (0.0)
Dose of cyclosporine used, median (IQR), mg/day	0 (0–0)	100 (0–125)	100 (0–100)	100 (75–125)
Uveitis affected eye, no. (%)				
One eye	254 (66.1)	144 (53.7)	211 (64.3)	166 (51.4)
Both eyes	130 (33.9)	124 (46.3)	117 (35.7)	157 (48.6)
BCVA in the worse-seeing eye, median (IQR), LogMAR	0.10 (0–0.52)	0.22 (0–1.0)	0.15 (0–0.70)	0.30 (0–0.82)
Anti-HBs (+), no. (%)	248 (64.6)	166 (61.9)	213 (64.9)	215 (66.6)
ALT level, median (IQR), U/L	18 (13–23)	18 (14–24)	18 (13–23)	18 (14–24)
AST level, median (IQR), U/L	18 (15–21)	18 (15–22)	17 (14–22)	18 (14–22)
Total bilirubin level, median (IQR), μmol/L	9.9 (7.7–12.9)	9.9 (7.3–12.6)	9.9 (7.6–12.6)	9.4 (7.2–12.4)
Creatinine level, median (IQR), μmol/L	70 (61–81)	70 (63–80)	70 (59–76)	70 (60–76)

*Values are reported according to the quartile (Q) of cumulative prednisone dose. The cumulative prednisone dose was categorised as: Q1 ≤300 mg; Q2 >300 mg but ≤3000 mg; Q3 > 3000 mg but ≤6750 mg; Q4 > 6750 mg.
ALT, alanine aminotransferase; Anti-HBs, antibody against hepatitis B surface antigen; AST, aspartate aminotransferase; BCVA, best corrected visual acuity; BMI, body mass index; LogMAR, logarithm of the minimum angle of resolution (higher logMAR scores indicate a worse visual acuity).

events than 10 per 100 person-years. After multivariable adjustment, quartiles of time-weighted average dose were independently associated with a monotonic increase in the event risk (HR per quartile increase: 2.15; 95% CI, 1.56 to 2.98), reaching an HR of 49.48 (95% CI, 6.24 to 392.48) in the top quartile as compared with the bottom one (table 4). There tended to be a stepwise increase in the event risk when given an increase in time-weighted average dose, whereby the risk was marginally higher at time-weighted average dose values of 21 mg/day (HR: 4.37; 95% CI, 1.00 to 19.06) or greater (figure 1 and online supplemental table S5). As the time-weighted average dose increased, a similar increasing trend in event risk and incidence rate was seen in subgroup analyses comparing patients with or without Anti-HBs positivity, using cyclosporine or not, and with baseline ALT level of ≤20 U/L or >20 U/L (online supplemental figure S3 and online supplemental table S6). Results indicated no effect modification according to Anti-HBs status (p=0.42 for interaction), use of cyclosporine (p=0.47 for interaction) and baseline ALT levels (p=0.82 for interaction) (online supplemental table S6). After adjustment for the full set of covariates, a significant protective effect in HBV reactivation or hepatitis flare was not observed for Anti-HBs positivity (HR: 0.99; 95% CI, 0.54 to 1.80), Anti-HBs level of ≥20 mIU/mL (HR: 0.82; 95%, 0.47–1.45) and even ≥100 mIU/mL (HR: 0.83; 95% CI, 0.44 to 1.55) (online supplemental table S7). The DAG identified several suitable minimally sufficient adjustment sets of covariates needed to account for confounding (online supplemental figure S4 and online supplemental table S8). Results of the sensitivity analysis with adjustment for these sets did not differ substantially from that of the primary analysis, where HRs were adjusted for the full set of covariates (online supplemental table S9).

DISCUSSION

Among patients with resolved HBV infection on corticosteroid therapy, we found an inverted V-shaped relationship rather than a positive correlation between cumulative prednisone dose and risk of HBV reactivation or hepatitis flare. Instead, time-weighted average prednisone dose independently showed a positive association with the event risk, which appeared to have reasonably

Table 2 Baseline demographic and clinical characteristics according to time-weighted average prednisone dose categories

	Time-weighted average prednisone dose*			
Characteristics	**Q1**	**Q2**	**Q3**	**Q4**
No. of participants	328	346	494	135
Age, median (IQR), years	47.5 (37–56)	48 (40–58.3)	47.5 (36–56)	47 (36–53)
Female, no. (%)	157 (47.9)	188 (54.3)	234 (47.4)	50 (37.0)
BMI, median (IQR), kg/m^2	23.2 (21.4–24.5)	23.2 (22.2–24.5)	23.2 (21.6–24.9)	23.2 (23.2–23.9)
Rural residents, no. (%)	102 (31.1)	123 (35.5)	215 (43.5)	65 (48.1)
Education level, no. (%)				
Primary school and less	71 (21.6)	81 (23.4)	141 (28.5)	47 (34.8)
Middle school	88 (26.8)	111 (32.1)	153 (31.0)	42 (31.1)
High school	68 (20.7)	72 (20.8)	101 (20.4)	31 (23.0)
College and higher	101 (30.8)	82 (23.7)	99 (20.0)	15 (11.1)
Smoking status, no. (%)				
None	229 (69.8)	229 (66.2)	313 (63.4)	68 (50.4)
Past	46 (14.0)	45 (13.0)	66 (13.4)	46 (34.1)
Current	53 (0.3)	72 (20.8)	115 (23.3)	21 (15.6)
Drinking alcohol, no. (%)				
None	192 (58.5)	215 (62.1)	326 (66.0)	63 (46.7)
Past	67 (20.4)	55 (15.9)	90 (18.2)	54 (40.0)
Current	69 (21.0)	76 (22.0)	78 (15.8)	18 (13.3)
Hypertension, no. (%)	30 (9.1)	44 (12.7)	45 (9.1)	13 (9.6)
Diabetes, no. (%)	18 (5.5)	15 (4.3)	16 (3.2)	8 (5.9)
Coronary heart disease, no. (%)	5 (1.5)	9 (2.6)	6 (1.2)	1 (0.7)
Malignancy, no. (%)	4 (1.2)	3 (0.9)	4 (0.8)	0 (0.0)
Dose of cyclosporine used, median (IQR), mg/day	0 (0–0)	100 (0–100)	100 (50–125)	100 (75–125)
Uveitis affected eye, no. (%)				
One eye	233 (71.0)	224 (64.7)	248 (50.2)	70 (51.9)
Both eyes	95 (29.0)	122 (35.3)	246 (49.8)	65 (48.1)
BCVA in the worse-seeing eye, median (IQR), LogMAR	0.10 (0–0.40)	0.15 (0–0.52)	0.30 (0.10–1)	0.30 (0–1.30)
Anti-HBs (+), no. (%)	207 (63.1)	217 (62.7)	347 (70.2)	71 (52.5)
ALT level, median (IQR), U/L	18 (13–23)	18 (13–23)	18 (14–24)	19 (14–26)
AST level, median (IQR), U/L	18 (15–21)	18 (15–22)	18 (15–22)	18 (14–21)
Total bilirubin level, median (IQR), µmol/L	9.9 (7.5–12.5)	9.9 (7.5–12.3)	9.9 (7.4–12.7)	9.8 (7.2–14.4)
Creatinine level, median (IQR), µmol/L	70 (61–78)	70 (61.7–77.3)	70 (60–78.3)	71 (66–79)

*Values are reported according to the quartile (Q) of time-weighted average prednisone dose. The time-weighted average prednisone dose was categorised as: Q1 ≤10 mg/day; Q2 >10 mg/day but ≤15 mg/day; Q3 >15 mg/day but ≤20 mg/day; Q4 >20 mg/day.
ALT, alanine aminotransferase; Anti-HBs, antibody against hepatitis B surface antigen; AST, aspartate aminotransferase; BCVA, best corrected visual acuity; BMI, body mass index; LogMAR, logarithm of the minimum angle of resolution (higher logMAR scores indicate a worse visual acuity).

explained the dose–response relationship between the extent of corticosteroid exposures and risk for HBV reactivation or hepatitis flare. Use of prednisone with a time-weighted average dose greater than 20 mg/day resulted in an incidence of HBV reactivation or hepatitis flare more than 10 per 100 person-years in patients with resolved HBV infection.

There has been controversy about key determinants of HBV reactivation or hepatitis flare during corticosteroid therapy. Earlier studies found that even a short episode of high-dose corticosteroids increased the risk of hepatitis flare.[23 24] The peak daily dose of corticosteroids was, therefore, assigned more importance as a predictor of hepatitis flares than drug duration.[23 25] However, a peak dose does not usually reflect the continuous exposure extent of corticosteroids. One observation showed that the risk of hepatitis flare correlated with the corticosteroid dose during long-term maintenance but not with the peak dose of pulse therapy.[26] Another study showed that chronic and high-dose treatment with corticosteroids each contributed significantly to HBV reactivation.[27] These findings indicate that both dose and duration would predict the outcome of HBV reactivation, but each only explains a limited proportion of variance in the risk.

Cumulative prednisone dose synthetically incorporates the effects of treatment dose and duration, reflecting the accumulation of corticosteroid exposure over a certain period of time. Nevertheless, with the increase of cumulative dose, the event risk exhibited a trend from ascent to descent. The V-shaped relationship was in line with previous observations on long-term and low-dose use of corticosteroids.[25 28] A low daily dose of corticosteroids, even if resulting in a relatively high cumulative dose over a long course of treatment, would not be expected to pose a substantial risk of HBV reactivation or hepatitis flare. Thus, the cumulative dose might not be a driving factor related to the negative consequences of HBV reactivation or hepatitis flare during corticosteroid use.

Our study further implied that time-weighted average dose would be a more reasonable indicator to characterise the positive association between corticosteroid use and risk for HBV reactivation or hepatitis flare. This dose–response relationship seemed to be independent of Anti-HBs status, use of cyclosporine and baseline serum ALT levels. Thus,

Table 3 Incidence of the primary composite outcome

Categories*	Cumulative prednisone dose	Time-weighted average prednisone dose
No. with event/total no.		
Q1	11/383	1/328
Q2	33/269	15/346
Q3	6/328	22/494
Q4	1/323	13/135
Crude incidence, 100py		
Q1	4.35	0.35
Q2	46.53	5.06
Q3	2.16	6.16
Q4	0.23	12.67
Inverse probability weighted incidence†, 100py		
Q1	9.68	0.75
Q2	48.00	4.89
Q3	3.33	5.64
Q4	0.17	16.67

*Values are reported according to the quartile (Q) of cumulative prednisone dose and time-weighted average prednisone dose. The cumulative prednisone dose was categorised as: Q1 ≤300 mg; Q2 >300 mg but ≤3000 mg; Q3 >3000 mg but ≤6750 mg; Q4 >6750 mg. The time-weighted average prednisone dose was categorised as: Q1 ≤10 mg/day; Q2 >10 mg/day but ≤15 mg/day; Q3 >15 mg/day but ≤20 mg/day; Q4 >20 mg/day.
†Each observation was weighted by the inverse of the probability of a patient being in each quartile. The probability was generated using the multinomial logistic regression with cyclosporine daily dose, age, sex, BMI, HBs antibody status, serum ALT level, residence, educational level, smoking, drinking, hypertension, diabetes, coronary heart disease, malignancies, uveitis laterality, BCVA in the worse-seeing eye, AST level, total bilirubin level and creatinine level as independent variables.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCVA, best corrected visual acuity; BMI, body mass index; HBs, hepatitis B surface; 100py, per 100 person-years.

assessment of time-weighted average prednisone dose would allow for the exploration of risk stratification with implications for HBV reactivation prevention strategies in

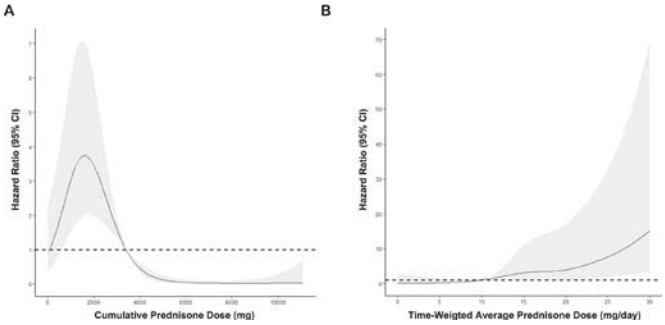

Figure 1 Relationship between the cumulative dose and time-weighted average dose of prednisone use and the primary composite outcome. HRs of the cumulative prednisone dose (A) and time-weighted average prednisone dose (B) for the primary composite outcome were estimated with a multivariable Cox regression analysis adjusted for cyclosporine daily dose, age, sex, BMI, HBs antibody status, serum ALT level, residence, educational level, smoking, drinking, hypertension, diabetes, coronary heart disease, malignancies, uveitis laterality, BCVA in the worse-seeing eye, AST level, total bilirubin level and creatinine level. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCVA, best corrected visual acuity; BMI, body mass index; HBs, Hepatitis B surface.

HBsAg-negative Anti-HBc-positive individuals. Our findings suggested that a time-weighted average dose of 20 mg/day would be a clinically meaningful cut-off value for risk stratification in this population, because greater doses were linked to an incidence of HBV reactivation or hepatitis flare in excess of 10 per 100 person-years, which could be classified into the high-risk group according to the previous guideline[7] and because greater doses were robustly associated with an increased event risk (where the 95% CI for the HR no longer included 1 when the dose was 21 mg/day or greater). Prophylactic Anti-HBV therapy may, therefore, be

Table 4 Risk for the primary composite outcome

Primary outcome	HR (95% CI)* Q2 vs Q1	Q3 vs Q1	Q4 vs Q1	Per quartile increase†	P value for trend†
Cumulative prednisone dose					
Univariate model‡	6.20 (2.98 to 12.92)	0.45 (0.17 to 1.23)	0.07 (0.01 to 0.51)	0.58 (0.45 to 0.75)	3.3×10^{-5}
Minimally adjusted model§	6.42 (2.81 to 14.65)	0.48 (0.17 to 1.41)	0.07 (0.01 to 0.53)	0.45 (0.32 to 0.63)	4.0×10^{-6}
Further adjusted model¶	5.95 (2.61 to 13.57)	0.48 (0.16 to 1.39)	0.06 (0.01 to 0.51)	0.46 (0.32 to 0.64)	5.0×10^{-6}
Fully adjusted model**	6.03 (2.60 to 14.01)	0.51 (0.17 to 1.52)	0.06 (0.01 to 0.49)	0.46 (0.33 to 0.65)	9.0×10^{-6}
Time-weighted average prednisone dose					
Univariate model‡	14.05 (1.86 to 106.35)	15.70 (2.12 to 116.52)	30.66 (4.01 to 234.39)	1.96 (1.43 to 2.67)	2.0×10^{-5}
Minimally adjusted model§	22.64 (2.96 to 173.36)	27.20 (3.58 to 206.72)	50.30 (6.40 to 395.63)	2.16 (1.59 to 2.95)	1.0×10^{-6}
Further adjusted model¶	21.50 (2.81 to 164.59)	26.30 (3.46 to 199.82)	48.87 (6.17 to 386.83)	2.17 (1.58 to 2.98)	2.0×10^{-6}
Fully adjusted model**	23.90 (3.09 to 184.65)	24.82 (3.23 to 190.54)	49.48 (6.24 to 392.48)	2.15 (1.56 to 2.98)	4.0×10^{-6}

*Values are reported according to the quartile (Q) of cumulative prednisone dose and time-weighted average prednisone dose. The cumulative prednisone dose was categorised as: Q1 ≤300 mg; Q2 >300 mg but ≤3000 mg; Q3 >3000 mg but ≤6750 mg; Q4 >6750 mg. The time-weighted average prednisone dose was categorised as: Q1 ≤10 mg/day; Q2 >10 mg/day but ≤15 mg/day; Q3 >15 mg/day but ≤20 mg/day; Q4 >20 mg/day.
†HRs per quartile increase and p values for linear trend were computed by modelling the factor as a continuous variable.
‡Crude HRs were estimated by using the univariate Cox regression analysis.
§HRs were adjusted for cyclosporine daily dose, age, sex and BMI.
¶HRs were adjusted for the minimally adjusted model, HBs antibody status and serum ALT level.
**Hazard ratios were adjusted for the further adjusted model, residence, educational level, smoking, drinking, hypertension, diabetes, coronary heart disease, malignancies, uveitis laterality, BCVA in the worse-seeing eye, AST level, total bilirubin level and creatinine level.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCVA, best corrected visual acuity; BMI, body mass index; HBs, hepatitis B surface.

needed for these high-risk patients according to the previous recommendation.[7]

A major advantage of our study design is that we have appropriately chosen patients with uveitis as the study population. This ocular disease is considered to be independent of HBV status and hepatic outcomes, and has a wide association with a variety of autoimmune and autoinflammatory diseases, including ankylosing spondylitis, juvenile idiopathic arthritis, Behçet's disease, inflammatory bowel disease, psoriasis and sarcoidosis.[13 14] Some uveitis subtypes, such as Fuchs uveitis syndrome and Posner-Schlossman syndrome, are usually treated with topical eye drops or even observation (accounting for roughly 25% in this cohort); other subtypes such as acute anterior uveitis associated with or without ankylosing spondylitis can be treated with topical therapies or shortly tapered systemic corticosteroids (accounting for roughly 30% in this cohort); while, some refractory subtypes, such as uveitis in Behçet's disease, juvenile idiopathic arthritis, Vogt-Koyanagi-Harada disease, inflammatory bowel disease, psoriasis and sarcoidosis, often require a long-term systemic corticosteroid therapy (accounting for roughly 45% in this cohort).[15] Therefore, the study population of uveitis has allowed us to naturally compare the effect of corticosteroids on HBV reactivation and also to explore the generalisability of time-weighted average dose in the analysis of different corticosteroid medication patterns. Therefore, findings on the relationship between time-weighted average dose and risk of HBV reactivation or hepatitis flare would be expected to be extrapolated to all those patients with resolved HBV infection who require corticosteroid therapies for various acute or chronic rheumatic diseases and connective tissue disorders. Prospectively and continuously documented dose and duration of prescription drugs have prevented misclassification of drug exposures due to dynamic changes in medication.

Our study has certain limitations. First, owing to the ethical considerations, the corticosteroid therapy was not randomly assigned, and unmeasured treatments may have residual confounding effects in this observational study. Several measures have been taken to minimise the confounding bias, including prespecified prohibition of other immunosuppressive drugs than cyclosporine, maintaining the cyclosporine dose throughout the study if used, adjustment for a detailed list of covariates, including cyclosporine dose, censoring data from those who subsequently initiated other immunosuppressive agents if any. Second, due to the fact that data on drug dose and duration were recorded in line with prescriptions and we applied an intention-to-treat design in data analysis, we could not correct for minor patient self-non-compliance. Third, our study was conducted in China, a hepatitis B high prevalence area, and thus, the results described here need to be further confirmed in countries with a lower incidence rate of HBV infection. Fourth, we noted an extremely high HR with a wide CI for the top quartile of time-weighted average prednisone dose (Q4: >20 mg/day) as compared with the bottom one (Q1: ≤10 mg/day). Such a condition may be due to the fact that the primary endpoint event of the bottom quartile was rare (only one event) and that there was a certain degree of deviation from linearity of the associations for quartile-related time-weighted average dose data. Therefore, the quartile-related HR may be misleading, and we further used the restricted cubic splines to characterise the non-linear relationship. Nevertheless, the association of the time-weighted average dose of 21 mg/day or greater with a high event risk remained essentially unchanged in cubic spline analyses. Finally, interpretation of composite endpoints used in this study remains difficult. We have observed the association between time-weighted average dose and risk for primary composite endpoints, but we could not precisely estimate the HR for each of the components. It has been shown that the course of HBV reactivation can be depicted as several phases according to the severity of the disease.[29 30] The Anti-HBV treatment initiated at the first sign of HBV reactivation would prevent the evolution of disease toward more severe phases. We, therefore, recognised that there was a competing risk among each component whose occurrence precluded the occurrence of the other primary event of interest, and that the HR for each component could not be precisely estimated. Moreover, this study might not be powered for detecting the each component, especially severe hepatitis, which is individually rare in corticosteroid users with resolved HBV infection. Nevertheless, the use of composite endpoints had several advantages. We noted that not all patients may follow the reactivation phases in a sequence and some severe events may rapidly occur within a few weeks (or days in some cases) in the progression of HBV reactivation.[1] The use of composite endpoints may avoid missing observation of events and result in an increase in event rates as well as statistical power compared with the use of a single endpoint. Moreover, all the components of the composite endpoints are of the similar nature of importance to patients, of which any occurrence indicates the need for further intervention and may serve as the basis for medical decision-making.

In conclusion, among patients with resolved HBV infection on corticosteroid therapy for acute or chronic immune-related diseases, time-weighted average prednisone dose but not cumulative dose has reasonably represented the extent of corticosteroid exposures and independently predicted a monotonic increase in the risk of HBV reactivation or hepatitis flare. These patients using a time-weighted average prednisone dose greater than 20 mg/day would be classified as the high-risk level for HBV reactivation or hepatitis flare, and prophylactic Anti-HBV therapy may, therefore, be needed for these high-risk patients.

Contributors ZZ designed the study. PY supervised the study. WL, LD, XF, GS, YG and QW collected clinical data. ZZ and WL analysed and interpreted the data. ZZ wrote the first draft of the paper. PY, ZZ and WL reviewed and edited the manuscript. PY served as the overall content guarantor. All authors provided a final review and approved the manuscript before submission.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Ethics Committee of the First affiliated Hospital of Chongqing Medical University (approval number: 20195001). Participants gave informed consent to participate in the study before enrolment. Study procedures were conducted in compliance with the provisions of the Declaration of Helsinki.

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Immunogenicity of the COVID-19 mRNA vaccine in adolescents with juvenile idiopathic arthritis on treatment with TNF inhibitors

Patients with rheumatic and musculoskeletal diseases (RMDs) on immunosuppressants are generally considered to be more prone to infections, and therefore, a vulnerable group for severe COVID-19 infection. However, current data are reassuring, indicating that immunosuppression, and especially, TNF inhibitor (TNF-i) treatment, is not a specific risk factor for severe or fatal disease.[1] On the other hand, treatment with rituximab is associated with more severe disease and less favourable outcome.[1] So far, adherence to personal protection measures and immunisation comprise the two available strategies for battling the COVID-19 pandemic.[2] In the adult population, it has been demonstrated that the vast majority of patients with RMDs using non-B-cell-depleting therapy who received two doses of the COVID-19 mRNA vaccine mounted a protective immune response.[3 4] Until recently, data regarding the immunogenicity of COVID-19 vaccination in adolescents with RMDs on immunosuppressants were lacking, since these individuals were excluded from the vaccine trials.[5] The purpose of this study was to evaluate the immunogenicity of the BNT162b2 COVID-19 vaccine in adolescents with juvenile idiopathic arthritis (JIA) on TNF-i treatment.

This single-centre study involved adolescents aged 16–21 years previously diagnosed with JIA (based on the International League of Associations for Rheumatology (ILAR) criteria)[6] and treated with TNF-i. All patients were in clinical remission (Juvenile Arthritis Disease Activity Score (JADAS) Score<2).[7 8] All participants received two doses of the COVID-19 vaccine (Pfizer-BioNTech) intramuscularly at 0 and 3 weeks from 15 April to 15 May 2021. COVID-19 vaccination was performed in the time intervals between the administrations of their immunosuppressive treatment. Follow-up visits were planned at 1 and 3 months. Blood samples for the evaluation of vaccine immunogenicity were collected from all of the subjects at the time of enrolment, as well as at 1 and 3 months after the second vaccine dose. Quantitative measurement of IgG antibodies to SARS-CoV-2 spike protein-1 was performed with a cut-off level of 100 rU/mL (Euroimmun Quantivac-Elisa-IgG assay). Data were analysed using SPSS V.28.0 software. Descriptive statistics were presented as counts/percentage for qualitative data and mean/SD or median/range for quantitative data. Groups were compared with Kruskal-Wallis test. A p value<0.05 was considered statistically significant.

A total of 21 adolescents (males: 5 (24%); females: 16 (76%)) were enrolled with a median age of 17 years (range:16–21 years). Eight (38%) patients had polyarticular JIA, 7 (33%) psoriatic JIA and six (29%) enthesitis-related arthritis. In particular, 10 (48%) were receiving adalimumab fortnightly; 11 (52%) were given etanercept once a week, whereas 15 patients (71%) were on concomitant weekly subcutaneous methotrexate (MTX). All patients were in clinical remission at the time of vaccinations. None of the participants discontinued TNF-i/MTX treatment at the time of vaccine administration or during the follow-up period. All subjects were seronegative at baseline. Seropositivity rate was 100%; all patients developed a sustained humoral response against SARS-CoV-2 at 1 and 3 months after vaccination (mean(±SD) anti-SARS-CoV-2 IgG levels 11 293 U/L±12 441 and 17 590 U/L±15400, respectively (p<0.05) (1 vs 3 months) (figure 1)). The type of JIA did not reveal any differences in the humoral response at 3 months post vaccination (p=0.894). Additionally, no statistically significant difference was detected on comparison of the immunogenicity between the different treatment arms (adalimumab vs etanercept) at 3 months (mean(±SD) anti-SARS-CoV-2-IgG level: 15 739 U/L±17 132 vs 19273 U/L±14270, (p=0.387)) or on comparison of TNF-i monotherapy versus combined therapy (TNF-i plus MTX) (mean(±SD) anti-SARS-CoV-2-IgG level: 16 480 U/L±14 602 vs 19393 U/L± 17496, (p=0.623)). None of the participants developed disease flare during the follow-up period.[9] None of the participants withdrew from the study due to vaccination adverse events.[9]

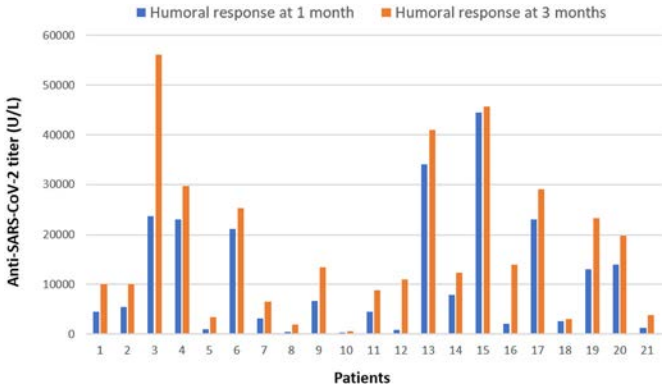

Figure 1 Humoral response against SARS-CoV-2 at 1 and 3 months after vaccination in adolescents with juvenile idiopathic arthritis on TNF inhibitor treatment.

This is a novel study demonstrating that mRNA vaccines develop and continue to accrue satisfactory immunogenicity at 1 and 3 months post immunisation in adolescents with JIA on TNF-i. Although our sample size was small and a restricted number of patients were included within each JIA type and treatment groups, it may be concluded that the vaccine assures an adequate humoral response against SARS-CoV-2, comparable with the immunogenicity of other vaccines studied in this specific population.[10 11] Likewise, this study indicated that it is not necessary to discontinue TNF-i/MTX before and after the vaccination. Further collaborative studies are required to determine long-term immunogenicity, real duration of immune protection and perhaps the need for a booster vaccine dose.

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Third COVID-19 vaccine dose with BNT162b2 in patients with ANCA-associated vasculitis

Humoral and cellular immune responses after standard two-dose COVID-19 vaccination are reduced in immunosuppressed patients with antineutrophil cytoplasmic antibodies associated vasculitis (AAV).[1–3] Emerging variants such as B.1.617.2 (delta) are of particular concern because of their higher transmissibility and partial immune escape.[4] AAV patients with lower neutralising antibody levels may become particularly susceptible to these variants of concern and additional booster vaccination may be required.

We performed a prospective observational study at three different German vasculitis centres to investigate humoral responses against the variant of concern B.1.617.2 after a third vaccine dose with BNT162b2 in 21 patients with AAV on immunosuppressive maintenance therapy. All individuals met the 2017 provisional American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria for AAV. We investigated antispike S1 IgG and surrogate neutralising antibodies a median (IQR) of 23 (21–58) days after standard two-dose COVID-19 vaccination, immediately before a third vaccine dose, as well as a median (IQR) of 21 (21–21) days after third vaccination (online supplemental material). The third vaccine dose was administered a median (IQR) of 103 (72–126) days after second vaccination. In addition, neutralisation activity against B.1.617.2 was analysed in vitro in SARS-CoV-2-infected VeroE6 cells after second vaccination and before and after the third vaccine dose (online supplemental methods).[5] Patients were also stratified according to whether or not they had received rituximab treatment as maintenance therapy in the last year. Baseline characteristics and individual immunosuppressive regimens are given in (online supplemental tables S1 and S2).

After second COVID-19 vaccine dose, the median (IQR) anti-S1 IgG index was 1.6 (0.1–3.0) and the median (IQR) per cent inhibition of surrogate neutralising antibodies 34 (31–70; figure 1A). A median (IQR) of 103 (72–126) days after the second vaccine dose, both anti-S1 IgG and neutralising surrogate antibodies decreased to 0.1 (0.1–1.8) and 9 (0–35), respectively, and a third vaccine dose with BNT162b2 was subsequently administered (figure 1A). Anti-S1 IgG and surrogate neutralising antibodies significantly increased to a median (IQR) index of 5.6 (0.5–150) and a median (IQR) per cent inhibition of 56 (4–94) 3 weeks after the third vaccine dose (for both $p<0.01$; figure 1A). Most importantly, after second vaccination, only 6/16 (38%) patients showed neutralising activity against B.1.617.2 and this number decreased to 3/16 (13%) directly before third vaccination (figure 1B). Even patients with detectable antibodies in commercially available anti-S1 IgG or surrogate neutralising assays had no neutralisation against B.1.617.2. The number of patients with neutralising antibody activity against B.1.617.2 significantly increased to 12/21 (57%) 3 weeks after the third vaccine dose with a median (IQR) ID_{50} of 40 (0–160) compared with 0 (0–20) after second vaccination and to 0 (0–0) before third vaccination ($p<0.05$ and $p<0.001$; figure 1B). Individual courses of anti-S1 IgG, surrogate neutralising and B.1.617.2 neutralising antibodies before and after third vaccination are shown in detail in online supplemental table S3.

Patients receiving rituximab maintenance therapy had significantly lower anti-S1 IgG, surrogate neutralising and B.1.617.2 neutralising antibody levels after third vaccination compared with patients not receiving rituximab treatment (online supplemental table S3; figure 1C). Of note, 12/13 (92%) patients without rituximab treatment showed neutralising activity against B.1.617.2, whereas none of those treated with rituximab showed neutralising activity after a third vaccine dose (figure 1C).

Both anti-S1 IgG index and neutralising surrogate antibody activity correlated well with the ID_{50} value of neutralising B.1.617.2 activity of patients with AAV (figure 1D). However, exceeding the cut-off value for detection in both commercially

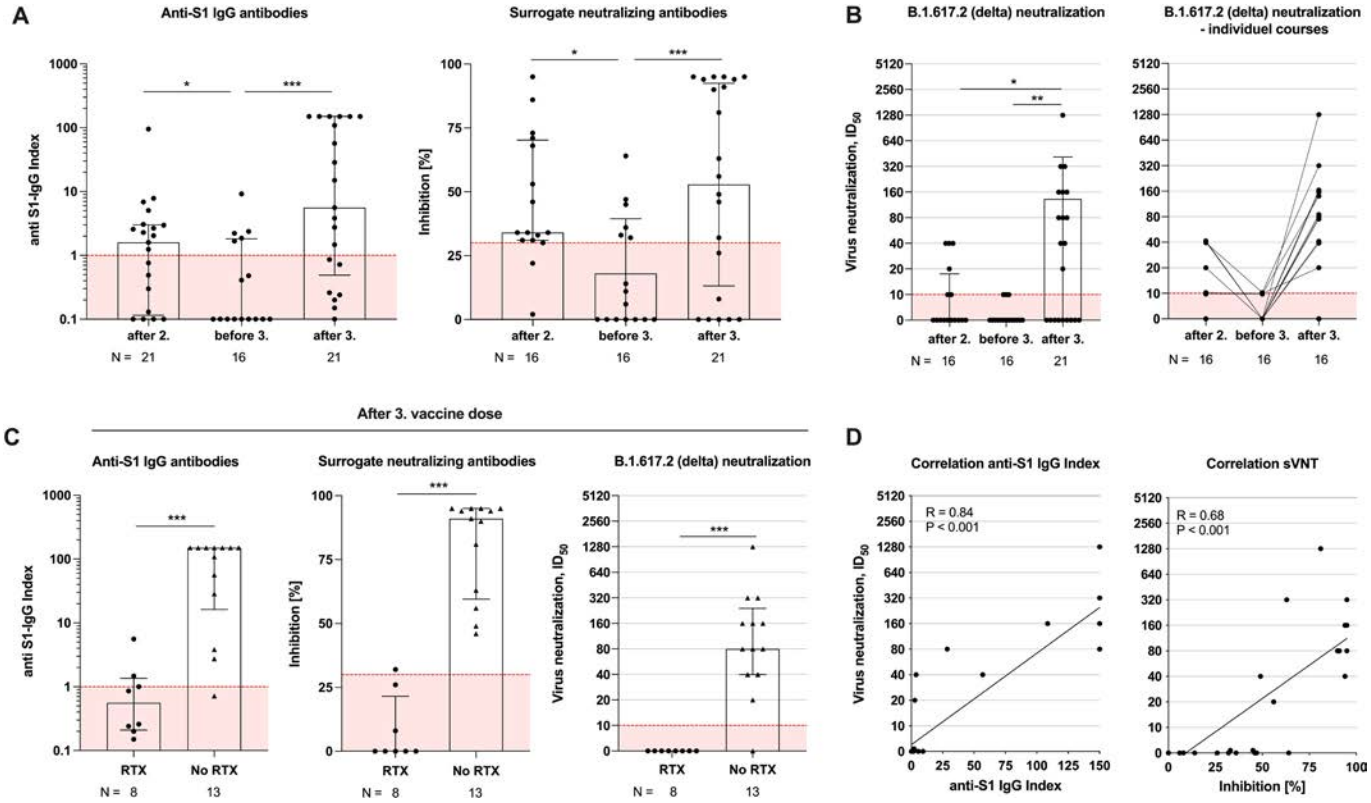

Figure 1 Humoral responses after a third COVID-19 vaccine dose with BNT162b2 in patients with ANCA-associated vasculitis (AAV) on maintenance therapy. (A) SARS-CoV-2 anti-S1 IgG and surrogate neutralising antibody levels were measured after the second COVID-19 vaccine dose (N=21 and N=16), immediately before a third vaccine dose with BNT162b2 (N=16) and after third vaccination (N=21) in patients with AAV on maintenance therapy. Anti-S1 IgG antibody levels are shown logarithmically as an anti-S1-IgG index. The dashed red line represents the cut-off for detection. A semiquantitative index of ≥1 was classified as positive. Surrogate neutralising antibodies are given as per cent binding inhibition. A cut-off of <30% binding inhibition (dashed red line) indicates the cut-off for detection of this assay. (B) Titers of neutralising antibodies against the B.1.617.2 (delta) variant were determined in a live virus SARS-CoV-2 infection assay using VeroE6 target cells and serial twofold serum dilutions after the second (N=16), directly before a third (N=16) and after (N=21) a third vaccine dose with BNT162b2. Neutralisation titers refer to the serum dilution that inhibits 50% of the infectivity (ID_{50}). The results of the three different time points in (A) and (B) were compared using Friedman's test for paired samples with Dunn's post-test. (C) Humoral responses of patients with AAV who had received a rituximab (monoclonal anti-CD20 antibody) dose <1 year before third COVID-19 vaccination (N=8) were analysed separately with the Mann-Whitney U test. (D) The correlation between the anti-S1-IgG index or the surrogate neutralisation assay and the neutralisation of B.1.617.2 (delta) was examined in patients with AAV using Spearman's correlation analysis, respectively. ANCA, antineutrophil cytoplasmic antibodies; RTX, rituximab; sVNT, surrogate neutralisation antibodies; *p<0.05; **p<0.01; ***p<0.001.

available assays did not necessarily imply neutralising activity against B.1.617.2 at the same time.

Local adverse events occurred significantly more often after third vaccine dose compared with the first or second vaccination (for both p<0.001; online supplemental figure S1). However, systemic adverse events occurred infrequently after all vaccine doses and no patient experienced a disease flare during follow-up (online supplemental figures S1 and S2).

Consistent with other studies on the immunogenicity of COVID-19 mRNA vaccines in immunosuppressed patients with autoimmune diseases, our data indicate that most individuals have detectable antibody levels in commercially available assays after standard two-dose vaccination, but at significantly lower levels as compared with healthy individuals.[1 6 7] Notably, patients treated with rituximab had particularly low seroconversion rates[6 7] without detectable neutralising antibody activity against B.1.617.2 in our study. In a first case series on a third vaccine dose in three patients with AAV treated with rituximab, the booster dose was only associated with detectable humoral response in one patient.[8] In our study, no patient treated with rituximab in the last year showed neutralising activity against B.1.617.2 after a third vaccine dose. However, in patients with AAV not treated with rituximab, a third mRNA vaccine dose resulted in significantly higher B.1.617.2 neutralisation compared with standard two-dose mRNA vaccination.

Summarised, this study suggests that immunosuppressed patients with AAV may not be adequately protected against B.1.617.2 after standard two-dose COVID-19 vaccination. A third vaccine dose with BNT162b2 induced a strong neutralising antibody activity against B.1.617.2 in most individuals; however, patients receiving rituximab maintenance therapy showed no humoral vaccine response even after a third vaccine dose.

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Herpes zoster reactivation after mRNA-1273 vaccination in patients with rheumatic diseases

The SARS-CoV-2 vaccination is one of the major strategies against the COVID-19 pandemic. The novel platforms of vaccines were developed to replace the time-consuming traditional vaccine manufacturing process, but this worldwide campaign raises new safety issues. A new panel of adverse events was recently reported at nation-wide registry levels.[1] However, the information is very limited in patients with rheumatic diseases, who potentially have increased risks of adverse events due to immune dysregulation or concomitant therapies.

We retrospectively collected the diseases, immunomodulators, types of vaccines and adverse events from patients receiving at least one dose of primary SARS-CoV-2 vaccine at rheumatology clinics of a tertiary referral centre in Taiwan. The data were analysed using the Fisher's exact test for dichotomous variables and Wilcoxon rank sum test for continuous variables. Between July 2021 and September 2021, 265 patients were enrolled, including patients with Sjogren's syndrome (n=49), rheumatoid arthritis (n=34), systemic lupus erythematosus (n=33), spondyloarthritis (n=21) and other rheumatic diseases (online supplemental table S1). Eighty-nine (33.7%) patients received ChAdOx1 nCoV-19 (AZD1222) vaccine (AstraZeneca/Oxford) and 176 (66.3%) received mRNA-1273 (Moderna) vaccines. The median (IQR) ages were 50 years (39, 60) in AZD1222 and 58 years (45, 67) in mRNA-1273 groups (p value<0.001). The overall adverse events were comparable in AZD1222 (18%) and mRNA-1273 groups (19%). The AZD1222 was associated with prolonged constitutional symptoms (6.7% vs 1.1%, p value=0.019), but the flare rates of rheumatic diseases (5.6% vs 6.2%) were similar in both groups. In addition, the rate of thromboembolic events (suggested by initial tender lesions evolving to ecchymoses and subsequent cerebral infarction occurred in one patient) was more in mRNA-1273 group than AZD1222 (5.7% vs 3.4%, p value=0.6), which is not reported as the major concerns in mRNA-1273.

Notably, herpes zoster reactivation occurred in 10 patients among mRNA-1273 group versus none in AZD1222 group (6.2% vs 0%, p value=0.019) (table 1). The median time from vaccination to herpes zoster attack was 10 days. Significantly, nine patients experienced the first herpes zoster event in their lives and multidermatome involvement was seen in five patients.

Table 1 Characteristics of herpes zoster reactivation individuals following mRNA-1273 vaccination

Diagnosis	Age (years)	Sex	Location of herpes zoster	Mucocutaneous complications	Day after vaccination/dose of primary vaccination	History of herpes zoster/prior VZV vaccination	Daily prednisolone dose (mg)	DMARDs
Spondyloarthritis	58.5	F	Left CN V2+IX	Nil	8/1	N/N	Nil	SSZ, HCQ, celecoxib
Systemic lupus erythematosus	53.9	F	Right T8	Nil	8/1	N/N	10	HCQ
Hashimoto thyroiditis	23.9	F	Right CN V3+IX	Severe mucositis with ulcers	9/2	N/N	Nil	RTX, HCQ
Spondyloarthritis	72.6	M	Right L4+L5	Toxic epidermal necrolysis	47/1	N/N	Nil	Etoricoxib
Rheumatoid arthritis	66.8	F	Left T7	Nil	7/1	N/N	5	HCQ, celecoxib, colchicine
Rheumatoid arthritis	80.7	M	Left CN V1+V3	Severe mucositis with ulcers	11/1	N/N	5	HCQ, colchicine
Spondyloarthritis	42.1	F	Right T3+T4	Nil	10/2	Y/Y*	Nil	HCQ
ANCA-associated vasculitis	74.9	M	Right S1	Nil	13/1	N/N	Nil	Nil
Rheumatoid arthritis	72.3	F	Left T4	Nil	51/1	N/N	Nil	MTX, HCQ
Mixed connective tissue disease	64.3	F	Left S1	Nil	47/1	N/N	Nil	SSZ, HCQ, celecoxib

*The patient received live-attenuated Zostavax vaccination.
ANCA, antineutrophil cytoplasmic antibody; CN, cranial nerve; DMARDs, disease-modifying antirheumatic drugs; HCQ, hydroxychloroquine; MTX, methotrexate; N, no; RTX, rituximab; SSZ, sulfasalazine; VZV, varicella-zoster virus; Y, yes.

Moreover, two patients were complicated with pemphigus-like oral mucositis and one patient developed toxic epidermal necrolysis (TEN). Most patients were successfully treated with valaciclovir except the three complicated patients who also received moderate-dose glucocorticoids.

Herpes zoster reactivates in cases of compromised cell-mediated immunity or viral arousal. Only one patient received rituximab 4 months ago with potential immunosuppression. The other patients received only conventional disease-modifying antirheumatic drugs or low-dose glucocorticoids. Based on the characteristics in those herpes zoster attacks, the pathogenesis might involve overactivating varicella zoster virus (VZV) by the mRNA-1273 vaccines.[2] Similar findings were reported in other studies[3] and herpes zoster was more common in patients receiving mRNA vaccines.[4] The possible hypothesis proposes that COVID-19 mRNA or vaccine adjuvants enhance strong T-cell responses[5] while compromising VZV-specific CD8+ T-cell immunity. In addition, vaccine mRNA or nanoparticle envelope may enhance the cytokines such as the type 1 interferons via Toll-like receptors signalling.[6] The severe mucositis and TEN might imply a fulminant activating VZV, which is one of the precipitating factors for erythema multiforme or TEN.

In this study, we reported the incidence of various adverse events, including the constitutional symptoms, disease flares, thromboembolic events and herpes zoster reactivation in different types of SARS-CoV-2 vaccines. The study is limited by the retrospective, single-centre design and the patients receiving AZD1222 were younger than the mRNA-1273 group. Nonetheless, our report discloses the potential risk of thromboembolism and herpes zoster reactivation by mRNA-1273 in patients with rheumatic diseases. More efforts are needed to clarify the safety and the possible pathogenesis of mRNA SARS-CoV-2 vaccines in autoimmune diseases.

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Hypoglycaemia following JAK inhibitor treatment in patients with diabetes

Janus kinase inhibitors (JAKi) are effective drugs for the treatment of several immune-mediated inflammatory diseases and are increasingly prescribed.

The Netherlands Pharmacovigilance Centre Lareb received an adverse drug reaction (ADR) report of a potential glucose lowering effect in a 54-year-old male patient with diabetes mellitus type 1 (DM1) using baricitinib (4 mg daily) for rheumatoid arthritis (RA).[1] Within 2 weeks after baricitinib initiation, this patient had to reduce the dosage of both insulin degludec (from 18 units to 14 units) and insulin aspart in order to prevent hypoglycaemia. Concomitant medication included methotrexate, tiotropium/olodaterol nebuliser and beclomethasone aerosol. When baricitinib was temporarily discontinued for 6 weeks due to a respiratory tract infection, the insulin dosages had to be increased, whereas insulin dosages needed to be reduced again after restarting baricitinib. The onset of glucose decrease shortly after initiation of JAKi treatment and recurrence after rechallenge with baricitinib suggests a causal relationship. Glucose lowering is not a labelled ADR and no warning for patients with diabetes is mentioned in the European or FDA product information of baricitinib, tofacitinib, upadacitinib or filgotinib. A comparable case has been published concerning a 71-year-old female patient with RA that was complicated by systemic sclerosis and DM1.[2] This patient was resistant to multiple disease-modifying anti-rheumatic drugs but was successfully treated with baricitinib, with concomitant use of prednisolone for 3 weeks and methotrexate. In addition to improvements in RA and skin sclerosis, the required daily dose of insulin decreased from 17 to 11 units and did not increase for up to 1 year. The glycated haemoglobin (HbA1c) level decreased from 57 mmol/mol to 46 mmol/mol.

Table 1 Suspected adverse drug reaction (ADR) reports indicating hypoglycaemia in patients with diabetes using a JAK inhibitor in the EudraVigilance database

	Tofacitinib N (%)	Baricitinib N (%)	Upadacitinib N (%)
Number of reports	20 (100)	19 (100)	4 (100)
Mean age, years, (range)	66.8 (56–83)	64.7 (48–80)	70.7 (65–74)
Female gender	17 (85)	14 (74)	4 (100)
Indication for JAKi			
Rheumatic disease	17 (85)	14 (74)	4 (100)
Unknown	2 (10)	3 (16)	–
Other*	1 (5)	2 (11)	–
Type of diabetes			
Diabetes mellitus type 1	3 (15)	6 (32)	1 (25)
Diabetes mellitus type 2	2 (10)	1 (5)	–
Not reported/type not specified	15 (75)	12 (63)	3 (75)
Reported ADR (MedDRA term)†			
Hypoglycaemia	7 (35)	13 (68)	2 (50)
Decreased blood glucose	13 (65)	7 (37)	2 (50)
No. of drugs suspected to cause the reaction			
Only JAKi	13 (65)	18 (95)	3 (75)
JAKi and one other drug	5 (25)	1 (5)	–
JAKi and two other drugs	1 (5)	–	–
JAKi and three other drugs	1 (5)	–	–
JAKi and four other drugs	–	–	1 (25)
Concomitant medication			
Insulin	8 (40)	12 (63)	–
Other antidiabetic‡	7 (35)	1 (5)	2 (50)
Methotrexate	4 (20)	1 (5)	1 (25)
Glucocorticoid	6 (30)	6 (32)	1 (25)
Other	11 (55)	10 (53)	3 (75)
Not reported	4 (20)	3 (16)	1 (25)
Reaction leading to hospitalisation	7 (35)	4 (21)	2 (50)
Time to onset after start JAKi:			
Within 1 month	8 (40)	6 (32)	1 (25)
2–6 months	4 (20)	2 (11)	3 (75)
More than 6 months	2 (10)	–	–
Not reported	6 (30)	11 (58)	–
Improvement after			
Drug withdrawal§	5 (25)	2 (11)	–
Dose adjustments§	1 (5)	1 (5)	–
Other¶	3 (15)	4 (21)	3 (75)

There were no reports of filgotinib.
*Tofacitinib: colitis ulcerative. Baricitinib: neurodermatitis, COVID-19.
†In one case of baricitinib, both hypoglycaemia and decreased blood glucose were reported
‡Tofacitinib: metformin: 3; glimepiride, pioglitazone and vildagliptin: 1; sitagliptin: 1; gliclazide, saxagliptin and metformin: 1; glimepiride and sitagliptin: 1. Baricitinib: metformin: 1. Upadacitinib: pioglitazone, glipizide and metformin: 1; sitagliptin and glimepiride: 1.
§Tofacitinib: tofacitinib withdrawn: 2, tofacitinib and insulin withdrawn: 1, tofacitinib withdrawn and insulin dose reduced (units unknown): 1, sitagliptin withdrawn: 1. Baricitinib: baricitinib withdrawn: 2, baricitinib dose reduced (unknown dosages): 1.
¶Tofacitinib: tofacitinib dose not changed: 3. Baricitinib: baricitinib dose not changed: 4. Upadacitinib: upadacitinib dose not changed: 2, action unknown: 1.
JAKi, Janus kinase inhibitors; MedDRA, Medical Dictionary for Regulatory Activities.

To further investigate the development of hypoglycaemia as potential ADR of JAKi, we collected and analysed ADR reports of tofacitinib, baricitinib, upadacitinib and filgotinib with Medical Dictionary for Regulatory Activities preferred terms 'Hypoglycaemia' or 'Blood glucose decreased' from EudraVigilance, the European Medicines Agency Pharmacovigilance database.[3] From initiation until 17 September 2021, EudraVigilance included 39 671 ADR reports concerning JAKi. Out of these, 43 reports concerned baricitinib, tofacitinib or upadactinib in patients with reported DM and/or with antidiabetic drugs as concomitant medication (table 1). In 9 out of 43 reports (21%), one or more other drugs were suspected

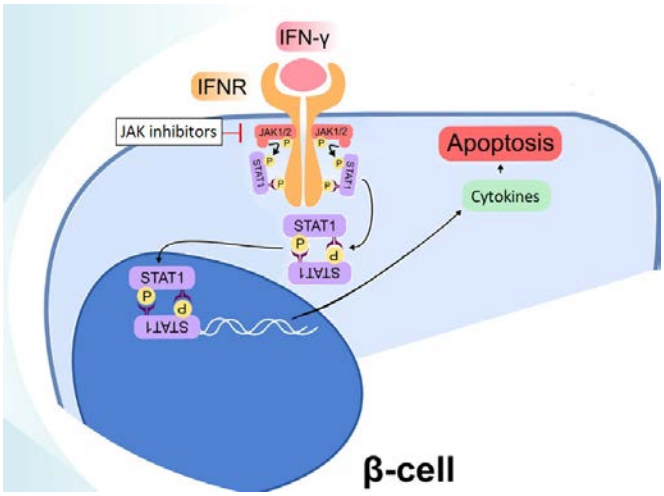

Figure 1 The JAK/STAT pathway involved in pancreatic β cells, based on figure 4 of Gurzov *et al*'s work.[5] IFN-γ, interferon-γ; IFNR, interferon receptor; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

to have contributed to the observed effect in addition to the JAKi, which included an antidiabetic in six cases, a corticosteroid in two cases and methotrexate in one case. Glucose levels after JAKi initiation were mentioned in 15 cases ranging from 1 to 5.5 mmol/L or a decrease from reference levels of 0.5 to 4 mmol/L. In 15 cases the event occurred within 1 month after JAKi initiation. In eight cases, glycaemic control improved after discontinuation or dose reduction of the JAKi or antidiabetic drug. Reduced dosages of fast-acting as well as long-acting insulin were described with dose reductions up to 30%. These reports varied in their extent of documentation, especially with respect to other factors that could affect glucose levels and insulin requirement such as tapering of corticosteroids, concomitant medication such as methotrexate or other antidiabetics, disease activity and concurrent infections, which was not consistently reported. However, the time to onset, the required insulin dose reductions after JAKi initiation and improvement after discontinuation suggest that JAKi may induce hypoglycaemia and may therefore reduce the need for antidiabetic medication in patients with diabetes.

These findings may be explained by the role of the JAK/signal transducer and activator of transcription (STAT) pathway in pancreatic islets. Previous studies showed evidence that the JAK1/2 and STAT1 pathway are involved in β-cell dysfunction in both DM1 and DM2.[4 5] Cytokines involved in pancreatic β-cell apoptosis are dependent on JAK1/2-STAT1 activation as a response to other cytokines, such as interferon-γ (figure 1). CXCL10 is a cytokine associated with β-cell apoptosis and is overexpressed in both DM1 and DM2.[6] Additionally, it has been demonstrated in preclinical models that DM can be reversed following JAKi treatment.[7–9] Consequently, the potential of repurposing JAKi for treatment of DM1/2 has been suggested and recently a phase 2 randomised placebo-controlled study investigating the efficacy of baricitinib in new onset DM1 has been started.[2 4 7–10] More detailed epidemiologic data or distinct pharmacologic studies that consider potential similarities and molecular differences of JAKi subtypes are needed to support our findings. Until the exact potential and risks of JAKi in DM1 and DM2 have been fully elucidated, physicians should be aware of the potential glucose lowering effect when starting a JAKi in patients with diabetes.

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Clinical characteristics of juvenile gout and treatment response to febuxostat

Gout is a common form of inflammatory arthritis caused by hyperuricaemia and deposition of monosodium urate crystals.[1] While risk factors and comorbidities associated with gout are well established in adults,[2 3] few studies have examined gout in children.[4] There is no treatment guideline for juvenile gout and little is known about the efficacy of urate-lowering therapy in children. Here, we describe the clinical characteristics of 111 patients with juvenile gout evaluated in our centre between 2016 and 2020. We also present data on the efficacy of febuxostat treatment in children.

Our cohort of patients with juvenile gout (age of onset ≤18 years) consisted of 107 males and 4 females. All patients met the 2015 ACR/EULAR Criteria for gout. The mean age of symptom onset was 15.2 years and the youngest patient was 9 years old (online supplemental table 1). Compared with adult gout cases (n=533) evaluated during the same period, body mass index was comparable between the groups (p=0.097). Hypertension and kidney stones were comorbidities of gout in adults but not children. Patients with juvenile gout were more likely to provide a family history of gout in first-degree or second-degree relatives (online supplemental table 1).

The most common site of gout attacks in children was finger joints while knee involvement was less prevalent compared with adults (figure 1A). The appearance of gout arthritis was difficult to distinguish from other forms of juvenile arthritis (figure 1B). Underscoring the importance of considering gout in the differential diagnosis of childhood arthritides, 51 patients (45.9%) would have fulfilled criteria for juvenile idiopathic arthritis (JIA) without confirmatory testing for gout (see online supplemental methods for further details on distinguishing juvenile gout and JIA). While the incidence of tophi was comparable between children and adults (28% vs 24%), tophi developed more rapidly in patients with juvenile gout (interval to tophi development: mean 1.5 years in children vs 7.5 years in adults; online supplemental table 1). Finger joints were the most common site for tophi development in children, compared with the metatarsophalangeal (MTP) joints in adults (online supplemental table 1). Birefringent crystals in the synovial fluid and tophi associated with juvenile gout are depicted in figure 1C and D.

Comparison of laboratory features revealed that patients with juvenile gout possessed higher serum uric acid levels than adults (mean 11.9 mg/dL vs 9.0 mg/dL; p=0.032) but less systemic inflammation, as reflected by lower erythrocyte sedimentation rate (mean 18 mm/hr vs 38 mm/hr; p<0.0001) and C-reactive protein levels (mean 9.5 mg/L vs 25.3 mg/L; p<0.0001).

Currently, there are no guidelines for the management of juvenile gout. Our patients with acute gout were treated with non-steroidal anti-inflammatory drugs and/or colchicine and counselled on dietary intervention. All patients with persistent hyperuricemia were offered urate-lowering treatment after acute

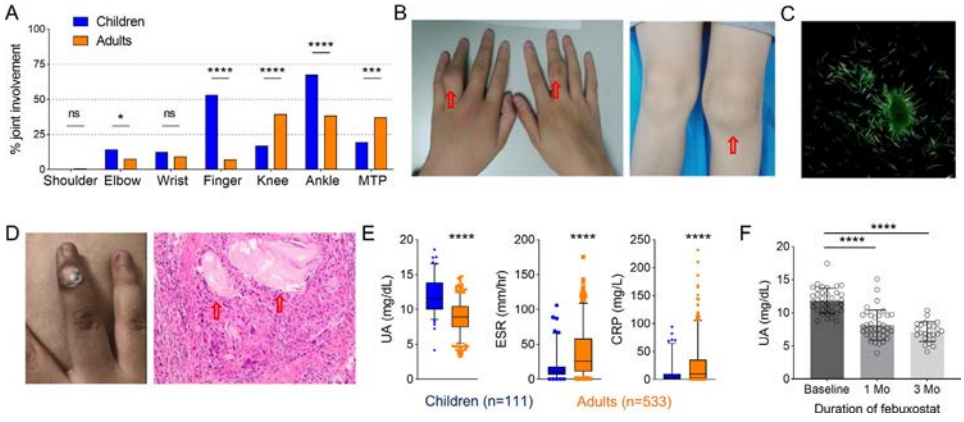

Figure 1 Characteristics of gout in children and efficacy of febuxostat therapy. (A) Comparison of joint involvement in adults (n=533) and children (n=111) with gout. (B) Representative depiction of affected fingers and knee joint in juvenile gout. Red arrows indicate affected joints. (C) Polarised light microscopy image of synovial fluid birefringent crystals from patient with juvenile gout. (D) Tophi in third distal interphalangeal joint of a patient with juvenile gout. Red arrows indicated tophi on H&E section. (E) Comparison of the levels of serum uric acid and inflammatory markers in adults and children with gout. Box indicates median and IQR and whiskers denote 5th–95th percentile. (F) Serum uric acid levels in patients with juvenile gout at baseline (n=36), 1 month (n=37) and 3 months (n=24) after initiation of febuxostat therapy. *P<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

gout attack was controlled. In our practice, the xanthine oxidase inhibitor febuxostat is the first-line treatment option for adults with gout due to the risk of allopurinol hypersensitivity.[5 6] We employed a similar approach for juvenile gout and collected data from 37 patients treated with febuxostat (40 mg once a day).

A significant reduction in serum uric acid levels was observed after 1 month of febuxostat treatment (median reduction 3.6 mg/dL; figure 1F). The improvement in uric acid levels was sustained after 3 months. Importantly, the frequency of gout arthritis flare reduced markedly after treatment initiation (pretreatment: 176 events in 679 patient-months; post-treatment: 14 events in 493 patient-months; $p<0.0001$).

Adverse effects were recorded for four patients treated with febuxostat. Transient transaminase elevation was noted in 3 cases and resolved without treatment discontinuation. One patient developed rhabdomyolysis within 3 weeks of starting febuxostat and fully recovered 10 days after treatment discontinuation.

Juvenile gout is an aggressive joint disease that should be considered in the differential diagnosis for childhood arthritides. We showed that febuxostat is well tolerated in adolescents and effectively reduced uric acid levels and gout attacks. In our experience, the prognosis for patients with juvenile gout is generally favourable with effective control the uric acid levels. Larger controlled studies are needed to better understand the natural history of juvenile gout and the safety and efficacy of various urate-lowering agents in children.

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Autoantibodies and interstitial lung disease in rheumatoid arthritis: towards a 'mix-and-match' approach?

We read with interest the article by Castellanos-Moreira *et al* who identified for the first time an association between anticarbamylated protein antibodies and interstitial lung disease (ILD) in patients with rheumatoid arthritis (RA).[1] The reported prevalence of ILD in RA ranges from 4% to 70% according to different cohorts and inclusion criteria. The clinical spectrum is rather broad ranging from mild reversible lung inflammatory disease to rapidly progressing fibrotic conditions with poor prognosis and frequent cause of death. On this basis, clinicians should be alert and promptly identify, classify and manage ILD according to its features and severity. However, whether a patient with RA will develop or not ILD, depends on several genetic, demographic, environmental and immunological factors that interact with each other and reliable markers able to predict ILD development are currently lacking.[2] The increasing knowledge on novel autoantibody specificities in RA could allow a better understanding of immunological mechanisms underlying different disease features[3] and, in this regard, the study by Castellanos-Moreira is of great relevance. However, some aspects need to be remarked. They observed an association of ILD and antibodies against two carbamylated antigens (fetal calf serum (FCS) and chimeric fibrin/filaggrin homocitrullinated peptide (CFFHP) in a regression model adjusted for age, disease duration, anticitrullinated proteins antibodies (ACPA), rheumatoid factor, sex and smoking cumulative dose. The population of patients was mainly constituted by females (79%) seropositive for ACPA (72%). What remains unclear, however, are the striking differences with the replication cohort with an OR almost three fold higher for anti-FCS and a lack of significant association between ILD and anti-CFFHP. It is interesting to note that gender distribution in the replication cohort is significantly different compared with the main cohort, with males being equally represented than females (F/M replication cohort 40/35, main cohort 141/38; χ^2p<0.0001). In addition, the cumulative smoking dose in the replication cohort is similar in patients with or without ILD, although a surprising trend towards higher values in non-ILD patients is observed. Conversely, in the main cohort, patients with ILD have a significantly higher smoking cumulative dose compared with those without ILD. As far as serology is concerned, differences can be seen between the two cohorts, with a higher prevalence of ACPA in the replication compared with the main one (ACPA+/ACPA− replication cohort 65/10, main cohort 128/51; χ^2p=0.01). Within each cohort, ACPA are equally distributed in patients with or without ILD. It would be interesting to see the individual ORs obtained at univariate analysis before building a model adjusted for the same variables in the two cohorts.

When performing a similar exercise and assessing the relationship between ILD and anticitrullinated alpha enolase peptide-1 (anti-CEP-1), we enrolled 252 RA patients (77% females, 66% anticyclic citrullinated peptide (anti-CCP) positive) and observed that anti-CEP-1 single positivity and anti-CCP/anti-CEP-1 double positivity, but not anti-CCP single positivity, were associated with ILD.[4] An increasing number of papers is supporting the hypothesis that it is a matter of which autoantibodies test positive and also how many specificities of the same antibody family coexist to be able to predict risk of developing RA, the response to treatment or the development of erosive disease.[5–7]

Such assessment in the cohorts tested by Castellanos-Moreira may help explaining the different results obtained in the two cohorts and ultimately facilitate the design of longitudinal studies aimed at understanding the predictive value of different antibody specificities assessed at the time of RA diagnosis for the future development of ILD. In the era of precision medicine, a mix-and-match approach combining test for antibodies with a diagnostic and/or a prognostic value may be a powerful tool to optimise the tailoring of follow-up and treatment strategies.

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Response to: 'Autoantibodies and interstitial lung disease in rheumatoid arthritis: towards a 'mix-and-match' approach' by Alunno *et al*

We welcome the comments by Alunno *et al* on our article about the association between anticarbamylated protein antibody (anti-CarP) specificities and rheumatoid arthritis-associated interstitial lung disease (RA-ILD).[1] The authors proposed a 'mix and match approach' consisting of the assessment of various antibody specificities at RA diagnosis with the aim of predicting the development of ILD.[2] We believe this hypothesis is reasonable, considering that different antimodified protein antibodies (AMPA) including anti-CarP, anticitrullinated and antiacetylated protein antibodies (ACPA and anti-AceP, respectively) have been associated with RA-ILD.[1 3 4] Furthermore, a greater number of coexisting specificities of a single AMPA have been found in patients with RA-ILD.[3 5]

The prevalence of ILD and its risk factors fluctuate between RA cohorts, partially due to differences in the screening methods, the population examined, and the defining criteria used. Although these factors were controlled in our study, there were differences in some baseline features between the main population and the replication sample, as pointed out by Alunno *et al*.[2] This may be due to the small sample size of the replication sample or because ILD screening and diagnosis was ultimately based on physician's criteria based on the ILD committee dictates from two hospitals. However, our final model was fitted after adjusting for these features and so they did not affect the results. Recently, Zhu *et al* reported a higher proportion of anti-CarP in Chinese patients diagnosed with RA-ILD compared to RA controls without ILD (53% vs 16%).[6] Their findings are consistent with and enhance the external validity of our observations. However, larger multiethnic studies are still required.

We consider the association between ILD and RA is a 'two-way street'. It should be considered that: (1) ILD may be present before or around RA onset in one in three patients[7] and (2) ACPA have been found in more than 20% of patients with idiopathic pulmonary fibrosis (IPF),[8] of whom approximately one-third subsequently may develop RA.[9] Thus, the 'mix and match approach' should be considered in RA and IPF. We believed a broader view (eg, multidisciplinary ILD committees) on the issues implied in the relation between ILD and RA should be considered in the design of future prospective collaborative studies.

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Increasing the threshold for patient global assessment in defining remission may have a different impact in patients with early and established rheumatoid arthritis

A significant proportion of patients with rheumatoid arthritis (RA) misses the target of disease remission solely because of the patient global assessment of disease activity (PGA) exceeding the cut-off of 1.[1–5] As PGA may also reflect non-inflammatory symptoms, its inclusion as a driver of intensification of immunosuppressive therapy is currently been questioned.[6] Complete omission of the patients' perspective, however, impairs functional outcomes and the ability to discriminate effective treatments from placebo.[7] As such, different thresholds for the PGA are being tested, with a recent proposal from randomised clinical trials suggesting a suitable cut-off of 2.[8]

Here, we evaluated the performance of modifying the Boolean definition of remission[9] by increasing the cut-off of the PGA to 2[8] in real life. Data were retrieved from 826 consecutive patients from two University Hospitals with an observation period of 12 months. Five hundred and thirty-five were patients with early RA (median (IQR) symptoms' duration 16 [9–28] weeks) started on methotrexate aimed at low disease activity.[10] Two hundred and ninety-one were established patients with RA (median (IQR) duration 6.7 [3.4–13.6] years) started on a biological drug (a tumour necrosis factor antagonist in 79.4% of the cases). In early RA, the rates of remission following PGA modification only slightly increased of +4.1% at 6 months and +4.3% at 12 months. Within remitters according to the Simplified Disease Activity Index (SDAI), simultaneous Boolean remitters increased from 65.7% at 6 months and 64.7% at 12 months with the original definition to 81.8% and 85.7% with the modified definition. However, modified Boolean remitters (original Boolean remitters excluded) were in SDAI remission in fewer cases compared with original Boolean remitters (40.9% vs 97.1% and 57.1% vs 96.7% at 6 and 12 months). As such, the concordance with SDAI remission was lower for modified compared with original Boolean remission at both time points (κ statistics 0.35, 95% CI 0.11 to 0.58 vs 0.74, 95% CI 0.67 to 0.82 and 0.52, 95% CI 0.28 to 0.75 vs 0.71, 95% CI 0.64 to 0.78). In contrast, in established RA, the increase in the remission rate was more pronounced (+7.3% and +12.5% at 6 and 12 months), and concordance with SDAI remission was higher compared with early RA (κ statistics 0.63, 95% CI 0.42 to 0.84 and 0.65, 95% CI 0.46 to 0.84 at the two time points). Patients in modified Boolean remission also showed different disease activity characteristics and functional outcomes in relation to disease duration (table 1). Indeed, in early RA, modified Boolean remitters at 6 months had significantly higher levels of C reactive protein (CRP) compared with original Boolean remitters. Furthermore, their Health Assessment Questionnaire (HAQ) at 12 months worsened of a clinically significant mean (SD) of 0.24 (0.31) points compared with functional stability in original Boolean remitters, and an HAQ ≤0.5 was observed in fewer cases. In contrast, in established RA, CRP levels, HAQ variations and the rate of good functional outcome (HAQ ≤0.5) at 12 months were comparable between modified and original Boolean remitters.

The inclusion of patients from a real-life clinical setting, with different disease duration, activity and treatment protocols hampers any comparison with published studies,[8] and our observations need confirmation in independent cohorts. However, our data suggest that a cut-off of the PGA of 2 increases the rates of remission without impacting on outcomes in patients with established RA. In contrast, in early disease, before changes in pain processing mechanisms have occurred,[11] the PGA may more strictly collect information on inflammatory-related symptoms, and even small increases of its cut-off may affect functional outcomes. Better understanding of the relationship between patient-reported outcomes and disease activity in the various phases of RA may thus be needed before introducing definitive changes in the current definition of remission.

Table 1 Comparison of disease characteristics and functional outcomes according to the original and the modified definition of remission

	Original Boolean remission	Modified Boolean remission	P value
6 months			
Early RA			
SJC28	0.5 (0.6)	0.6 (0.5)	0.77
TJC28	0.1 (0.4)	0.4 (0.7)	0.08
VAS pain 0–100	3.9 (7.7)	16.7 (7.2)	**<0.001**
HAQ 0–3	0.11 (0.25)	0.18 (0.30)	0.26
ESR, mm/1 h	13.6 (10)	15.8 (9.9)	0.41
CRP, mg/dL	0.26 (0.22)	0.43 (0.32)	**0.01**
Established RA			
SJC28	0.1 (0.2)	0.2 (0.6)	0.21
TJC28	0.2 (0.4)	0.3 (0.5)	0.34
VAS pain 0–100	5.8 (6.1)	13.6 (6.8)	**<0.001**
HAQ 0–3	0.16 (0.27)	0.35 (0.29)	**0.007**
ESR, mm/1 h	15.7 (12)	19 (13.5)	0.27
CRP, mg/dL	0.32 (0.23)	0.31 (0.25)	0.80
Functional outcomes at 12 months			
Early RA			
HAQ variation from 6 to 12 months	0.02 (0.34) paired t-test p=0.61	0.24 (0.31) paired t-test **p=0.004**	**0.02**
HAQ ≤0.5	93%	72.2%	0.05
Established RA			
HAQ variation from 6 to 12 months	0.02 (0.26) paired t-test p=0.56	−0.09 (0.29) paired t-test p=0.20	0.12
HAQ ≤0.5	88.1%	88.9%	0.75

Data are reported as means and SD unless otherwise stated.
Data are shown for non-overlapping remission groups. That is, the group in modified Boolean remission does not include patients in original Boolean remission.
Bold indicates statistically significant p values (p <0.5).
CRP, C reactive protein; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; RA, rheumatoid arthritis; SJC28, swollen joint count on 28 joints; TJC28, tender joint count on 28 joints; VAS, Visual Analogue Scale.

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Response to: 'Increasing the threshold for patient global assessment in defining remission may have a different impact in patients with early and established rheumatoid arthritis' by Bugatti *et al*

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We thank Bugatti *et al* for their interest in our paper and for sharing their results on applying a 2 cm cut-off for the patient global assessment (PGA) criterion of the Boolean remission criteria for rheumatoid arthritis (RA) within the setting of a real-world database,[1] which confirm the data presented in our recent report based on a clinical trial population.[2] An appropriate definition for remission is of utmost relevance to prevent structural progression or functional deterioration, regardless of the treatment regimen used; in fact, such a definition is clearly needed in clinical practice, where today a significant proportion of patients is able to attain very good control of disease activity.[3–6] The currently used provisional ACR/EULAR remission should be revisited to no longer be provisional.[7] Ideally, the definition of remission should be applicable to all people with RA independent of disease duration.

Since more patients fulfil the index-based remission definition (15% in early RA, and 6.8% in established RA at 6 months) compared with Boolean remission (12.4% and 5.9%, respectively), in our original study we investigated at which cutpoints of the PGA in Boolean criteria there was best agreement with index-based remission definition (simplified disease activity index (SDAI) ≤3.3). While Bugatti *et al* have shown many similarities in the performance of our proposed 2 cm PGA cut-off using real-world data to those we reported using trial data,[2] they did not analyse their data in a way that permits them to assess agreement of different Boolean definitions with the SDAI definition of remission, and were therefore not enabled to draw respective conclusions. In terms of analysing agreement, Bugatti *et al* needed to look at one group that met original Boolean definitions and another that met the expanded definition (this latter group also includes those who meet the original Boolean definition). Bugatti *et al* rather, as noted in a footnote to their table 1, examined the stratum that had higher PGA assessments separately. Given the close association of PGA with pain and function,[8] it is expected that those with higher PGA's would have more pain and have worse health assessment questionnaire (HAQ) scores.

We have been conservative in the interpretation of comparative analyses in non-overlapping remission groups (using the different PGA cut-points) since sample size was rather small. The exact number of people in Boolean 2.0 remission is unfortunately not depicted in Bugatti *et al* nor is the rate of people in Boolean remission. Taking into account that the transition of the PGA cut-off to 2 cm led to an increase of a bit more than 4% of remitters makes it possible to convey that only few patients could be accounted to the discrete group of Boolean 2.0 remitters, which may affect performance of kappa statistics applied to this stratum.

We did not recommend a Boolean definition using solely this stratum but rather an expanded Boolean definition that included those who met the original definition and liberalised this definition to include those with slightly higher PGAs. Bugatti *et al* should have performed agreement analyses using this expanded definition, a definition that includes those who met the original Boolean definition, as it would apply in the clinical setting.

Overall, these published findings should make us more confident that in trials as well as clinical practice a PGA cut-off of 2 out of 10 can be used in evaluation of Boolean remission.

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Withdrawal of low-dose prednisone in inactive SLE patients: Is there another alternative?

I read with great interest, the recently published article in your journal titled 'Withdrawal of low-dose prednisone in systemic lupus erythematosus (SLE) patients with a clinically quiescent disease for more than 1 year: a randomised clinical trial' by Mathian *et al*.[1] In this article, the authors conclude that the maintenance of long-term 5 mg prednisone in SLE patients with inactive disease prevents relapses. In the recent update of European League Against Rheumatism (EULAR) recommendations for the management of SLE,[2] the experts say that the 'treatment in SLE should aim at remission or low disease activity and prevention of flares in all organs, maintained with the lowest possible dose of glucocorticoids (GC)'. However, in my opinion, a daily dose of prednisone of 5 mg might not be the lowest possible dose in patients with long-term inactive SLE, and there is an alternative strategy that consists of the progressive reduction of this dose, which has not been considered. I believe that the one-time withdrawal of 5 mg/day is too abrupt and it could favour the appearance of flares. In contrast, many patients could benefit from a more gradual reduction. In this situation, the protocol of our unit is to decrease 1.25 mg of prednisone every 2–3 months until it is suspended, or in case of relapse or flare, we maintain the previous effective dose for a longer time and then lower more slowly. The aim of this strategy is to decrease the accumulated dose of GC in order to prevent irreversible organ damage (Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI)) associated with its use. In this study, no significant differences in SDI and adverse effects were found between those who discontinued prednisone and those who maintained a daily dose of 5 mg after 1 year of follow-up. However, this period may be too short, as some studies suggest that sustained low doses of GC may be associated with increased SDI.[3] In summary, the alternative of a progressive dose reduction of prednisone in patients with long-term inactive SLE should be explored before deciding on indefinite maintenance of a daily dose of 5 mg.[4]

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Response to: 'Withdrawal of low-dose prednisone in inactive SLE patients: is there another alternative?' by Sabio

We thank Dr Sabio *et al* for his interest in our study.[1 2] Dr Sabio in his letter suggests that the one-time withdrawal of 5 mg/day of prednisone is too abrupt and could have favoured the appearance of systemic lupus erythematosus (SLE) flares. Dr Sabio describes the practice of his unit which is to decrease by 1.25 mg of prednisone every 2 to 3 months until a complete stop. In the event of a relapse or flare, an effective dose is maintained for a longer period of time and then slowly lowered. The opinion that an abrupt cessation of a very low dose of glucocorticoids (GCs), such as 5 mg of prednisone per day, during a period of remission would be in itself a factor favouring the relapse of SLE and, in consequence, would require a slow and gradual tapering of this treatment is shared by many physicians. However, to our knowledge, no studies to date do support this hypothesis in SLE. In an observational study reporting a gradual GC withdrawal in SLE, about a quarter of the patients relapsed, similar to the number reported in our study.[3] Furthermore, although one should be careful in drawing a comparison between diseases, it has been reported that continued administration of a very low dose of prednisone or prednisolone to patients with rheumatoid arthritis with a low disease activity status provided better disease control than GC withdrawal, even in case of slow tapering.[4 5] It is important to also consider that in our study the vast majority of patients, following the interruption of GC intake, remained on long-term treatment with hydroxychloroquine and thus, indeed, in daily practice SLE treatment is almost never abruptly stopped.

The issue raised by Dr Sabio is of major importance and emphasises that clinicians should in the future (1) determine whether clinical characteristics and biomarkers could help to identify patients with SLE who are at a lower risk of relapse and therefore would benefit from discontinuation of their chronic maintenance treatment and (2) challenge, in academic clinical trials, the modalities of therapeutic de-escalation in patients in remission, in particular in view of the low number of studies that have been reported in this field.[6] New knowledge on the disease will undoubtedly lead to a better understanding of its treatment. Our study, by concluding that maintenance of 5 mg prednisone is superior to its withdrawal in order to prevent flares in patients with clinically quiescent SLE, provides a rationale that we think paves the way for future studies, aiming to improve patient management.

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External validation of EULAR/ACR classification criteria for idiopathic inflammatory myopathies

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We have read with great interest the article published by Jinnin *et al*, which was an external validation of sensitivity and specificity of the EULAR/ACR (European League Against Rheumatism / American College of Rheumatology) classification criteria for idiopathic inflammatory myopathies with a Japanese cohort. The title and article claim that this is the first external validation study. While this may be true for a Japanese cohort, our intention is to alert the readers to the earlier published validation studies that preceded this and help highlight the complete body of evidence that should be considered.

The idiopathic inflammatory myopathies (IIMs) are a group of uncommon disorders with a potential for significant mortality and morbidity. Progress in the understanding and management of these disorders has been hampered by the lack of validated criteria on which appropriate studies and clinical trials can be based. The classification criteria by the joint European League Against Rheumatism / American College of Rheumatology (EULAR/ACR) consortium[1] were developed to address this problem. The criteria were developed from analysis of data collected over 10 years from 976 IIM and 624 non-IIM patients from multiple centres. An important acknowledged limitation was that the external validation did not include controls or comparators and was therefore able to consider sensitivity but not specificity. Further limitations were the low frequency of myositis-specific antibody (MSA) and MRI testing prompting the authors to call for further validation studies.

The report by Jinnin *et al*[2] is therefore a valuable contribution particularly as it addresses a specific population (Japanese), which was minimally represented in the original study.

The claim made in the title and body of the report that this is the first external validation study is however incorrect. There are other external validation studies[3–7] that precede it and that were on public record prior to the final October 2019 revision date of the report.

Hočevar *et al*[3] and Zhang *et al*[6] had reported their findings in 2018 and May 2019, respectively, and we reported our validation study in Australian patients initially as a preliminary analysis to a domestic audience in May 2018.[4] The final complete analysis was presented to an international audience at the October 2018 American College of Rheumatology Annual Scientific meeting[5] and published in ACR Open Rheumatology online in August 2019.[7]

We found that in our cohort the EULAR/ACR criteria had very high specificity but lower sensitivity, and lower optimal sensitivity and specificity cut points than that suggested in the EULAR/ACR report. We also explored the effect of MRI and an extended panel of myositis-associated antibodies and MSAs and showed in a logistic regression model that including them as covariates of the EULAR/ACR criteria improved the ability to discriminate between IIM and non-IIM patients.

The confidence with which clinicians may use newly developed classification criteria is determined in part by their validation in other populations and ideally drawing on patients from different geographic locations and ethnicity. Hence validation of the initial EULAR/ACR classification criteria for IIM in an Asian population[2] is an important contribution. Our intention here is to alert the readers to the earlier published validation studies that preceded this and help highlight the complete body of evidence that should be considered.

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Response to: 'External validation of EULAR/ACR classification criteria for idiopathic inflammatory myopathies' by Luu *et al*

We would like to thank Luu *et al*[1] for their comments on our recent publication in the *Annals of the Rheumatic Diseases*, entitled 'First external validation of sensitivity and specificity of the European League Against Rheumatism (EULAR)/American College of Rheumatology (ACR) classification criteria for idiopathic inflammatory myopathies with a Japanese cohort'.[2]

We are pleased to know similar validation studies of the new criteria[3–7] have been carried out to strengthen our contention. Although Luu *et al*[1] argue that earlier studies have been published before ours, they were not found with the PubMed search, using the name of the new criteria and validation as keywords, at the time of the submission of our manuscript in April 2019. Presumably, so did the reviewers of our manuscript. Before our publication, Luu *et al* gave their presentation in scientific meetings in 2018,[3 4] which was followed by an official publication in the middle of 2019.[5] It is common that presentations at scientific meetings precede the final and formal publication. The meeting abstracts do not appear in the literature databases, reflecting their possible scientific immaturity. In this regard, we gave oral presentations of our study at the meetings including the 13th International Workshop on Autoimmunity and Autoantibodies held in 2016. Nevertheless, we would like to point out that they made precious suggestion: inclusion of MRI or an extended antibody panel should improve the accuracy of the criteria.

A single-centre study reported by Hočevar *et al*[6] was small in scale and retrospective but valuable. In a correspondence report, they raised the possibility of low sensitivity of the new criteria.

A larger retrospective report by Zhang *et al*,[7] published in May 2019, also described that the new criteria showed high sensitivity and specificity. We agree with their suggestion that 'other DM-associated rashes, such as technician's hand, shawl sign and V area rash, may be included in the classification tree to improve the performance of the criteria in the future'.

Taken together, all of the above studies are greatly informative, suggesting overall high performance of the new criteria and possible difference in sensitivity and specificity among different ethnicities. They also show us what to be done in the future to improve the criteria.

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Successful treatment of plasma exchange for refractory systemic juvenile idiopathic arthritis complicated with macrophage activation syndrome and severe lung disease

We read with great interest the recent article by Saper *et al*[1] describing high mortality of systemic juvenile idiopathic arthritis (sJIA) patients affected by parenchymal lung disease (LD). LD with sJIA has also been associated with macrophage activation syndrome (MAS).[2] While both MAS and LD complicating sJIA are known risk factors for mortality, an effective therapeutic strategy has not been established.[3 4] The present case report highlights an exacerbated LD complication in an sJIA patient treated successfully with additional plasma exchange (PE).

A 5-year-old boy was diagnosed with sJIA when presenting with arthritis, prolonged fever and a skin rash. His white cell count (WCC, 8.5×109/L), C-reactive protein (CRP, 4.8 mg/dL), ferritin (467 ng/mL) and interleukin (IL)-18 (25 453 pg/mL) levels were elevated on diagnosis. Initial treatment of oral prednisolone at 18 mg/day and oral methotrexate at 6 mg/week was insufficient.

Oral cyclosporine was started followed by tocilizumab, but clinical remission was still not achieved. He had no respiratory symptoms, but slight pneumonia on chest CT (figure 1A) during biologic agent therapy change screening. He was switched to canakinumab 10 months after the onset of sJIA.

Two months after starting canakinumab, he developed new fever, arthritis and a mild cough. Vital signs were as follows: temperature, 38.2°C; respiratory rate, 20/min, pulse oximetry, 98% SpO2; room air. A chest X-ray revealed a silhouette sign of the left diaphragm. On admission, blood tests showed a WCC of 10 000/μL with a marked raise in glutamic-pyruvic transaminase (GPT) (63 UI/L), lactate dehydrogenase (LDH) (723 UI/L), ferritin (404 ng/mL) and CRP (3.7 mg/dL). A chest CT (figure 1B) revealed the peripheral septal thickening in the right lobe, and the peripheral septal thickening with pleural thickening in the left lower lobe. A relapse of sJIA with acute pneumoniae was suspected. The patient was treated with administrations of methyl-prednisolone pulse therapy and intravenous immunoglobulin and cyclosporine, followed by oral prednisolone. Moreover, intravenous therapy with meropenem, vancomycin and liposomal amphotericin B was provided. All culture results were negative. (1,3)-beta-D-glucan was within normal limits. Interferon-gamma release assays (IGRAs) and cytomegalovirus (CMV) antigenemia were not detected. His dyspnoea and fever persisted. Additional blood tests showed a WCC of 24 400/μL with GPT (240 UI/L), LDH (1603 UI/L), and hyperferritinemia (32 577 ng/mL), CRP (5.5 mg/dL) and IL-18 (149 269 pg/mL. He also developed hepatosplenomegaly, suggesting progression to MAS. A third chest CT showed progression of consolidation with pleural effusion in both lower lobes, and ground-glass opacities are detected in both upper lobes. (figure 1C) Therefore, PE therapy was performed eight times. After his respiratory condition and fever improved, a progressive pancytopenia occurred. We administered granulocyte-macrophage colony stimulating factor and his neutropenia resolved. At the 2-month imaging follow-up, the chest CT showed decreased degree of peripheral septal thickening and pleural thickening on the left lower lobe (figure 1D). He has remained well, arthritis as well as respiratory condition, for 5 years, and prednisolone (PSL) dose has been reduced to 9 mg/day with infliximab.

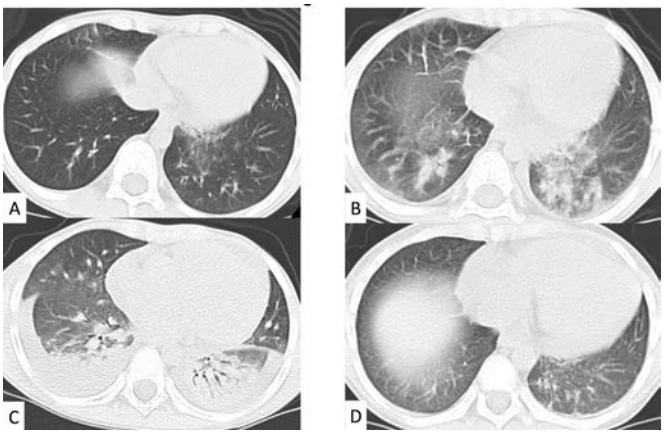

Figure 1 (A) Chest CT showed slight pneumonia. (B) A chest CT revealed the peripheral septal thickening in the right lobe, and the peripheral septal thickening with pleural thickening in the left lower lobe. (C) A chest CT showed consolidation with pleural effusion in both lower lobes, and ground-glass opacities are detected in both upper lobes. (D) Chest CT showed decreased degree of peripheral septal thickening and pleural thickening on the left lower lobe.

Hypercytokinemia plays a key role in the pathogenesis not only of MAS but also LD as complications of sJIA.[2 5] This case was refractory, and clinical remission could not be achieved, despite using IL-1/IL-6 inhibitors. Treatment guidelines and algorithms for MAS in sJIA still require thorough development, especially when conventional treatments are ineffective.[3 4] However, after initiation of PE, the patient improved in this study. PE was effective because it rapidly decreased circulating cytokine levels, such as IL-18.[6] The present case demonstrated that combining immunosuppression and PE can be a useful therapeutic strategy for LD and MAS complicated by hypercytokinemia in patients with sJIA.

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Response to: 'Successful treatment of plasma exchange for refractory systemic juvenile idiopathic arthritis complicated with macrophage activation syndrome and severe lung disease' by Sato *et al*

We were very interested to read the correspondence from Dr. Sato and colleagues describing a case of systemic juvenile idiopathic arthritis (sJIA) with associated diffuse lung disease.[1] The patient in this new report shares a number of features with cases occurring after exposure to interleukin (IL)-1 or IL-6 inhibitors (n=46) that we recently detailed in an international case series.[2] Lung disease complicating sJIA remains a clinical challenge with significant mortality, and we thank the authors for sharing their experience.

One of the typical features of this type of lung disease, as illustrated by this case report, is minimal respiratory symptoms despite significant abnormalities on chest CT. The predominant chest CT pattern in our series, as observed in this case, is septal thickening with or without ground-glass opacities, sometimes together with peribronchovascular consolidation. Also like the patient in this report, a majority of cases in our series had refractory sJIA. However, a significant number (~40%) responded to initial treatment, indicating that refractory sJIA is not a prerequisite for lung disease.[2] Another characteristic, shared by a subset of cases in our series and the current case, is the development of overt macrophage activation syndrome (MAS) at lung disease detection without a prior history of MAS. This finding raises the still unanswered question as to whether parenchymal lung disease stimulates MAS or whether a shared pathway of innate immune dysfunction drives both this new lung disease and MAS.

There are limited data on the efficacy of plasmapheresis for the treatment of MAS and other secondary hemophagocytic lymphohistiocytoses,[3] and this is the first use of this approach in sJIA with lung disease to our knowledge. The possibility that circulating factors drive MAS and lung disease in the reported patient is suggested by his positive response to plasma exchange. Whereas a number of cytokines are strongly implicated in MAS,[4 5] cytokines contributing to the development of lung disease have been suggested but not yet confirmed.[6 7] Notably, a set of circulating proteins, including chemokines associated with T-helper 2 responses, are uniquely found in sera from patients with sJIA and lung disease compared with active sJIA or active MAS.[8] In addition, in our series, progressive lung disease was observed in 17/18 children who had achieved inactive sJIA (on medication), arguing for independent causal factors and possible evolution to a more lung-targeted process.[2]

Prior to the recognition of lung disease, a subset of cases in our series (online supplementary table S8[1]) developed drug reaction with eosinophilia and systemic symptoms (DReSS), an often severe, delayed hypersensitivity reaction. The implicated drugs were IL-1 inhibitors. A patient with sJIA, described by Bader-Meunier *et al* in a recent letter to this journal,[9 10] developed DReSS to canakinumab (monoclonal antibody to IL-1β) before lung disease diagnosis. Our ongoing collection of cases of sJIA with lung disease (unpublished) includes patients with DReSS to IL-1 inhibitors and patients with DReSS to tocilizumab (anti-IL-6 receptor). In the case described by Sato *et al*, the patient was exposed first to tocilizumab and subsequently to canakinumab. The ensuing MAS and liver enzyme elevation are consistent with a delayed-type drug hypersensitivity reaction, which also can include lung involvement and pancytopenia.[11 12] Importantly, stopping canakinumab and removing residual drug by plasma exchange may have contributed to the improved lung status in this child. Cessation of the implicated drug is key to controlling DReSS progression, along with treatment of any ongoing inflammation that persists after drug withdrawal.[13]

Given the multiple treatments used in this case (and other cases of sJIA with lung disease studied to date), it is difficult to assign sources of improvement with certainty. There are reports of patients with sJIA with lung disease improving after discontinuation of the cytokine inhibitors, as well as during their continued use.[1 2 6 9] These varied outcomes may reflect disease heterogeneity, treatment approaches that suppress hypersensitivity reactions or other factors. A key question remains: is parenchymal lung disease that emerges during treatment of sJIA with IL-1 or IL-6 inhibitors triggered by severe delayed drug hypersensitivity, by lung-specific MAS activity or something else? Management decisions hinge on this issue. Organised, controlled testing of treatment strategies, including drug withdrawal and plasma exchange, will contribute to understanding pathogenesis and to identification of best clinical practices.

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Is non-industrial society undergoing an energy balance transition predisposed to accumulate abdominal adipose tissue and susceptible to knee osteoarthritis?

We read with deep interest a recent article published in this journal by lan Wallace *et al*,[1] who found that individuals born under conditions of energetic scarcity who later encounter greater energy abundance are predisposed to accumulate abdominal adipose tissue, making them susceptible to knee osteoarthritis (OA) at lower levels of body mass index (BMI). We really appreciate the work which was done by the authors. However, some worthwhile issues need to be explored.

First, one of the major findings by the authors was that individuals in non-industrial societies undergoing an energy balance transition are inclined to accumulate abdominal adipose tissue and tend to have 'low-BMI, large-abdomen phenotype'. We fully agree with the authors that the Tarahumara had lower BMI compared with the Framingham, as evidence showed in figure 2A.[1] However, figure 2B,[1] the comparison of abdomen sizes in a given weight, was unable to prove that the Tarahumara had a large-abdomen phenotype, and instead, we think a density plot of abdomen size would help to define this issue more convincingly. On the other hand, the differences in abdomen size between Tarahumara and Framingham exhibited in figure 2B[1] could result from the differences in height between the two peoples. Whether or not having experienced energy balance transition, a short person is more likely to have a larger abdomen size than a tall person in a given weight. Additionally, the authors failed to collect data from Tarahumara women, which might lead to overestimation of abdomen size of the Tarahumara, considering that men and women tend to have different fat deposit locations when gaining weight. Overall, by data of this study, it might be not appropriate to conclude that the Tarahumara are predisposed to accumulate abdominal adipose tissue.

Second, the authors did not mention the unexpected negative correlation between probability of knee OA and abdomen size in the Framingham, which was showed in figure 3C,F.[1] Given the known strong association with obesity and knee OA,[2] it is more likely that rising abdomen size will lead to an increase in OA prevalence.

Third, the authors emphasised the contribution of chronic low-grade systemic inflammation to knee OA pathogenesis in the Tarahumara. However, according to the previous study, surrogates for mechanical stress were suggested to be the most important risk factors for OA in weight-bearing joints.[3] Thus, in addition to energy balance transition, the Tarahumara's active lifestyles could be responsible for their high susceptibility to OA.

We respect the great contributions of the authors and we would also be very interested in the authors' response to these issues.

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Response to: 'Is non-industrial society undergoing an energy balance transition predisposed to accumulate abdominal adipose tissue and susceptible to knee osteoarthritis?' by Yu *et al*

Many thanks to Yu and colleagues[1] for their interest in our recent paper on knee osteoarthritis (OA) susceptibility among the Tarahumara, an indigenous population of subsistence farmers in Mexico undergoing rapid lifestyle changes that promote positive energy balance.[2] We welcome their constructive comments and are happy to respond.

As Yu *et al* point out, a key prediction of the model we proposed for knee OA risk among non-industrial societies is that people born under conditions of limited energy availability are prone to accumulate and maintain excess abdominal adipose tissue if they later experience chronic positive energy balance.[3 4] As a result, such people are vulnerable to developing a relatively low body mass index (BMI) but high abdominal adiposity,[5] which we hypothesised puts them at greater risk of knee OA for a given BMI.

Yu *et al* do not disagree that the Tarahumara men we studied had significantly lower BMIs, on average, than the individuals in our comparative sample, urban American men from Framingham, Massachusetts. However, they question whether the Tarahumara had relatively larger abdomens and were prone to accumulating and maintaining abdominal adipose tissue. Average overall body size among the Tarahumara was much smaller than among Framingham individuals, which we needed to account for when comparing abdomen sizes between the two groups. In our paper, we showed that after controlling statistically for body weight and age, Tarahumara's abdomen circumferences were significantly larger than those of Framingham individuals. If both body weight and stature are accounted for, a similar result is obtained: After controlling for body weight, stature and age, Tarahumara abdomen circumferences (adjusted mean, 107.0 cm; 95% CI 105.2 to 108.2 cm) were, on average, larger ($p<0.0001$) than those of Framingham individuals (adjusted mean, 100.2 cm; 95% CI 99.7 to 100.8 cm). Thus, for their body size, the Tarahumara indeed tended to have larger abdomens compared with Framingham individuals.

Two additional lines of evidence further illustrate the propensity of the Tarahumara to accumulate and maintain abdominal adipose tissue under conditions of chronic positive energy balance. First, adipose tissue distribution can be compared among the Tarahumara and Framingham individuals by assessing the ratio of abdomen circumference to hip circumference (figure 1A). After controlling for age, Tarahumara abdomen-to-hip ratios (adjusted mean, 1.01; 95% CI 1.00 to 1.02) were, on average, 2.7% higher (95% CI 1.6% to 3.8%; $p<0.0001$) than those of Framingham individuals (adjusted mean, 0.99; 95% CI 0.98 to 0.99). Controlling for age as well as stature, average Tarahumara abdomen-to-hip ratios (adjusted mean, 1.02; 95% CI 1.00 to 1.04) were 3.4% higher (95% CI 1.2% to 5.7%; $p=0.0030$) than those of Framingham individuals (adjusted mean, 0.99; 95% CI 0.98 to 0.99). The Tarahumara thus had a greater concentration of adipose tissue in their abdomens. Second, anthropometric data we collected from the Tarahumara can be compared with measurements reported by researchers of the Tarahumara working roughly a half century ago, prior to the lifestyle changes that are currently promoting positive energy balance.[6 7] Compared with the Tarahumara in our study, Tarahumara men living in the 1970s had abdomens that were, on average, 13 cm thinner (t test: $p<0.0001$)(figure 1B). However, triceps skin fold thickness was not markedly different among Tarahumara men in the 1970s compared with the Tarahumara in our study (t test: $p=0.76$)(figure 1C), indicating that recent shifts toward chronic positive energy balance have led to greater increases in abdominal than peripheral adiposity.

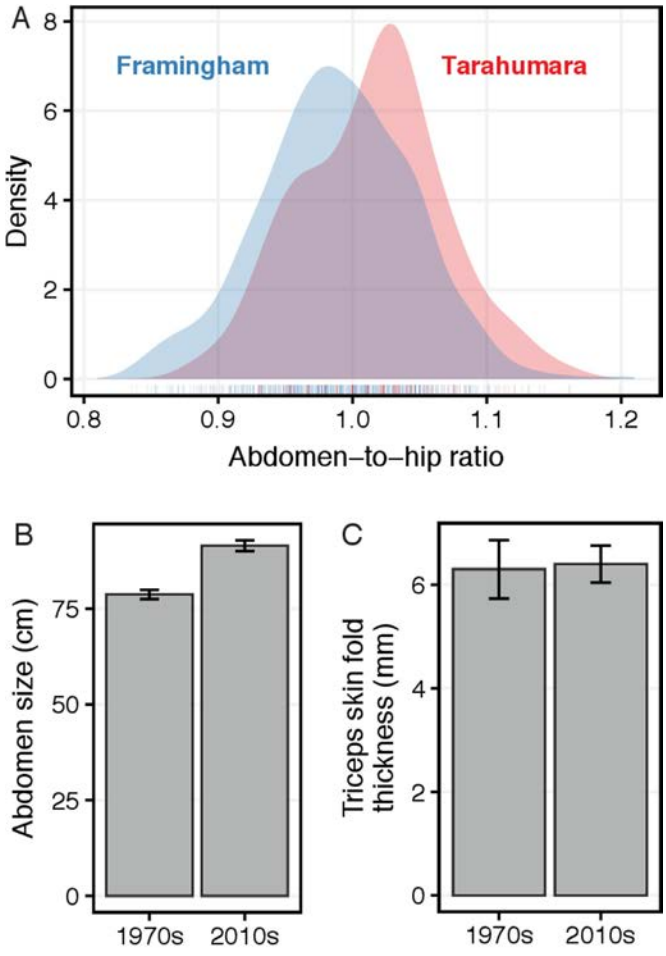

Figure 1 Evidence of the Tarahumara's propensity to accumulate and maintain abdominal adipose tissue under conditions of chronic positive energy balance. (A) Density plot of the ratio of abdomen circumference to hip circumference among the Tarahumara and Framingham individuals in our study. (B) Abdomen size among the Tarahumara in our study compared with a sample of Tarahumara men (n=77) studied in the 1970s.[6] (C) Triceps skin fold thickness among the Tarahumara in our study compared with a sample of Tarahumara men (n=108) studied in the 1970s.[7] Bars in (B) and (C) are group means and whiskers are 95% CIs.

The second issue raised by Yu *et al* concerns our finding that the probability of knee OA increased more markedly with greater abdomen size among the Tarahumara than Framingham individuals, after controlling for body weight and age. Specifically, Yu *et al* consider it unexpected that knee OA probability was not more positively related to abdomen size among the Framingham individuals. This should not be surprising, however, since it has been reported previously that, after adjustment for body weight, abdomen size is not associated with knee OA among Framingham individuals.[8] Studies of other populations have yielded similar findings.[9 10] This is almost certainly because abdomen size and body weight are typically highly correlated and, among some populations, potentially measures of the same risk factor.

Figure 2 Tarahumara subsistence farmer working in a field. Photo by David Ramos and used here with permission.

Intriguingly, however, this is evidently not true for the Tarahumara, for whom abdomen size was a strong risk factor for knee OA independent of body weight. In our paper, we hypothesised that this is because, under conditions of energetic abundance, the adipocytes of people whose metabolic phenotype is adapted to energetic scarcity secrete higher concentrations of proinflammatory adipokines, which has been suggested by experimental studies.[11 12] However, this hypothesis requires further testing.

Finally, Yu *et al* wonder whether the Tarahumara's physically active lifestyles may have been a stronger determinant of knee OA than abdominal adiposity. Lacking good data on physical activity from the Tarahumara, it is difficult to assess this hypothesis rigorously. In our paper, we provided evidence that the Tarahumara are not more prone to either injury-related knee OA or generally greater joint tissue degeneration throughout life compared with Framingham individuals. Conceivably, the postures that the Tarahumara adopt during farming could cause harmful loading of their knees. For example, frequent squatting, kneeling and lifting have been shown to be associated with increased knee OA risk in post-industrial societies,[13] and such behaviours are not uncommon among the Tarahumara (figure 2) or other non-industrial societies.[14 15] Yet, in a previous study in which knee OA levels were compared between prehistoric subsistence farmers in North America and those of modern urban Americans, prevalence of knee OA was found to be half as high among the prehistoric individuals as the modern individuals.[16] Thus, it is unclear whether the activities involved in subsistence farming are inherently bad for knees. Ultimately, as we stated in our paper, we suspect that if Tarahumara activity patterns affected knee OA risk, it was not primarily because their knees sustained loads that were excessively high, frequent or abnormal, but that due to lifestyle changes that promote positive energy balance, knee loading in many individuals occurred in the context of chronic low-grade systemic inflammation that weakened their joint tissues. But this hypothesis remains to be tested.

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Use of tanezumab for patients with hip and knee osteoarthritis with reference to a randomised clinical trial by Berenbaum and colleagues

Publication spin, in the context of randomised clinical trials, is defined as 'use of specific reporting strategies, from whatever motive, to highlight that the experimental treatment is beneficial, despite a statistically nonsignificant difference for the primary outcome, or to distract the reader from statistically nonsignificant results' (p. 2059).[1] In our view, a secondary but clinically important alternative type of publication spin is reliance on statistically significant findings without regard to potential clinical implications of the estimated effects. The American Statistical Association (ASA) has commented on this issue, stating a statistically significant effect does not inform its size or importance.[2] A later editorial more explicitly states that conclusions not be based solely on statistical significance.[3] We believe the recently published trial by Berenbaum and colleagues[4] meets our secondary definition of publication spin and does not meet the recommendation endorsed by the ASA.

Berenbaum and colleagues conducted a three-arm phase III randomised clinical trial of two doses of subcutaneously delivered tanezumab (2.5 vs 5 mg) as compared with placebo, applied to participants with symptomatic hip or knee osteoarthritis (OA).[4] This trial follows two similarly designed trials conducted on persons with symptomatic hip OA[5] or knee OA.[6] Primary outcomes of the 2020 trial[4] were the WOMAC Pain Scale (numeric rating version with scores ranging from 0 to 50 with higher scores equating to more severe pain with activity) and the WOMAC Physical Function Scale (scores ranging from 0 to 170 with higher scores equating to more difficulty with activity). The third primary outcome was the five-item Patient Global Assessment of OA with scores ranging from 1 to 5 with higher scores equating to worse self-reported symptoms and activity limitations.

The abstract reported the following results: 'At week 24, there was a statistically significant improvement from baseline for tanezumab 5 mg compared with placebo for WOMAC Pain (least squares mean difference ±SE –0.62±0.18, p=0.0006), WOMAC Physical Function (–0.71±0.17, p<0.0001) and PGA-OA (–0.19±0.07, p=0.0051). For tanezumab 2.5 mg, there was a statistically significant improvement in WOMAC Pain and Physical Function, but not PGA-OA' (p. 1). The conclusion was 'tanezumab 5 mg statistically significantly improved pain, physical function and PGA-OA, but tanezumab 2.5 mg only achieved two co-primary end points' (p. 1).

Treatment effects on primary outcomes were assessed by comparing changes from baseline to a 24-week follow-up. Mean differences among the three arms was less than one point for all primary outcomes. For WOMAC Pain, mean differences were 0.61 or less compared with placebo, for WOMAC Physical Function, 0.71 points or less compared with placebo and for the global rating measure, 0.18 points or less. Given that the WOMAC Pain Scale ranges from 0 to 50 points and the WOMAC Physical Function Scale ranges from 0 to 170, changes of 0.71 points or less are, in our view, clinically irrelevant. The findings indicate the expected difference is smaller than a patient shifting a response on a single item by one point. We found no discussion of the potential clinical impact of these findings in the paper.

Effects on six secondary outcomes were reported, but our concern with these estimates was that the trial registry listed 59 secondary outcomes with most of these including multiple measures compared over multiple time points. The large number of outcomes tested leads to inflated type I error rates and questions if results were chosen based on the significance.

The adverse effects of tanezumab were quantified in a variety of ways. The abstract emphasised more serious adverse events including rapidly progressive OA and joint replacement. The investigators reported that 'rapidly progressive osteoarthritis (RPOA) was observed in 1.4% (4/283) and 2.8% (8/284) of patients in the tanezumab 2.5 mg and tanezumab 5 mg groups, respectively and none receiving placebo. Total joint replacements (TJRs) were similarly distributed across all three treatment groups (6.7%–7.8%)' (p, 1). Given the hypothesised mechanism of action of tanezumab,[7] the rare but serious complication of rapidly progressive OA in the active arms and the lack of clinical meaningfulness of the findings, our interpretation differs from that of the investigators. This study indicates to us that tanezumab does not show promise as an effective treatment alternative to more traditional medication for painful knee or hip OA.

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Response to: 'Use of tanezumab for patients with hip and knee osteoarthritis with reference to a randomised clinical trial by Berenbaum and colleagues' by Riddle and Perera

We thank Riddle and Perera[1] for their interest in our publication.[2] In our article, we adhered to recognised statistical methods and appropriate association with meaningfulness. We agree with the American Statistical Association guidance that a statistically significant effect does not inform its size or importance,[3] and therefore, a change needs to be associated with clinical impact.

In this study (ClinicalTrials.gov: NCT02709486), Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) Pain subscale and Physical Function subscale were measured using an 11-point numeric rating scale (specifically, the WOMAC NRS3.1 V5 US). As specified in the WOMAC User Guide, each WOMAC subscale is to be summed and can be normalised on a 0–10 scale for the numeric rating scale. Therefore, in this study, the five questions for the Pain subscale and the 17 questions for the Physical Function subscale were summed and averaged, respectively, to determine each mean subscale score. This differs from subscale scores that are not normalised and reported as 0–50 (Pain)[4] or 0–170 (Physical Function) from the 11-point WOMAC numeric rating scale, as suggested by Riddle and Perera.

Our interpretation of the data in the discussion was deliberately cautious in this article, noting that the effect size as assessed by WOMAC Pain was modest and that there were limitations in the trial. We reported that, based on the change from baseline in WOMAC Pain at week 24, the effect size (placebo-adjusted least squares mean change divided by model-based SD) for tanezumab 2.5 mg and 5 mg dose regimens was 0.24 (0.46/1.93) and 0.32 (0.62/1.93), respectively. These are both above the suggested lower threshold for meaningfulness of 0.20, proposed as the lower bound for a small effect (or change in pain).[5] In the discussion, we specifically noted the possibility of a reduced treatment response to tanezumab compared with earlier tanezumab studies that used intravenous administration, and that an administration-route effect cannot be excluded, but there were also differences in patient populations.

The details of analyses for this study were prespecified in the statistical analysis plan before unblinding. With respect to efficacy outcomes, we prioritised multiple outcomes as coprimary, key secondary and (other) secondary outcomes, and applied a gatekeeping approach to control the family-wise type I error of 0.05 for primary and key secondary outcomes. In other words, we evaluated the success of this clinical trial based on the results of these coprimary and key secondary outcomes from an efficacy perspective while controlling the family-wise type I error of 0.05, and other secondary outcome results (eg, point estimate, CI, nominal p value) were evaluated as supportive information. The six secondary endpoints reported in this article were the three key secondary endpoints and three additional secondary endpoints related to one of the key secondary endpoints.

We believe this study demonstrated tanezumab has a sufficiently favourable risk–benefit profile in patients with moderate-to-severe, difficult-to-treat osteoarthritis, for whom acetaminophen, nonsteroidal anti-inflammatory drugs and opioids were inadequate or unsuitable. A long-term, active-controlled study (NCT02528188) will provide more data to further characterise the risk–benefit of tanezumab in patients with osteoarthritis.

Francis Berenbaum ,[1] **Francisco J Blanco** ,[2] **Ali Guermazi**,[3] **Kenji Miki**,[4] **Takaharu Yamabe**,[5] **Lars Viktrup**,[6] **Rod Junor**,[7] **William Carey**,[7] **Mark T Brown**,[5] **Christine R West**,[5] **Kenneth M Verburg**[5]

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JAK inhibitors as promising agents for refractory Takayasu arteritis

I read with great interest the article 'Successful remission with tofacitinib in a patient with refractory Takayasu arteritis complicated by ulcerative colitis' by Kuwabara *et al*.[1] In this article, the authors reported a patient with both Takayasu arteritis (TAK) and ulcerative colitis (UC) who was successfully treated with tofacitinib, an inhibitor of Janus kinase (JAK). The critical message of this report is that, although inhibitors of tumour necrosis factor α and interleukin (IL)-6 failed to induce remission, symptoms and arterial inflammation on imaging were promptly ameliorated by tofacitinib.[1] Because our group reported the efficacy of JAK inhibitors on experimental large-vessel vasculitis in mice for the first time,[2] I would like to comment on this report.

First, UC is not an uncommon complication in TAK, with a complication rate of approximately 6%.[3 4] However, it has been reported that TAK patients with UC have a different genetic background from TAK patients without UC in HLA-B52:01 positivity, and that the age of TAK onset in the former group is younger than that in the latter.[4] Thus, it may be possible that JAK inhibitors are more likely to be effective in patients with both diseases. It is necessary to test whether JAK inhibitors are efficacious in TAK patients without UC as well.

Second, a genome-wide association study revealed *IL-12B* as a susceptibility gene in TAK, and IL-12 plays a critical role in T helper 1 (Th1) differentiation.[5 6] In addition, patients with TAK have a higher serum concentration of IL-23 than healthy individuals, and IL-23 promotes IL-17 production by $CD4^+$ T cells.[7 8] Both IL-12 and IL-23 are critically involved in the pathophysiology of TAK and activate JAK2 and Tyk2.[9] Because tofacitinib primarily inhibits JAK1 and JAK3, baricitinib, an inhibitor of JAK1 and JAK2, may be a better option in some patients.

Third, the authors seem to believe that the efficacy of tofacitinib is mediated by blocking Th1-derived and Th17-derived cytokines. However, JAK inhibitors, including tofacitinib, target not only $CD4^+$ T cells but also macrophages and natural killer cells,[2 10] which have recently emerged as a promising target in TAK.[11] Because TAK is a multifactorial disease in which many cytokines and cell populations interact with each other in the disease mechanism, multicytokine blockade with JAK inhibitors rather than single cytokine inhibition may be reasonable.

Whether JAK inhibitors can be considered as first-choice immunosuppressive agents added to glucocorticoids (GCs) or even an alternative to GCs for TAK remains unclear. However, although there are some concerns with JAK inhibitors, such as herpes zoster and malignancies, given the significant burden of GCs on patients with TAK, I believe this issue should be discussed in the future.

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Response to: 'JAK inhibitors as promising agents for refractory Takayasu arteritis' by Watanabe

We thank Watanabe for his interest in our case report showing that tofacitinib, a janus kinase (JAK) inhibitor, successfully induced a remission of Takayasu arteritis (TAK) complicated by ulcerative colitis (UC)[1] and for providing some meaningful comments to supplement our discussion.[2] Watanabe wonders if JAK inhibitors can be effective in TAK patients without UC. In addition, he is concerned that types of JAK inhibitors may affect the efficiency in treating TAK. We agree that the issues should be evaluated in a large-scale case series and clinical study. According to a limited number of case reports, JAK inhibitors were effective in TAK regardless of coexisting UC and types of JAK inhibitors. Sato *et al* reported a similar case of TAK with UC successfully treated with tofacitinib (JAK1/JAK3 inhibitor).[3] On the other hand, Régnier *et al* reported that ruxolitinib or baricitinib (JAK1/JAK2 inhibitors) clinically improved TAK without UC in three patients.[4] They also demonstrated that the improvement was along with decrease of Th1/Th17-related cytokines and correction of effector/regulatory T-cell imbalance. However, as suggested by Watanabe, JAK inhibitors could modulate innate immunity composed of macrophages and natural killer cells in TAK.

For the management of refractory TAK, EULAR recommends considering tumour necrosis factor (TNF) inhibitor or tocilizumab,[5] although the evidence for TNF inhibitor depends on only open-label studies,[6] and a randomised controlled trial of tocilizumab failed to achieve its primary endpoint.[7] Tocilizumab rapidly normalises IL-6-driven serum inflammatory markers despite sustained vessel inflammation.[1 8] It can be confusing when the disease activity of TAK is correctly assessed. Thus, additional treatment options with clear evidence have been desired for TAK. To conclude, we believe that further studies should be conducted in refractory TAK for evaluating the efficacy and safety of JAK inhibitors as a promising agent.

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