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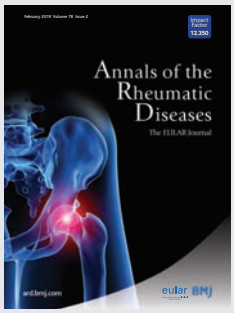

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Tapering Janus kinase inhibitors in rheumatoid arthritis with low disease activity or remission: reality or dream?

Jasvinder A Singh[1,2,3]

In this issue of the journal, Takeuchi *et al* presented results of a baricitinib long-term extension study of patients who received baricitinib 4 mg for ≥15 months and maintained a clinical disease activity index (CDAI) low disease activity (LDA; CDAI <10) or remission (CDAI ≤2.8) for ≥3 months.[1] Patients with rheumatoid arthritis (RA) were randomised to baricitinib tapering to 2 mg daily dose versus continuing the baricitinib 4 mg daily.

Patients in this study had a mean (SD) age of 54 (12) years, 75% were female, 75% were anticyclic citrullinated protein antibody (ACPA) positive, 75% were rheumatoid factor positive, 46% on concomitant glucocorticoids, 82% were on concomitant methotrexate, one-third each had previously failed one or two traditional disease-modifying antirheumatic drugs (DMARDs), but only 13% previously failed a biologic. Patients had one swollen and one tender joint count, and the CDAI score was 3.6 (SD, 2.8), just before tapering.

The rates of LDA (67% vs 80%) and remission (33% vs 40%) at 48 weeks and non-serious infection rates (24.9 vs 30.6) were lower and relapse (CDAI score >10; 37% vs 23%) and rescue rates (18% vs 10%) higher in baricitinib 2 mg (tapering) vs 4 mg (continuing) daily dose groups. Among the rescued patients, most people who lost response (up to two-thirds) could regain the LDA or remission within 24 weeks after rescue to baricitinib 4 mg daily, 67% for the 2 mg group vs 54% for the 4 mg group. Compared with 4 mg daily dosing, baricitinib dose reductions to 2 mg daily were associated with statistically significant increase in CDAI, simplified disease activity, disease activity score (DAS) and earlier relapse. Among DMARD-incomplete responder (IR) patients who had achieved remission at step-down baseline, the majority maintained remission in both dose groups, 56% vs 68% in the baricitinib 2 mg vs 4 mg group. The authors acknowledge major study limitations, including the lack of radiographs, only a 48-week follow-up and smaller numbers for important subgroups of patients (ie, DMARD-naïve, biological DMARD IR).[1] The authors did not perform analyses of specific patient or disease characteristic/s predicting the risk of losing LDA or remission during baricitinib tapering.

SO, WHAT ARE THE STUDY IMPLICATIONS FOR BARICITINIB TAPERING IN RA?

This study showed in patients with RA (primarily with previous conventional DMARD failure) being treated with baricitinib 4 gm daily dose who were concurrently on methotrexate (MTX) (mean dose 15 mg/week) with/without glucocorticoids, tapering to baricitinib 2 mg dose led to statistically significantly lower LDA rates up to 48 weeks follow-up, that is, 10%–13% fewer patients had LDA in the group tapering baricitinib to 2mg dose compared with those continuing at 4mg dose. Up to two-thirds of the patients with RA who relapsed could regain their LDA or remission within 24 weeks after rescue with baricitinib 4 mg dose. In those with previous conventional DMARD failure who were in remission at baseline, 15% fewer and 12% fewer of those being tapered maintained remission at 24 and 48 weeks, respectively, compared with 4 mg dose continuers.

To me, this indicates that patients in remission with baricitinib 4 mg daily dose who have taken this medication for >1 year can attempt baricitinib dose tapering while continuing their MTX (±glucocorticoids) regimen, with some risk (10%–20%) of loss of LDA or remission state, but two-thirds regain that state with baricitinib dose escalation. This is an important finding since patients frequently consider and try RA medication tapering or discontinuation (with and without provider knowledge). The cost of lifelong therapy with biologic or Janus kinase inhibitors in RA is high, and there are few associated risks of treatment. This study provides robust data to support baricitinib tapering in patients who desire it, with some risk of loss of remission state.

WHAT ARE THE STUDY IMPLICATIONS FOR DMARD/BIOLOGIC TAPERING IN RA? WHERE DOES THIS FIT IN THE SPECTRUM OF EVIDENCE?

Edwards *et al* performed a systematic review of 52 papers of biologics across various rheumatic conditions and concluded that remission is typically not sustained in patients who discontinue biologic therapy.[2] The relapse rates and flare in people discontinuing biologic was moderate to high in people with early RA (48%–54%) and established RA (2%–84%).[2] In many cases, an acceptable disease activity could be regained on retreatment; however, 19%–100% of the patients regained disease remission,[2] a very wide range that represents significant clinical uncertainty. In another systematic review of 11 studies of biologics in RA, the authors found that dosing down of biologic may be an option in many patients who have achieved remission or LDA.[3] A key limitation of the current evidence is the inability to predict which patient with RA will succeed in DMARD/biologic tapering without flare and without the loss of current LDA/remission state.

A recent review highlighted potential factors associated with successful tapering, but the evidence for each factor is based on one to few studies. The presence of deep remission state (DAS28 of 2.2 or lower) prior to tapering DMARDs in people with remission, shorter duration of RA (early RA), a longer duration of remission state, a more rapid response to DMARDs, absence of serum markers of inflammation (acute-phase reactants, cytokines and metalloproteinases) and ACPA negativity may each be associated with a higher likelihood or remission maintenance with tapering of biologic and/or synthetic DMARDs.[4] The presence of synovitis detected by the ultrasound was a predictor of failure of successful tapering of biologics in three studies.[5–7]

The 2015 American College of Rheumatology (ACR) guideline for the treatment of RA *conditionally recommended* tapering of biologics, traditional DMARDs or Janus kinase inhibitor (only tofacitinib was approved for RA at the time of guideline formulation) versus not tapering the

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respective medications in patients with RA in disease remission[8] and *conditionally against* tapering these medications in with those with LDA. The ACR RA guideline defined DMARD/biologic tapering as scaling back therapy one medication at a time, by reducing dose or dosing frequency and recommended conducting it slowly and carefully, watching for increased disease activity and flares.[8] The ACR guideline also recommended that even in remission, all the drugs should not be stopped at the same time, a **strong recommendation**.[8] The 2017 European League against Rheumatism (EULAR) recommendations for the management of RA state that if a patient with RA is in deep remission after tapering glucocorticoids, then biologic can be tapered especially if it is combined with a traditional DMARD.[9] Thus, both ACR and EULAR guidelines for RA management allow for gradual tapering of DMARD/biologic in people with remission, watching carefully for disease flare and the loss of remission state.

WHAT IS THE TAKE HOME MESSAGE?

Tapering of DMARDs, biologics and Janus kinase inhibitors in patients with RA is a reality in those in sustained, deep RA remission, and on combination therapy with traditional DMARD. This decision should be made in line with patient values and preferences, balancing cost/safety against the possibility of RA flare and loss of RA remission which can be regained in the majority by restarting or increasing the dose, but not all patients. Thus, the uncertainty of the potential loss of remission must be acceptable to patients attempting Janus kinase inhibitor tapering and a shared decision-making approach is critical, to avoid mismatched expectations. A key factor to consider is that if the patient is on concomitant glucocorticoids, they should be tapered first, considering the risk–benefit ratio. As more evidence is generated with longer follow-up studies, patients and providers can make more informed decisions about tapering biologics and/or DMARDs. But, let's not forget concomitant glucocorticoids, which should be tapered first. More research is need to address if biologic or Janus kinase inhibitor drug holidays would be preferable to their discontinuation.

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Management of antiphospholipid syndrome

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ABSTRACT

Antiphospholipid syndrome, also known as 'Hughes Syndrome', is an autoimmune disease characterised by a set of clinical manifestations, almost all of which are direct or indirect sequelae of a hypercoagulable state involving the venous, and to a lesser extent the arterial vasculature. The incidence and prevalence of antiphospholipid syndrome are estimated at approximately 5 de novo cases per 100 000 per year and 40–50 cases per 100 000 individuals, respectively. The clinical spectrum of antiphospholipid syndrome involves haematological (thrombocytopaenia, venous thrombosis), obstetrical (recurrent pregnancy loss), neurological (stroke, transient ischaemic attack, migraine, seizures, cognitive dysfunction, chorea, transverse myelitis, multiple sclerosis), cardiovascular (cardiac valve disease), dermatological (livedo reticularis and racemosa, skin ulceration and necrosis), renal (glomerulonephritis, renal thrombotic microangiopathy) and orthopaedic (avascular necrosis of bones, non-traumatic fractures) manifestations, among others. In addition to the classical antiphospholipid antibodies, namely anticardiolipin antibodies and lupus anticoagulant, new autoantibodies and antibody complexes of different immunoglobulin subtypes (IgA, IgG, IgM) are now recognised as significant contributors to the pathogenesis of antiphospholipid syndrome. Anticoagulation remains the cornerstone in the management of antiphospholipid syndrome; nevertheless, new drugs and therapeutic strategies are being tested, and some have been found effective for the primary and secondary thromboprophylaxis in antiphospholipid syndrome.

INTRODUCTION

The literature on antiphospholipid syndrome (APS) has been undergoing an exponential growth since its first description in 1983[1] (initially known as anticardiolipin syndrome). Several case reports and series of patients with recurrent spontaneous miscarriages, thromboembolic events and positive serology were documented before 1983 and prepared the ground for the discovery of APS.[2–5] The original description of APS included a clinical triad of recurrent miscarriages, central nervous system disease and recurrent deep venous thrombosis (DVT) in patients with systemic lupus erythematosus (SLE) with seropositive anticardiolipin antibodies (aCL) and lupus anticoagulant (LAC).[1] Since then, numerous clinical manifestations and laboratory findings have been consistently added to the list of classification and non-classification criteria of the disease. International, large-scale epidemiological studies[6] and clinical trials[7 8] provided evidence-based knowledge about APS. Many meetings, workshops and symposia were organised, most notably the Sapporo workshop in 1998[9] and the Sydney workshop in 2004,[10] which culminated in an international consensus on the classification criteria of APS. Recently, the enthusiasm and commitment of APS experts have led to the establishment of the APS Alliance for Clinical Trials and International Networking (APS ACTION), the primary goal of which is to further the understanding and management of the disease.[11]

CLINICAL PRESENTATION OF APS

APS has an expanding range of clinical manifestations. Although up to 5% of the population might be positive for antiphospholipid antibodies (aPL),[12] only a small fraction are diagnosed with APS. On the other hand, aPL prevalence rates in non-APS patients with stroke, myocardial infarction, DVT and pregnancy morbidity are much higher, reaching 13%, 11%, 9.5% and 6%, respectively.[13]

Thrombosis and pregnancy morbidity are the two hallmarks of APS.[14] Although venous thromboembolism is the most frequent manifestation, thrombotic events in APS may also occur in virtually any vascular bed, with the cerebral circulation being the arterial territory most commonly affected, usually in the form of stroke or transient ischaemic attacks. The most common obstetrical manifestation of APS is recurrent early miscarriage, usually before 10 weeks of gestation (WG). Placental insufficiency in later gestation periods, manifested as fetal growth restriction, early (<34 WG) pre-eclampsia and fetal death, is characteristic of APS, and its occurrence should prompt the evaluation of the mother for the presence of aPL.

APS has been associated with many other clinical features,[15] most of them, though not all, with a putative thrombotic pathogenetic substrate. These include, livedo reticularis, epilepsy, heart valve lesions or thrombocytopaenia, among others. The most severe and fortunately infrequent form of APS is the so-called catastrophic APS (CAPS), characterised by widespread small vessel thrombosis with multiorgan failure and high associated mortality.[16] Boxes 1 and 2 depict the criteria and extra-criteria clinical manifestations of APS, respectively.

LABORATORY PRESENTATION OF APS

The documentation of aPL positivity has been an essential component in the APS diagnostic—not only classification—approach for patients who are suspected to have the disease. aCL, LAC and anti-β2 glycoprotein-I antibody (anti-β2GPI) should test repeatedly positive at medium-to-high titres. During the last decade, a huge body of basic and clinical research on this topic unveiled several novel autoantibodies,[17] of which the exact role in APS pathogenesis and significance in clinical risk

Box 1 A comprehensive list of organ system-based, criteria clinical manifestations of antiphospholipid syndrome

Criteria clinical manifestations of the antiphospholipid syndrome.
Neurological:
- Cerebral venous thrombosis
- Multi-infarct dementia
- Stroke
- Transient ischaemic attack

Ophthalmic:
- Amaurosis fugax
- Optic neuropathy
- Retinal artery/vein thrombosis

Endocrine:
- Adrenal infarction

ENT:
- Nasal septum ischaemia/perforation

Cardiac:
- Intracardiac thrombus
- Myocardial infarction

Obstetrical:
- ≥1 unexplained fetal* death ≥10 WG
- ≥1 premature birth* at *or* <34 WG due to:
  - Severe pre-eclampsia
  - Eclampsia
  - Severe placental insufficiency
- ≥3 unexplained consecutive spontaneous abortions† at *or* <10 WG

Pulmonary:
- Pulmonary embolism
- Pulmonary artery thrombosis

Gastrointestinal:
- Budd-Chiari syndrome
- Oesophageal ischaemia
- Hepatic vein thrombosis
- Mesenteric ischaemia
- Pancreatic infarction
- Splenic infarction

Renal:
- Renal artery/vein thrombosis

Dermatological:
- Digital gangrene

Vascular‡:
- Arterial/Venous thrombosis (upper extremity)
- Arterial thrombosis (lower extremity)
- Deep vein thrombosis
- Jugular vein thrombosis
- Subclavian vein thrombosis
- Superficial venous thrombosis/thrombophlebitis

*Confirmed normal morphology.
†Absence of maternal anatomical/hormonal and maternal/paternal chromosomal abnormalities.
‡Any other vessel is at risk of developing thrombotic disease.
ENT, ear, nose and throat; WG, weeks of gestation.

Box 2 A comprehensive list of organ system-based, extra-criteria clinical manifestations of antiphospholipid syndrome

Extra-criteria clinical manifestations of the antiphospholipid syndrome.
Neurological:
- Acute encephalopathy
- Cerebellar ataxia
- Chorea
- Cognitive dysfunction (in the absence of cerebral thrombosis)
- Epilepsy and seizures
- Guillain-Barre syndrome
- Hemiballismus
- Migraine
- Multiple sclerosis-like lesions
- Sensorineural hearing loss
- Transverse myelitis

Cardiac:
- Angina
- Cardiac valve disease
- Valve thickening
- Valve dysfunction
- Cardiomyopathy

Obstetrical:
- Late pre-eclampsia
- Late premature birth
- Placental abruption
- 3 non-consecutive miscarriages
- 2 unexplained miscarriages
- ≥2 unexplained in vitro fertilisation failures

Pulmonary:
- Alveolitis with alveolar haemorrhage
- Fibrosing alveolitis
- Pulmonary hypertension

Renal:
- Glomerulonephritis:
  - Membranous
  - Proliferative
- Thrombotic microangiopathy

Dermatological:
- Livedo reticularis
- Livedo racemosa
- Pseudovasculitic lesions
- Skin ulceration and necrosis
- Splinter haemorrhages

Vascular:
- Accelerated atherosclerosis
- Arterial stenosis (renal, coeliac, cerebral and so on)

Haematological:
- Evans syndrome
- Haemolytic anaemia
- Thrombocytopaenia

Musculoskeletal:
- Arthralgia
- Arthritis
- Avascular necrosis of bone
- Bone marrow necrosis
- Non-traumatic fractures

assessment are not clearly elucidated yet. Many new antibodies have been proposed so far; antidomain I β2GPI (anti-β2GPI DI) and anticomplex phosphatidylserine-prothrombin (anti-PS/PT) are the two most promising to become clinically relevant aPL.[18] Moreover, while aPL positivity has always been critical to diagnose APS, a new entity—seronegative APS (SNAPS)—was introduced in 2003.[19] Patient candidates for the diagnosis of SNAPS show several clinical manifestations suggestive of APS, with persistently negative aCL, anti-β2GPI and LAC, but not for

the extra-criteria aPL. It has been shown in a recent study that around one-third of such patients are seropositive to at least one alternative aPL, including anti-PS/PT in 12% of patients but not anti-β2GPI DI, which was however positive in 27% of 'seropositive' patients.[20] Another small series tested 40 patients with APS meeting the clinical and serological criteria (group 1) for five extra-criteria aPL, namely IgG, IgM and IgA anti-β2GPI DI, IgA aCL and IgA anti-β2GPI, and compared the results with another group of patients meeting the clinical but not the serological criteria for APS (group 2). Interestingly, 62.5% of patients in group 1 were positive for at least one extra-criteria aPL, whereas 10% of group 2 were positive for one of the extra-criteria aPL. Specifically, three patients (7.5%) were positive for IgG anti-β2GPI DI and one patient (2.5%) was positive for IgA anti-β2GPI.[21] The authors in this study defined the normal cut-off value of IgG anti-β2GPI DI as 10 absorbance units (AU); however, the positive values were actually borderline (16 AU, 15.3 AU and 22.2 AU) and may not be taken for granted as positive values since these new assays, including IgA anti-β2GPI, are still used as research kits and normal cut-off values are yet to be defined.[22] Noteworthy, a much higher prevalence of anti-β2GPI DI is reported in patients with seropositive APS (45.4%),[23] and domain I seems to be much more prevalent (66%) compared with other domains (IV/V) of anti-β2GPI (22%).[24] Intriguingly, it seems that the anti-β2GPI epitope specificity profile (domain I vs domain IV/V) may predict future APS complications. Higher risk of recurrence of vascular and obstetrical APS was found to be associated with anti-β2GPI DI[23 25 26] but not as much with domain IV/V,[24 26] and a ratio of antidomain I to antidomain IV/V of more than or equal to 1.5 was found to be predictive of systemic autoimmunity.[24]

aPL: from disease markers to risk assessment

In an attempt to assess the risk of developing the clinical manifestations related to vascular thrombosis and pregnancy morbidity in patients with APS, the first Risk Scale for the diagnosis of APS was proposed in 2011.[27] The study was successful in pointing out that triple aPL positivity substantially increases the risk of APS, and LAC is more strongly associated with APS diagnosis compared with other aPL.[27] This pilot model was followed by the Antiphospholipid Score model, which is calculated by a formula based on the relative risk or OR of having clinical manifestations of APS for different aPL testing methods.[28] While these two models were solely based on aPL as disease markers, a quantitative model, the Global APS Score, was developed to account for other independent, yet significant conventional cardiovascular risk factors that are related to thrombosis and pregnancy morbidity, namely hyperlipidaemia and arterial hypertension.[29] As for long-term survival and quality of life of patients with APS, a new disease-specific cumulative damage index in patients with thrombotic APS has been proposed to evaluate principal APS manifestations that are associated with a worse prognosis and organ damage.[30]

MANAGEMENT

The management of APS has been in continuous evolution over the last 30 years. Over this time, we have learnt that a number of different variables, such as the aPL profile (number of different positive antibodies, serum levels of aPL and persistence of positivity over time), the site of thrombosis (arterial vs venous vs small vessel disease) and the concurrence of additional cardiovascular risk factors, may substantially modify the clinical profile and thus the occurrence of both first and recurrent thrombosis. The last consensus guidelines were published back in 2011, after the 13th International Congress on aPL, held in Galveston in 2010.[31] Updated consensus recommendations by the European League Against Rheumatism Task Force are now under way. The current trend is to design tailored treatment strategies taking into account individual risk assessments.[32] Figure 1 depicts a management plan for persistently aPL-positive patients.

Primary thromboprophylaxis

Despite the lack of appropriate studies addressing its efficacy, cardiovascular risk control is considered the necessary background to any pharmacological—primary or secondary—thromboprophylaxis.[31] Low-dose aspirin (LDA) at 75–100 mg/day is recommended in high-risk, aPL-positive patients[31] and was proven to have similar efficacy, although safer, compared with a combination primary thromboprophylactic regimen of LDA and low-intensity warfarin.[33] The results of a recent meta-analysis including 11 observational studies reinforce the role of LDA as a preventive therapy in asymptomatic aPL-positive individuals (HR: 0.50; 95% CI 0.27 to 0.93); the effect was significant for arterial thrombosis (HR: 0.48; 95% CI 0.28 to 0.82) and borderline for venous thrombosis (HR: 0.58; 95% CI 0.32 to 1.06). Significant risk reductions were seen among asymptomatic aPL carriers, patients with SLE and women with obstetrical APS.[34] A second meta-analysis using individual patient-level data, including 5 of the above 11 cohorts, confirmed the global protective role of LDA against thrombosis (HR: 0.43; 95% CI 0.25 to 0.75). Similar effects were seen after adjustment for gender, age, centre, presence of cardiovascular risk factors, type of aPL and treatment with hydroxychloroquine (HCQ). Furthermore, the risk reduction was the same (0.43) for patients with and without SLE.[35] The role of HCQ in preventing thrombosis has been shown in several studies involving patients with lupus with and without aPL.[36 37]

Therefore, primary thromboprophylaxis with LDA is recommended for asymptomatic individuals and women with purely obstetrical APS with high-risk aPL profile, particularly in the presence of cardiovascular risk factors. HCQ is the primary prophylactic agent in patients with SLE, in whom the addition of LDA should be considered in patients with persistently positive aPL, more so in those triple-positive and with cardiovascular risk factors. Also, preventive measures including thromboprophylaxis with low molecular weight heparin (LMWH) should be taken in high-risk individuals such as postsurgical, postpartum and immobilised patients.

Secondary thromboprophylaxis

The initial management of thrombotic events in patients with APS is frequently similar to the general population until persistent aPL has been demonstrated. According to current recommendations, patients with venous thromboembolism are best treated with standard-intensity oral anticoagulation at a target international normalised ratio (INR) of 2.0–3.0.[31] The duration of anticoagulation, however, has been a subject of debate; some authors have suggested 3–6 months of anticoagulation in patients with a first venous thromboembolic event who are known to have a transient/reversible precipitating factor and in whom aPL becomes negative over time.[38] However, recent data in 241 patients with a first unprovoked venous thromboembolism have shown a borderline significantly increased risk for recurrent events after stopping anticoagulant therapy in those with aPL (HR: 1.8; 95% CI 0.9 to 3.6).[39] Compared with aPL-negative patients, the rates of recurrence further increased in patients with the same type

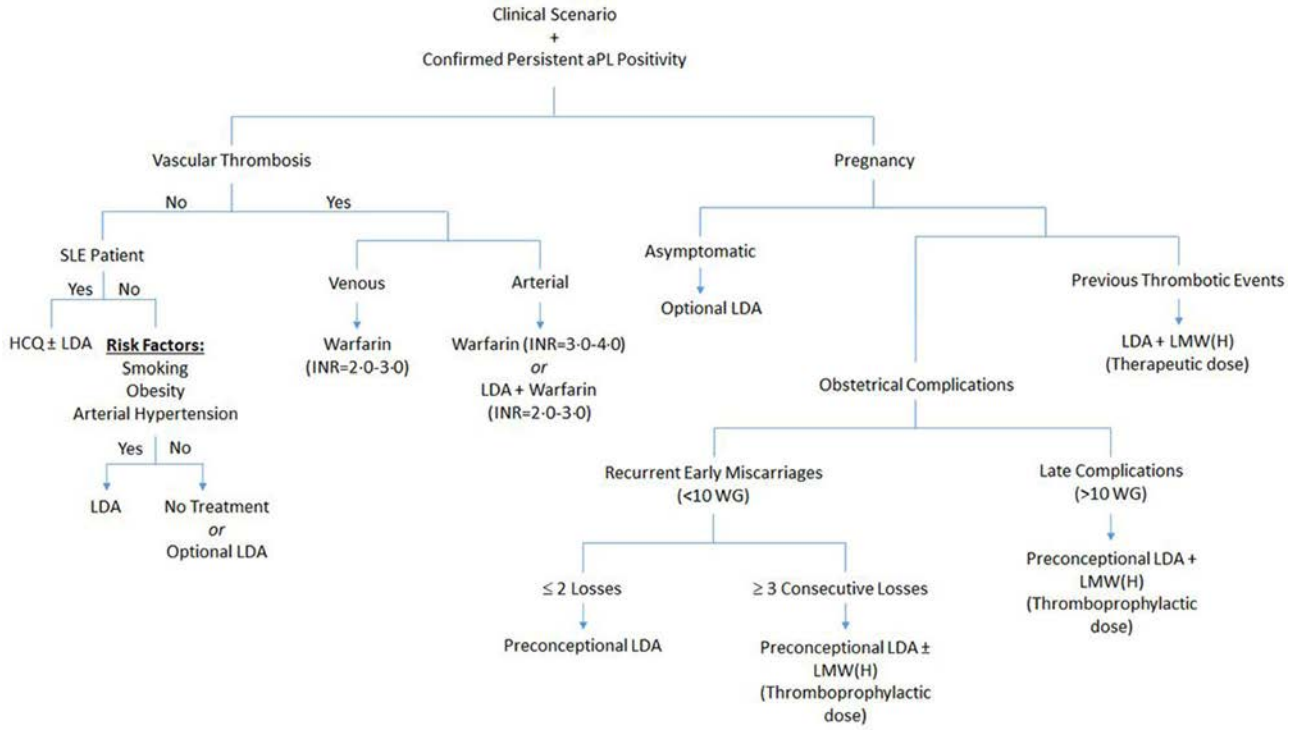

Figure 1 Management plan for persistently aPL-positive patients. The management plan of persistently aPL-positive patients consists of two arms: (1) primary/secondary thromboprophylaxis and (2) prophylaxis against and treatment of obstetrical complications. Patients with arterial thrombosis are considered high risk and should be managed with more aggressive anticoagulation or a combination of anticoagulation and LDA. HCQ is recommended in aPL-positive SLE patients. Preconceptional LDA with or without LMW(H) at thromboprophylactic doses is/are recommended in aPL-positive, pregnant women presenting with obstetrical complications, whereas therapeutic doses are reserved for those with history of thrombotic events. aPL, antiphospholipid antibodies; HCQ, hydroxychloroquine; SLE, systemic lupus erythematosus; LDA, low-dose aspirin; LMW(H), low molecular weight heparin/heparin; INR, international normalised ratio; WG, weeks of gestation.

of positive aPL on the two occasions tested (HR: 2.7; 95% CI 1.1 to 6.7) and in patients with two or three types of aPL on the same or different occasions (HR: 4.5; 95% CI 1.5 to 13.0). The increased risk of recurrence was independent of the results of the D-dimer test. Thus, these results support long-term anticoagulation after a first episode of venous thromboembolism in patients with persistently positive aPL.

For patients with APS and arterial thrombosis, the Galveston guidelines recommended either high-intensity anticoagulation at a target INR of 3.0–4.0 or combined therapy with LDA plus anticoagulation at a target INR of 2.0–3.0.[31] However, the degree of agreement on this specific point was low. New data on this topic come from a recent retrospective study by Jackson *et al*[40] on 139 patients with APS from the cohorts of the New York Presbyterian Hospital and APS ACTION presenting with arterial thrombosis. The authors found that dual therapy with LDA plus anticoagulation (most of them at an INR of 2.0–3.0) decreased the rate of recurrent events: 6.9% compared with 23.7% and 37.2% on anticoagulant and antiplatelet therapy alone, respectively; and increased the time to recurrence of thrombosis: 16.3 years compared with 7.3 and 3.4 years on anticoagulant and antiplatelet therapy alone, respectively.[40] These data support the use of dual anticoagulant and antiplatelet therapy in this controversial setting.

Therefore, patients with APS presenting with venous thromboembolism and persistent aPL should receive long-term oral anticoagulation at a target INR of 2.0–3.0; for those presenting with arterial events, oral anticoagulation at a target INR of 3.0–4.0 or combined therapy with LDA plus oral anticoagulation at a target INR of 2.0–3.0 are our preferred treatment options.

Direct oral anticoagulants and other drugs

Long-term anticoagulation with direct oral anticoagulants (DOACs) such as direct factor Xa inhibitors (rivaroxaban, apixaban and edoxaban) and direct thrombin inhibitors (dabigatran) offers some advantages compared with anticoagulation with vitamin K antagonists in terms of a better drug–drug interaction profile, a fixed dosing protocol without the need for blood level monitoring and a narrow therapeutic range. The US Food and Drug Administration and the European Medicines Agency have approved DOACs for thromboprophylaxis and treatment of several venous thromboembolic diseases including DVT and pulmonary embolism.[41–44] The recently published Rivaroxaban in APS study showed that thrombin generation markers are not increased with rivaroxaban compared with warfarin in patients with APS who had previous venous thromboembolism. Taking also into account the absence of clinically significant bleeding, the study concluded that rivaroxaban could be efficacious and safe in this subgroup of patients with APS.[7] Nevertheless, the study results cannot be extrapolated to patients with APS with arterial or venous thrombosis who require higher intensity anticoagulation, according to the authors. The Rivaroxaban in Thrombotic APS is a multicentre, randomised, controlled, open-label study, non-inferiority trial comparing rivaroxaban (20 mg/day) with warfarin (INR 2.5) with respect to cumulative incident, arterial or venous, thrombosis in patients with triple aPL positivity. After randomising 59 patients to the rivaroxaban arm and 61 patients to the warfarin arm, the study was terminated due to occurrence of more events in the rivaroxaban group; thromboembolic events and major bleeding occurred in 12% and 7% in

the rivaroxaban arm compared with 0% and 3% in the warfarin arm, respectively. Noteworthy, seven arterial events were documented in the rivaroxaban group versus none in the warfarin group.[45] Another study, the Rivaroxaban in APS Pilot Feasibility Study (ClinicalTrials.gov: NCT02116036), prospectively follows patients with definite APS with known history of venous thromboembolism, with or without arterial thrombosis, on rivaroxaban (20 mg/day) for thrombosis (minor, major or fatal bleeding) as a secondary endpoint. Seventy-nine patients were identified and will be followed for 1 year, and no thrombotic events have occurred until now, although an unexplained hepatitis occurred in one patient. The Apixaban for Secondary Thrombosis Prevention in APS study is a phase IV pilot, prospective, open-label, randomised, blinded trial studying the efficacy and safety of apixaban (2.5 mg twice a day; then increased to 5 mg twice a day based on a recommendation by the data safety monitoring board (DSMB)) compared with warfarin (INR 2.0–3.0) in secondary thromboprophylaxis in patients with history of APS. After the enrolment of 30 patients, the DSMB re-evaluated the data and recommended to exclude patients with prior arterial thrombosis and to perform brain MRI for all candidates to exclude prior silent stroke.[46] A recent study by Malec and colleagues[47] revealed a 6% risk of recurrent venous thromboembolism in patients with APS treated with DOACs, the data of which were dissected by Yazici *et al*,[48] who interpreted the results in the opposite way and argued for following the recommendations of the APS Treatment Trends Task Force.[49 50] The role of DOACs in the management of thrombotic APS manifestations is still not clear, and there are concerns regarding their role in arterial thrombosis. The recent 15th International Congress on aPL Task Force on Treatment Trends states that there is insufficient evidence to make recommendations at this time regarding the use of DOACs in APS.[49]

Preliminary studies on statins (fluvastatin; 20 mg and 40 mg per day for 1 and 3 months, respectively) showed benefit in preventing thrombus formation in patients with APS,[51 52] but their current use in the treatment of APS is limited to patients with hyperlipidaemia. The 15th International Congress on aPL Task Force on Treatment Trends suggested that statins may be used in patients with APS with high risk for cardiovascular events and in those with recurrent thrombosis despite adequate anticoagulation.[49]

Patients with immune-mediated thrombocytopaenia (platelet count less than 20 ×10^9/L) and haemolytic anaemia may benefit from glucocorticoids with or without intravenous immunoglobulins as first-line treatment, whereas azathioprine, cyclophosphamide and mycophenolate mofetil may be used as second-line therapies.[53]

Several other drugs such as B-cell inhibitors (rituximab and belimumab) and other immunosuppressants, intravenous immunoglobulins, corticosteroids, complement inhibitors (eculizumab), integrin inhibitors, adenosine 2A receptor agonists (defibrotide), cilostazol, protease activator receptor (Par) antagonists, Toll-like receptor antagonists and tissue factor inhibitors are still under investigation for their potential benefit in the APS therapeutic plan.[8 22 49 50 54–57] In particular, aPL/β2GPI receptor blockers may have a future role in the management of refractory obstetrical APS. 1N11 monoclonal antibodies can inhibit the binding of β2GPI to its receptors on the trophoblast surface.[58] In the same context, non-complement fixing antibodies (CH-2 deleted antibody)[59] and synthetic peptides (TIFI)[60] can also target β2GPI domains I and V, respectively, thus preventing the binding of aPL and β2GPI.

More preclinical studies and controlled trials are needed to elucidate the true role of these novel therapies in the management of APS.

The 15th International Congress on aPL Task Force on Treatment Trends suggested that the mammalian target of rapamycin inhibitors may have a role in the treatment of aPL-positive patients with microthrombosis, and clopidogrel (or other adenosine diphosphate (ADP) $P2Y_{12}$ receptor antagonists) can be considered as an adjunctive therapy in some patients with APS with arterial thrombosis refractory to conventional treatment.[49]

CATASTROPHIC APS

CAPS is an acute to subacute, severe life-threatening variant of APS, characterised by rapid onset of systemic, multiple organs small vessel thromboses, which may lead to dysfunction, and often failure, of the involved organs if not diagnosed early and managed promptly. Three criteria must be met for a definite diagnosis, based on the preliminary CAPS criteria initially proposed by Asherson *et al*[61] in 2003: (1) aPL positivity, (2) multisystem organ dysfunction/failure during a 1-week period and (3) histopathological confirmation of small vessel occlusion. The pathophysiology of CAPS is not clearly defined yet; however, some authors hypothesise that a precipitating factor, including but not limited to surgery and infections, may cause an acute endothelial injury, which initiates a cycle of cytokine overproduction and systemic inflammatory responses, ultimately leading to large-scale microangiopathy and small vessel thromboses.[16 62] Although CAPS is very rare occurring in less than 1% of patients with APS,[6] the treatment must be initiated as soon as possible to overcome the high mortality (up to 48%[16]) associated with CAPS.

The 14th International Congress on aPL Task Force report on CAPS recommended the use of triple therapy, a combination of full-dose anticoagulation, high-dose glucocorticoids and plasma exchange; intravenous immunoglobulins and cyclophosphamide may be added to the regimen in the presence of an infection and concomitant autoimmune disease such as SLE, respectively.[63] Rituximab may be used as an initial adjuvant therapy if microangiopathic haemolytic anaemia is present, as an alternative adjuvant therapy when anticoagulation is contraindicated, or as a second-line therapy in refractory cases.[8 63] The recent 15th International Congress on aPL Task Force on CAPS highlighted the effectiveness of adding eculizumab to standard triple therapy with or without thrombotic microangiopathy. The Task Force group also stressed on the importance of choosing the best steroid dose and tapering schedule, the best replacement fluid during plasma exchange, and the best therapeutic dose and time to administer intravenous immunoglobulins, and discussed the role of rituximab and new anticoagulants in the management of CAPS.[64]

OBSTETRICAL APS

Preconception planning in women with APS should include complete profiling of aPL using standardised tests, and these patients may benefit from long-term, preconceptional aspirin, which may increase the likelihood of pregnancy and embryo implantation, favourable fetal outcomes, as well as achieving successful live births in >70% of pregnancies.[65 66] Dual antiplatelet and anticoagulation therapy is generally recommended in pregnant patients with APS, although some patients with recurrent early miscarriages (<10 WG) may benefit from LDA alone. Due to their teratogenicity, oral anticoagulants must be stopped as early as possible on confirmation of pregnancy

(within the first 6 WG) and replaced with LDA plus LMWH at prophylactic or therapeutic doses in women without and with history of thrombotic events, respectively[50 67] (figure 1). During the postpartum period, women who do not have risk factors for thrombosis and who did not receive antenatal thromboprophylaxis may benefit from LMWH for 7-10 days only; if additional risk factors for thrombosis are present or if women were treated with LMWH during pregnancy, thromboprophylaxis should be extended to 6 weeks.[65]

For women with refractory obstetrical APS, several alternatives have been proposed based on observational case series: HCQ,[68 69] prednisone 10 mg/day up to week 14,[70] pravastatin 20 mg/day in cases of severe placental insufficiency with pre-eclampsia as soon as the complication is detected,[71] and intravenous immunoglobulins (2 g/kg per month) and/or plasma exchange.[69 72] In our opinion, the latter is only recommended in patients with severe thrombotic APS and very selected cases of obstetrical APS.

CONCLUSION

Over the last 35 years, APS has been recognised as a major, treatable condition in obstetrical medicine, neurology, cardiology, rheumatology and in most other branches of medicine. The recognition of the many non-thrombotic manifestations of APS has added to the importance of separating APS diagnosis from classification. Patients with strong clinical features suggestive of APS but with negative standard tests—the so-called 'Seronegative' APS—are now being identified using non-criteria laboratory tests, and the role of extra-criteria aPL is being investigated. The current treatment of APS is still largely confined to aspirin, clopidogrel, heparin and warfarin. The introduction of DOACs in the treatment of APS has been predictably cautious, and it is too early to generalise.

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Clinical trials in children and adolescents with systemic lupus erythematosus: methodological aspects, regulatory landscape and future opportunities

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ABSTRACT

Childhood-onset systemic lupus erythematosus (cSLE) is rare in many regions of the world, including Europe. Access to approved medications for cSLE is currently limited, among others, due to a lack of high-quality evidence from clinical trials. The objectives of the study were to evaluate the current regulatory framework regarding medication approvals, delineate barriers to clinical trial conduct, and strategies to improve access to new medications for cSLE. Relevant methodological and regulatory aspects, epidemiological data, study designs and outcome measures are reviewed, and the results of a survey among Paediatric Rheumatology International Trials Organisation/Pediatric Rheumatology Collaborative Study Group investigators are presented. Laws and regulations in the USA and Europe necessitate that novel medicines are studied in paediatric populations, if similar or the same diseases in adults have been found to benefit from them. Regulatory agencies consider cSLE the paediatric form of SLE in adults. For medicines that have been found safe and effective in adult SLE, paediatric extrapolation strategies can limit the number and complexity of studies needed to support the labelling of these medicines for use in cSLE. In this setting, specialised research networks, validated outcome measures, stakeholder input, study designs as well as statistical methods successfully used in other uncommon diseases will help improve study efficiency in an effort to enhance the speed with which new drugs for cSLE can be studied. Open-label pharmacokinetic-pharmacodynamic studies are preferred by paediatric rheumatologists over double-blind parallel designs for cSLE trials. Appropriate infrastructure, outcome measures and sufficient numbers of patients are available for the testing of new medicines for children with cSLE.

INTRODUCTION

Systemic lupus erythematosus (*SLE*) is a multiorgan inflammatory disease characterised by autoantibody production secondary to immune dysregulation, involving both the innate and adaptive immune systems. The underlying causes of SLE have not been fully elucidated, and there is large phenotypic variability. Most common SLE features include mucosal ulcerations, alopecia, various skin eruptions, arthritis or arthralgia and fatigue. Neuropsychiatric involvement and glomerulonephritis with SLE markedly worsen its prognosis.

In 1948, the US Food and Drug Administration (FDA) approved the first drug to treat lupus: aspirin. This was followed by the FDA approval of corticosteroids and hydroxychloroquine.[1 2] However, after a long hiatus, belimumab received marketing authorisation by the FDA and the European Medicines Agency (EMA) for the treatment of adult patients with active, autoantibody-positive SLE despite standard therapy in 2011.[3]

There are no drugs that have been approved by either FDA or EMA for childhood-onset SLE (cSLE), that is, children and adolescents with SLE with disease onset prior to age 18 years.[4] Lack of approved drugs for cSLE leads to delays in providing proper care in a time where physicians are restricted in prescribing off-label medications, especially if their price is high.

This report summarises methodological aspects, study designs and outcome measures relevant for the study of cSLE; the results of an international survey of paediatric rheumatologists regarding clinical trials in cSLE are presented; and a framework is offered for the practical conduct of future studies performed in children as part of regulatory requirements in the USA and Europe.

DIFFERENCES AND SIMILARITIES OF SLE WITH ONSET DURING ADULTHOOD AND CSLE

The development of clinical and laboratory manifestations with SLE and cSLE is thought to be mediated by environmental and lifestyle factors in genetically predisposed individuals. Monogenic genetic causes of cSLE are especially common among individuals with disease onset by 5 years of age.[5] In those with disease onset beyond age 5 years but still early in life, there is likely a higher load of the aforementioned factors and, possibly, pathological changes of immune system development.[5–8] Compared with individuals with disease onset during adulthood, children with cSLE have more commonly multiorgan disease, acute disease onset and ongoing active inflammation over time. As such, lupus nephritis is estimated to be at least 30% and neuropsychiatric involvement about 25% more common in cSLE than with adult-onset disease[9]; this necessitates the more frequent chronic use of corticosteroids and immunosuppressive drugs in cSLE, and there are higher rates of hospitalisations,

which likely damage acquisition and mortality in cSLE, with a large variability worldwide.[6 10] Despite these dissimilarities, there are no laboratory abnormalities, signs or symptoms that only occur in cSLE but not in SLE with onset during adulthood.

EPIDEMIOLOGY OF CSLE

There are considerable differences in phenotypic presentations, incidence and prevalence rates of SLE around the world.[11 12] About 10%–20% of a global lupus population has had disease onset during childhood.[13] The estimated prevalence of cSLE is at 9.73 (95% CI 9.38 to 10.08) per 100 000 persons in the USA,[13] which compares with the prevalence of juvenile idiopathic arthritis (JIA) at 44.7 (95% CI 39.1 to 50.2) per 100 000 persons.[14] In Europe, JIA is more common at a prevalence of 70.2 (95% CI 62.9 to 78.1) per 100 000 persons,[15] and cSLE is even less prevalent with estimates of 4.3 (95% CI 1.4 to 14) per 100 000 persons.[16 17] Isolated subcutaneous or discoid lupus occurs rarely in children,[18 19] and the disease is at least five times more common among girls.[13]

REGULATION OF DRUGS FOR CHILDREN WITH PAEDIATRIC RHEUMATIC DISEASES

The Best Pharmaceuticals for Children Act (BPCA)[20] and the Pediatric Research Equity Act are especially relevant to paediatric drug development in the USA.[21] The latter requires new drugs and biologic therapies to be tested in children, provided there is a paediatric disease that is similar to a non-orphan disease occurring in adults.[22] BPCA provides pharmaceutical companies with an additional 6 months of market exclusivity for the adult indications after the completion of drug studies in children performed at the request of FDA.[23] A key document that FDA requires pharmaceutical companies to develop is the *Pediatric Study Plan* (*PSP*) on completion of phase 2 studies and before the initiation of phase 3 studies in adults.[24] In the European Union (EU) the EMA is responsible for the scientific evaluation, supervision and safety monitoring of medicines in 28 EU member states, as well as the countries of the European Economic Area. In 2006, the European Parliament passed legislation relating to paediatric drug testing and approval, similar to that in place in the US pharmaceutical companies are required to submit a *Paediatric Investigational Plan* (*PIP*) to the *Paediatric Committee,*[25 26] irrespective of whether the adult disease is regarded an orphan disease or not. Both the US and the EU paediatric legislation has been a great success in that several medications received licensing for use in children, especially in paediatric rheumatology and infectious diseases.[27] EMA and FDA regulations require that age-appropriate preparations of medication are made available.[28 29] For off-patent medicines, EMA may grant a Paediatric Use Marketing Authorisation, with patent protection for 10 years, if an indication together with an appropriate dosage form or formulation specifically devised for children has been developed.[30]

CONSIDERATIONS OF ETHICAL PRINCIPLES AND USE OF PREVAILING KNOWLEDGE IN CSLE DRUG DEVELOPMENT

For enhanced acceptance of data generated in paediatric global drug development programmes by regulatory agencies, such as EMA an FDA, and to ensure timely access to medicines for children, the *International Council on Harmonisation* (*ICH*) of Technical Requirements for Pharmaceuticals for Human Use has recently updated its E11 guideline entitled, '*Clinical Investigation Of Medicinal Products In The Pediatric Population*'.[31 32]

Besides ethical principles for research in paediatrics as are summarised in figure 1, the ICH stresses that children should

Figure 1 Ethical principles of medication studies in children.

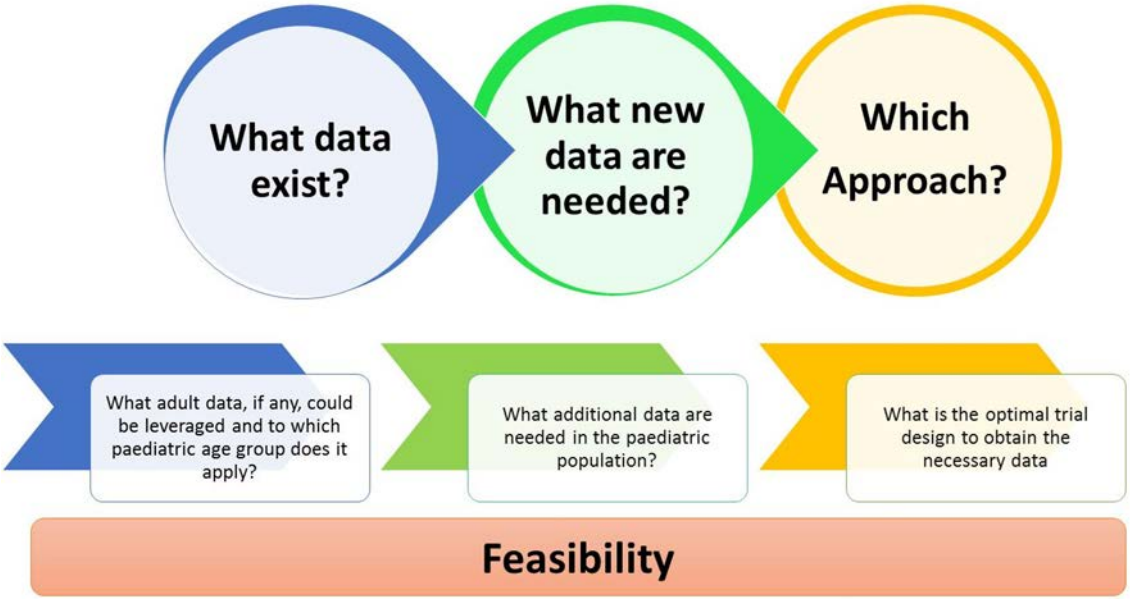

Figure 2 Factors influencing paediatric extrapolation strategies. The paediatric extrapolation from adult to paediatric diseases assumed similar pathoaetiology and response to therapy are present. Existing data are appraised to determine which new data are needed to determine safety and efficacy of a medicinal product in childhood-onset systemic lupus erythematosus (cSLE). The approach to acquiring the needed information about a given medicinal product is determined accordingly, also considering feasibility and stakeholder input.

not be enrolled in a clinical study unless necessary to address an important paediatric public health need; and that risks and benefits of research participation need to be carefully assessed. This also implies that paediatric drug development programmes are cognisant of study feasibility and consider paediatric extrapolation (figure 2).

'*Paediatric extrapolation*' is defined as an approach to providing evidence in support of effective and safe use of drugs in the paediatric population when it can be assumed that the course of the disease and the expected response to a medicinal product is sufficiently similar in the paediatric and the reference (adult or other paediatric) population.[32] Thus, previously acquired data from studies in adults with SLE and other diseases, information collected from other paediatric populations and preclinical data are all considered in the extrapolation concept for cSLE.[33] While input from clinicians and clinical trial experts has been commonly sought in the past, the importance to also include the patient perspective and experience is now recognised.[34] Based on the appraisal of the available body of knowledge, feasibility and stakeholder input, the paediatric drug development programme (PIP, PSP) will be aimed at closing a specific knowledge gap to help determine whether a medicinal product would be beneficial when used in cSLE or not.

LUPUS-SPECIFIC RECOMMENDATIONS FOR THE STUDY OF MEDICATIONS FOR CHILDREN AND ADULTS BY REGULATORY AGENCIES

Specific guidance documents relevant to studies in SLE have been issued by FDA and EMA.[35 36] Only the EMA document provides guidance for trials in lupus nephritis. The FDA guidance document states that the most feasible and ethical approach to test a new medicine in SLE is likely to add a new medicine to current standard of care therapy. Considering study size, FDA guidance also states that the most realistic approach is to conduct a superiority study.[35] In general, the minimum duration of an SLE trial is 1 year for evaluating the endpoint of reduction in disease activity, complete clinical response or remission, reduction in flare/increase in time to flare and maintenance of response. FDA recommends the use of the American College of Rheumatology (ACR) Classification Criteria[37] for identifying SLE subjects for trials, while the respective EMA guidance document[36] also supports the use of the newer Systemic Lupus International Collaborating Clinics (SLICC) Classification Criteria for SLE.[38] Both classification criteria sets have been validated for use in cSLE.[39 40] Interestingly, the EMA specifically states that its guidance paper does not apply to subsets of SLE, for example, neuropsychiatric SLE and secondary antiphospholipid syndrome, in lieu of difficulties in making a diagnosis and/or the absence of validated efficacy assessment tools. Nonetheless, EMA encourages the inclusion of patients with these SLE subsets in trials.[36]

Similar to the EMA guidance document,[36] there is a short paragraph (section 7) in the FDA Guidance document that speaks specifically to cSLE.[35] Herein, EMA and FDA concur that, compared with adult-onset SLE populations, there is an increased male-to-female ratio, a higher prevalence of kidney and neuropsychiatric involvement as well as faster accrual of damage in cSLE. Biomarker use is encouraged by both agencies in studies of new medicines for SLE and cSLE.[28 29 33 35 36] Further, EMA's guidance paper points out the rarity of cSLE and that a waiver could be granted for children under the age of 5 years.[36] *Waivers* may also be granted when a paediatric development is not needed or appears inappropriate. Regulatory agencies often provide a *deferral,* that is, cSLE studies are postponed until there are sufficient data to demonstrate the efficacy and safety of a medicinal product in adults with SLE.

As can be deduced from the above, the current medication development for children with cSLE relies heavily on evidence around medication safety and efficacy in adults. Notably, safety of medicines, when used in children, can never be fully extrapolated from adult data, as the impact of a medication on growth and development cannot be studied in adults. This mandates generally the conduct of long-term postauthorisation studies and establishment of patient registries in cSLE.

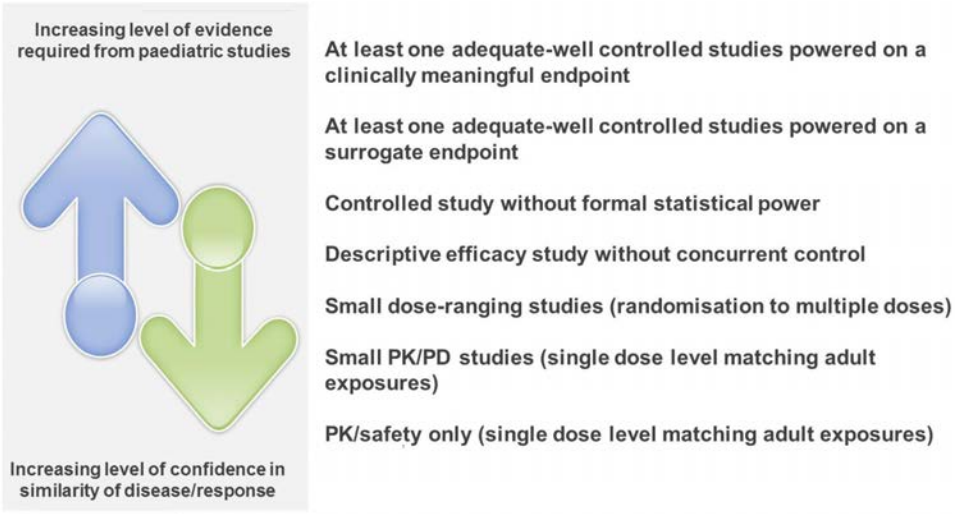

Figure 3 Extrapolation approaches—possible design of paediatric studies based on the results of the paediatric drug development must be sufficient to support the therapeutic benefits and appropriate dosing of a medicinal product. Paediatric extrapolation assessment will inform about appropriate approaches to studying safety and efficacy of a medicinal product when used in childhood-onset systemic lupus erythematosus (cSLE). Approaches may change over time as new data (learning) become available. Feasibility of conducting medication studies in cSLE will need to be considered. PK/PD, pharmacokinetic-pharmacodynamic study.

TRIAL DESIGNS FOUND SUCCESSFUL FOR THE STUDY OF RARE DISEASES SUCH AS CSLE

The cSLE drug development programme deemed necessary by EMA (PIP) and FDA (PSP) will depend on the results of paediatric extrapolation considerations, feasibility and differences in-between cSLE and SLE relevant for a given medicinal product's method of action (figure 3).

The prototypic approach to testing medication efficacy is a parallel-arm, double-blinded, placebo-controlled randomised controlled trials with a fixed target sample size. This approach will face considerable challenges when used in cSLE as is exemplified by the double-blinded, placebo-controlled trial of belimumab in cSLE (NCT01649765). Although the concept of assembling a placebo cohort is ideal from a scientific point of view to assess drug efficacy while minimising sample, as opposed to an active arm comparison, the aforementioned trial required a 12-month exposure to placebo infusions of children as young as 5 years, and use of advanced analytic methods was not planned. As a result, enrolment was markedly delayed. The study also lacked power to assess differences in major safety events or differences in efficacy compared with adult SLE. This raises ethical concerns which, coupled with feasibility issues, have already delayed the access of children to belimumab.

Recently, much attention has been paid to *adaptive clinical trials*, that is, trials with prospectively planned modifications to the study design while preserving the scientific validity and

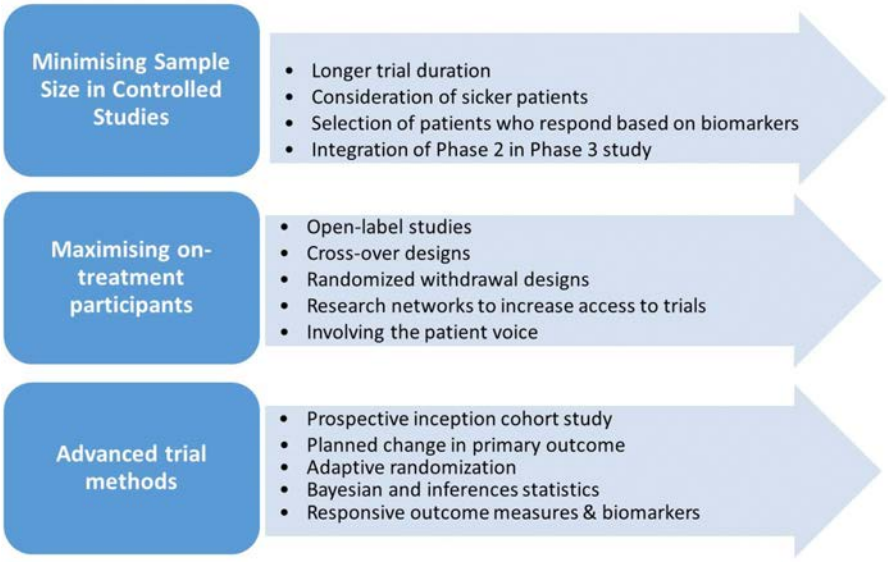

Figure 4 Strategies for successful clinical trials in rare diseases. Successful trials of medications in rare or uncommon disease must consider limited numbers of patients who can be enrolled in a study. Access to patients may be increased through research networks and by considering the input of all stakeholders, that is, families, patients, clinicians treating patients and clinical trialists. Maximising on-treatment time will further enhance the amount of information regarding the drug effect and increases interest in research participation. Advanced trial methods can further increase the efficiency of a trial.

integrity of that trial. Using adaptive design elements in clinical trials is expected to enhance the proficiency with which drug efficacy is shown.[41 42] Indeed, adaptive design elements are often used in studies of uncommon diseases. Examples include *adaptive randomisation* to minimise imbalance in baseline covariates among treatment groups and/or increase the proportion of patients assigned to the presumed more effective treatment, while reducing overall trial enrolment (*response-adaptive randomisation*). In *sequential adaptive trials*, data are analysed intermittently to guide decisions on termination when safety concerns, futility, efficacy or a combination of these factors is demonstrated. Trials that are stopped early because of important interim results require fewer patients.

Other clinical trial strategies that have been favoured in medication studies of rare diseases are summarised in figure 4.[43] They include seamless phase 2–3 designs where phase 2 and phase 3 studies are combined so that some patients can participate in both phases. Study designs where all study participants receive active study drug help enhance recruitment.[43] The same holds true for studies that offer a high proportion of participant's active drug over prolonged time periods. This can, for example, be achieved by using a randomised withdrawal design (RWD),[43] or in observational studies where all patients receive active study drug. However, for observational studies, issues of confounding will need to be carefully addressed to delineate drug effectiveness by advanced statistical methods, such as propensity scores, or 'new-user' studies: inception cohorts permit investigators to establish clear temporality among study variables, that is, baseline confounders, exposures and outcome events that occur after entry to the cohort. Cross-over designs are also often used for the study of rare diseases. Although this design was successfully employed for the study of rilonacept in familial Mediterranean fever,[44] it is unlikely to be useful in cSLE, among others, due to the variability of disease manifestations over time. Notably, many of the aforementioned clinical trial strategies have been successfully used in other paediatric rheumatic diseases.[45–47]

VALIDATED CORE OUTCOME AND RESPONSE MEASURES TO QUANTIFY THE EFFECTS OF NEW MEDICINES

Another method to reduce sample size requirements in rare disease studies is through the selection of accurate outcome measures, that is, surrogate and biological markers that are highly sensitive, specific and responsive to change. Indeed, performing medication studies in children without well-validated outcome measures may be considered unethical. In the EMA and FDA guidance documents for SLE,[35 36] reference is made to the cSLE core set domains which were established by the Paediatric Rheumatology International Trials Organisation (PRINTO, www.printo.it), in collaboration with the Pediatric Rheumatology Collaborative Study Group (PRCSG, www.prcsg.org). The five cSLE core set domains are disease activity, renal function, patient well-being, physician's global assessment of cSLE activity and health status. EMA endorses the use of the PRINTO/ACR Response Criteria,[48] while the FDA considers their use as exploratory, pending additional validation studies. Since the time of the publication of the FDA guidance document, additional validation of the PRINTO/ACR Response Criteria has occurred[49] which may suggest that there is consensus of both agencies for the use of the PRINTO/ACR Response Criteria in cSLE clinical trials. Despite its apparently lower accuracy compared with the PRINTO/ACR Response Criteria, the Systemic Lupus Responder Index is appropriate for use in cSLE.[49] There are also validated criteria for flare of global disease with cSLE.[50 51] Regulatory agencies consider the avoidance of disease flares important,[35 36] and flare criteria are essential for medication studies using an RWD. Table 1 summarises some of the important measures of disease activity, disease damage and patient-reported outcomes that are validated for use in cSLE.

Table 1 Important outcome measures for cSLE medicine studies

Name of instrument or criteria	Disease-specific scale (yes/no)	Same (similar) in adults with SLE	Reference
Disease status measures			
SLEDAI*	Yes	Yes	79
SLAM	Yes	Yes	79
BILAG† Index	Yes	Yes	79
ECLAM	Yes	Yes	80
Inactive disease status	Yes	No	81
SDI	Yes	Yes	73
Paediatrics SDI	Yes	Yes	82
PedANAM-CPS	No	No	61
RAIL	Yes	Yes	58
Response measures			
SRI	Yes	Yes	49
PRINTO/ACR criteria for improvement	Yes	No	48 83
BILAG flare tool	Yes	Yes	51
Lupus flare score	Yes	No	50
Lupus nephritis response measure	Yes	(Yes)	54 84 85
CLASI	Yes	Yes	86
Patient-reported outcomes			
CHAQ	No	(HAQ)	87
CHQ‡	No	(SF-36)§	52
PedsQL Generic Core Scale	No	No	53
PedsQL Rheumatology Module	No	No	53
PedsQL Fatigue Scale	No	No	88
Fatigue Disability Index	No	No	88
SMILEY	Yes	No	89

*SLE Disease Activity Index, version 2000 and SELENA SLEDAI.
†British Isles Lupus Activity Group Index, versions 1984 and 1994.
‡Child Health Questionnaire, version P50.
§Medical Outcome Survey, Short Form 36.

ORGAN-SPECIFIC ASSESSMENTS OF CSLE RESPONSE TO THERAPY

Specific organ system involvement with cSLE differentially impacts health-related quality of life and/or importantly influences long-term prognosis. Accordingly, particular attention needs to be paid to evaluating the organ-specific impact of medicinal products.[52 53] Organ-specific outcome measures for musculoskeletal manifestations, cutaneous lupus manifestation, neuropsychiatric cSLE and paediatric lupus nephritis should be collected. Consensus has been achieved among North American and European paediatric rheumatologists of how to capture changes in paediatric lupus nephritis activity.[54 55] More recently, urine biomarkers of lupus nephritis have been discovered and validated for use in children and adults. The Renal Activity Index for Lupus quantifies lupus nephritis activity based on levels of several protein biomarkers measured in the urine.[56–58] For capturing joint inflammation with cSLE, the assessment of number of joints with active arthritis and joints with limited range of motion, as done for JIA,[59] has face validity. Despite the

variability of neuropsychiatric manifestations with cSLE, cognitive ability is generally considered a global measure of brain health. A standardised battery of age-appropriate formal neuropsychiatric tests has been defined for cSLE,[60] and the Pediatric Rheumatology Assessment Metrics software (PedANAM, Vista LifeSciences, Oklahoma, USA) is available for estimating cognitive ability.[61–63] Notably, there is no highly specific diagnostic test for neuropsychiatric SLE in children and adolescents.

SPECIAL ISSUES AROUND STUDY DESIGNS OF CSLE

To avoid potential delays in drug availability for children, studies in cSLE should commence soon after efficacy and sufficient safety is demonstrated in phase 3 trials in adults for an agent in a new drug class, and after phase 2 completion for drugs within a class of medicines with proven efficacy in adults with SLE.

There is agreement among paediatric rheumatologists regarding principles of therapeutic and disease monitoring standards for cSLE.[64 65] This is relevant for the design of cSLE trials, together with current principles of off-label medication use and standards of medical care.[66–68] Regulatory agencies support the enrolment of adolescent patients with cSLE in adult SLE trials. However, differing standards of medical care between cSLE and adults with SLE exist,[54 64] and enrolling adolescents into adult SLE trials is likely difficult, based on the experience gained in JIA.

In line with suggestions from regulators, global enrolment stratified by country of origin and race seems ideal to enable sensible subanalyses to test for potential differences in drug efficacy based on racial, ethnic or regional factors and gender effects in cSLE.[8 28 29 69] Consistent access to high-quality data from well-designed geographically matched cSLE and SLE registries to define standards of care and provide evidence of similar response to current treatments across all ages of patients with lupus would much facilitate any clinical trial design of cSLE.

Enrolment of patients with cSLE into traditional double-blind, placebo-controlled parallel design studies is not favoured by paediatric rheumatologists. This is supported by the results of recent surveys regarding hypothetical future medication trials in cSLE. Survey respondents were 192 PRINTO and 161 PRCSG investigators from over 40 countries, who participated in several trials of the two networks (table 2). Over 70% of the survey responders preferred an open-label PK/PD study while only 21%–30% favoured a blinded, parallel, placebo-controlled study. Despite enthusiasm about testing new medicines for cSLE and the potential to enrol over 1000 study participants with active cSLE over a 12-month period, prolonged placebo exposures were only agreeable to a minority of the PRCSG/PRINTO survey responders.

If regulators deem blinded or controlled studies necessary to address the existing gap in scientific knowledge about a new medicine for cSLE, then trial designs seem advantageous that have proven to be successful when used in other rare diseases.[70 71] These Pediatric Study Plans: Content of and Process for Submitting Initial Pediatric Study Plans and Amended Initial Pediatric Study Plans Guidance for Industry include RWD trials (see online supplementary figure 1) which provide instant open-label active study drug to all patients during the lead in phase (part 1) and limit potential placebo exposure to patients who have experienced improvement during part 1 of the study, whereas children who fail to respond to study drug are generally discontinued from the study prior to randomisation.

The primary endpoint of an RWD study is the proportion of patients with 'disease flare' or the time to 'disease flare' in part 2. Therefore, patients remain in the blinded part 2 only as long as their disease continues to demonstrate at least a similar level of cSLE control as was present at the time of randomisation. RWD trials used in paediatric rheumatology only require mild to moderate worsening during part 2 for a patient to again receive active drug in part 3 of the trial. Seemingly, the overall burden of disease activity rather than short minor to moderate global flares of cSLE carries a sizeable risk for disease damage.[72–74] Nonetheless, appropriate trial discontinuation rules, provision of rescue medication and close patient follow-up will all be needed to avoid long-lasting sequelae of flare.

Table 2 Survey among paediatric rheumatology investigators for study in active cSLE with controlled lupus nephritis

Survey detail	PRCSG	PRINTO
Number of responders/survey recipients	161/289 (56%)	192/462 (42%)
Number of countries surveyed	3	39
Willingness to participate to a double-blind, placebo-controlled trial	43/161 (27%)	98/192 (51%)
Estimated number of patients observed in 1 year with:		
SLEDAI ≥10, ages 5+ to 11 years	120–186	242–520
SLEDAI ≥10, ages 12–17 years	520–733	543–1014
SLEDAI ≥10, no severe* lupus nephritis, ages 5+ to 11 years	51–70	147–329
SLEDAI ≥10, no severe* lupus nephritis, ages 12–17 years	101–163	310–609
Estimated number of patients who can be enrolled in a *double-blind, placebo-controlled parallel study (study A)*		
Ages between 5+ and 11 years	51–70	99–259
Ages 12–17 years	101–163	207–470
Open-label PK/PD study (study B)		
Ages between 5+ and 11 years	41–68	129–294
Ages 12–17 years	117–175	257–544
Responders who prefer study A† design	4/19 (21%)	28/93 (30%)
Responders who prefer study B† design	15/19 (79%)	65/93 (70%)

cSLE, childhood-onset systemic lupus erythematosus; PK/PD, pharmacokinetic-pharmacodynamic study; PRCSG, Pediatric Rheumatology Collaborative Study Group; PRINTO, Paediatric Rheumatology International Trials Organisation; SLEDAI, SLE Disease Activity Index.

*Severe lupus nephritis 2 or more grams of daily proteinuria or glomerular filtration rate <50 mL/min/1.73 m^2).

†Limited centres willing to participate either to study A or study B.

cSLE, childhood-onset systemic lupus erythematosus;PK/PD, pharmacokinetic-pharmacodynamic study;PRCSG, Pediatric Rheumatology Collaborative Study Group;PRINTO, Paediatric Rheumatology International Trials Organisation;SLEDAI, SLE Disease Activity Index.

Despite its attractiveness, the RWD has its shortcomings which will need to be carefully considered.[71 75] They include carry-over effects which may reduce the likelihood of flaring in patients switched from placebo. RWD trials are more difficult for testing medications with prolonged biological effects or drug which are only given intermittently, such as rituximab. Further, RWD trials only provide indirect evidence of drug efficacy, given that flare rather than response to therapy is the primary outcome.

Chronic use of corticosteroids remains a major concern, given the well-known detrimental effects on growth and pubertal development of children beyond the side effects encountered by adults.[76] Therefore, steroid tapering needs to be integrated in clinical trial designs to address concerns about prolonged corticosteroid exposures with cSLE. This may be achieved by a sufficiently long open-label lead period of an RWD trial to enable tapering to a relatively safe dose of 0.2 mg/kg/day or 10 mg/day (whichever is lower). Corticosteroid tapering cannot be easily implemented and adequately interpreted in parallel study design.

For the study of paediatric lupus nephritis in particular, exposure to placebo seems problematic when there is high renal activity. This is because prolonged uncontrolled lupus nephritis activity and proteinuria, presumably more common with receiving placebo, is a potent risk factor for poor prognosis.[77]

Adaptive design elements, such as early randomisation of responders and patients who tapered corticosteroids successfully, or adjustment of sample size based on response rates should all be considered for added efficiency when studying new medicines in cSLE.

SUMMARY AND INTERPRETATION

There is a dire need to develop new medicines for the treatment of cSLE in general and lupus nephritis in particular. Based on prior experience,[78] specialised paediatric research networks that offer experience in clinical trial design and execution have coordinating centres that are proficient in overseeing clinical trial operations and are familiar with the validated assessments will be essential for the successful completion of cSLE trials. Sufficient numbers of patients with cSLE are available and experienced investigative teams are in place to perform pivotal studies with the scientific rigour needed to support subsequent market authorisation for general cSLE, associated skin, joint and kidney disease. Blinded studies should omit traditional parallel-arm placebo designs. It is noted that use of extrapolation plan including data from other sources, particularly from adult trials, together with open-label and innovative design studies will be more expedient in providing children with cSLE access to new medications.

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Collaborators The authors are officers of the PRINTO-PRCSG networks.

Contributors HIB generated the first draft of the manuscript. All four authors were involved in the feasibility survey (table 2), critically reviewed the manuscript and approved its final version.

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CLINICAL SCIENCE

Dose reduction of baricitinib in patients with rheumatoid arthritis achieving sustained disease control: results of a prospective study

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ABSTRACT

Objectives This study investigated the effects of dose step-down in patients with rheumatoid arthritis (RA) who achieved sustained disease control with baricitinib 4 mg once a day.

Methods Patients who completed a baricitinib phase 3 study could enter a long-term extension (LTE). In the LTE, patients who received baricitinib 4 mg for ≥15 months and maintained CDAI low disease activity (LDA) or remission (REM) were blindly randomised to continue 4 mg or taper to 2 mg. Patients could rescue (to 4 mg) if needed. Efficacy and safety were assessed through 48 weeks.

Results Patients in both groups maintained LDA (80% 4 mg; 67% 2 mg) or REM (40% 4 mg; 33% 2 mg) over 48 weeks. However, dose reduction resulted in small, statistically significant increases in disease activity at 12, 24 and 48 weeks. Dose reduction also produced earlier and more frequent relapse (loss of step-down criteria) over 48 weeks compared with 4 mg maintenance (23% 4 mg vs 37% 2 mg, p=0.001). Rescue rates were 10% for baricitinib 4 mg and 18% for baricitinib 2 mg. Dose reduction was associated with a numerically lower rate of non-serious infections (30.6 for baricitinib 4 mg vs 24.9 for 2 mg). Rates of serious adverse events and adverse events leading to discontinuation were similar across groups.

Conclusions In a large randomised, blinded phase 3 study, maintenance of RA control following induction of sustained LDA/REM with baricitinib 4 mg was greater with continued 4 mg than after taper to 2 mg. Nonetheless, most patients tapered to 2 mg could maintain LDA/REM or recapture with return to 4 mg if needed.

Key messages

What is already known about this subject?

► Professional guidelines in rheumatoid arthritis (RA) suggest that in patients who achieve sustained remission with disease modifying antirheumatic drug (DMARD) therapy, consideration should be given to attempting DMARD taper. Clinical studies evaluating this treatment strategy for targeted synthetic DMARDs (tsDMARDs) are lacking.

What does this study add?

► This randomized, blinded substudy within an ongoing Phase 3 extension trial evaluated dose taper of baricitinib, an inhibitor of Janus Kinase (JAK) 1 and 2, from 4 mg to 2 mg oncedaily in patients who had achieved sustained disease control (low disease activity or remission) with the 4 mg daily dose. The results indicated that while 4-mg was the more efficacious dose, many patients could maintain control of disease activity following dose taper to 2-mg, and for those who did not, disease control could be recaptured with return to 4-mg if needed.

How might this impact on clinical practice or futured evelopments?

► This study provides robust data to inform the use of baricitinib according to professional treatment guidelines regarding consideration of DMARD taper following inductionof sustained disease control in RA.

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INTRODUCTION

Treatment goals in rheumatoid arthritis (RA) include achieving remission (or at least low disease activity (LDA) in patients with long-standing disease), preventing accrual of joint damage, maximising physical function and improving quality of life.[1] Once this state is achieved and maintained, both the American College of Rheumatology and the European League Against Rheumatism recommend reducing the dose of biologic (b), targeted synthetic (ts) and conventional synthetic (cs) disease-modifying antirheumatic drugs (DMARD) in their guidance documents.[2 3] While overall burden of drug intake and societal or individual costs is thereby reduced, the important aspect in the course of tapering RA therapy is maintenance of sustained disease control.

Dose reduction or even cessation of bDMARD therapies has been the focus of several trials in the current decade.[4–8] While many patients can sustain their improved clinical state with cessation or reduction in dose, flare-ups occur in a significant number of patients. Reintroduction of therapy is associated with recapture of the state prior to dose reduction in most but not all patients. However, almost half of csDMARD-treated patients who flare following cessation of therapy do not regain their previous state of remission.[9] The consequence of cessation

or reduction in dose of tsDMARDs following attainment of LDA or remission is unknown.

Baricitinib, a selective Janus kinase 1 and 2 inhibitor, modulates signal transduction of a variety of cytokines involved in the immune-inflammatory response.[10] Baricitinib is approved for the treatment of moderately to severely active RA in adults in over 40 countries including European countries, USA and Japan. Approval was based on the results of four pivotal phase 3 studies (RA-BEGIN, RA-BEAM, RA-BUILD and RA-BEACON) and a single long-term extension (LTE) study (RA-BEYOND).[11–14] Within RA-BEYOND, a randomised, double-blind substudy evaluated the effects of baricitinib dose reduction from 4 mg to 2 mg in patients who had achieved sustained disease control on the 4 mg dose. Herein we report results of this substudy.

METHODS

Study design

The baricitinib phase 3 programme included four pivotal studies. At entry into the programme, patients were ≥18 years old with moderately to severely active RA and had an inadequate response to methotrexate (NCT01710358, RA-BEAM), had an inadequate response or intolerance to ≥1 csDMARD (NCT01721057, RA-BUILD) or ≥1 bDMARD (NCT01721044, RA-BEACON), or had received no or minimal csDMARDs (NCT01711359, RA-BEGIN).[11–14] Patients who completed any of the pivotal studies were eligible to enter RA-BEYOND (NCT01885078), which is an ongoing study designed to evaluate long-term safety and efficacy of baricitinib in patients with RA. Patients were not eligible for participation in RA-BEYOND if they demonstrated laboratory abnormalities or significant uncontrolled medical conditions that, in the opinion of the investigator, posed a risk to the administration of baricitinib.

Patients receiving 4 mg or 2 mg baricitinib at the conclusion of an originating study remained on the same dose in RA-BEYOND. Patients receiving placebo or an active comparator at the end of the originating study were switched to baricitinib 4 mg upon entry. Patients and investigators remained blind to the original treatment assignment. Patients could continue to receive background non-investigational open-label csDMARDs, non-steroidal anti-inflammatory drugs or corticosteroids they were receiving at completion of the originating study. For patients originating from RA-BEAM, RA-BUILD or RA-BEACON, rescue therapy (open-label baricitinib 4 mg and/or addition or increase in dose of csDMARD) was allowed for any patient who had a clinical disease activity index (CDAI) score >10 at or after 3 months following enrolment in RA-BEYOND. For patients originating from RA-BEGIN, rescue therapy (addition of csDMARD) was provided at any time according to the discretion of the investigator.

Patients in RA-BEYOND were eligible to participate in the step-down substudy if they had been receiving baricitinib 4 mg for ≥15 months (including time in the originating study) and achieved sustained LDA (defined by CDAI score ≤10 for patients from RA-BEAM, RA-BUILD, RA-BEACON) or remission (CDAI ≤2.8 for patients from RA-BEGIN) at two consecutive visits ≥3 months apart. This instrument was used for inclusion (and rescue where applicable) as it does not require a laboratory result, and therefore permitting immediate determination of eligibility at study visits. Prior rescue in an originating study or RA-BEYOND excluded patients from step-down eligibility.

Patients meeting eligibility for participation in the substudy were randomised 1:1 (stratified by geographic region and originating study) to continue on baricitinib 4 mg or to step-down to 2 mg. Randomisation occurred via an interactive web-based system without knowledge of the investigator or patient. Patients receiving baricitinib 2 mg also received 4 mg placebo-to-match. Patients receiving baricitinib 4 mg also received 2 mg placebo-to-match, including those patients entering RA-BEYOND from RA-BEGIN and RA-BEAM in which only the 4 mg dose was investigated. Within the step-down substudy, investigators could provide rescue (to open-label baricitinib 4 mg±escalation of background csDMARD) at any time for patients who failed to retain LDA or remission or at any time for DMARD-naïve patients from RA-BEGIN.

The study was designed by the sponsor, Eli Lilly and Company, an academic advisory board including non-Lilly authors of this manuscript, and Incyte. It was conducted in accordance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice guidelines. All patients provided written informed consent before the first study procedure in RA-BEYOND. Consent for participation in the step-down substudy was obtained at the time of entry into RA-BEYOND. Lilly or its representatives provided data, laboratory and site monitoring services. All authors participated in data analysis and interpretation, reviewed drafts and final manuscript and provided critical comment. The authors vouch for the veracity and completeness of the data and data analyses.

Data cut-offs and unblinding

As RA-BEYOND is 10 years in duration, the study was designed to allow multiple data cuts and analyses (online supplementary figure S1). The sponsor was first unblinded to patient allocation during August 2015 to prepare for submission of the license application to regulatory agencies. The step-down substudy is an ongoing process where new patients continuously enter the study once the criteria are met. Additional data cuts have occurred periodically for regulatory reporting purposes. Importantly, investigators and patients continue to remain blinded to dose in the step-down substudy. The data cut-off date used to prepare this manuscript was 1 September 2016, when a substantial number of patients had entered the substudy at least 48 weeks before the cut-off, and thus had the opportunity to provide approximately 1 year of data after randomisation. Unless otherwise specified, the analyses presented focus on this September 2016 48-week analysis set.

Efficacy

The prospectively defined primary endpoints for the substudy were (1) the proportion of patients who maintained a CDAI score of ≤10 in the DMARD-inadequate responder (IR) population (from RA-BEAM, RA-BUILD and RA-BEACON) after 3 months of treatment with baricitinib 2 mg daily compared with patients continuing treatment with 4 mg daily; and (2) time to relapse (defined as a CDAI score >10) after randomisation to baricitinib 2 mg or continuation on 4 mg in this population. These endpoints were included as secondary objectives in the RA-BEYOND protocol. Outcomes in the DMARD-naïve population from RA-BEGIN were assessed separately (due to the distinct inclusion requirement for CDAI remission in this group) and defined prospectively as exploratory endpoints. These outcomes included (1) the proportion of patients who maintained a CDAI score of ≤2.8 after 3 months of treatment with baricitinib 2 mg daily compared with patients continuing

on 4 mg, and (2) time to relapse (defined as CDAI score >2.8) after randomisation to baricitinib 2 mg or continuation of 4 mg.

Additional analyses included assessments of change from the time of step-down randomisation in composite scores and their components, and analyses using differing definitions of relapse (ie, loss of CDAI categorisation at the time of randomisation, need for rescue). Evaluations in distinct patient populations of interest were also conducted, including patients in CDAI remission at step-down randomisation, csDMARD-IR patients from RA-BEAM and RA-BUILD, bDMARD-IR patients from RA-BEACON, larger pools of randomised patients who had shorter minimum periods (≥12 weeks, ≥24 weeks) before the present data cut-off and patient data from the initial sponsor unblinding at the August 2015 cut-off.

Safety

The occurrence and severity of all adverse events (AE) were recorded and included step-down-emergent AEs (events occurring after randomisation into the substudy), serious AEs (including infections) and AEs leading to discontinuation. An independent data safety monitoring committee oversaw the conduct of all phase 3 studies evaluating baricitinib in patients with RA, including this LTE study.

Statistical analyses

The modified intention-to-treat (mITT) population for the substudy included all patients who were randomised into the step-down substudy ≥48 weeks prior to the data cut-off date and had received ≥1 dose of study drug after randomisation. There was no prospective assessment of sample size and statistical power for efficacy analysis in the substudy. However, for a sample size of approximately 245 patients in each treatment group, there will be about 70%–90% of power to detect a true difference of 10% between groups using a two-sided Fisher's exact test based on a significance level of 0.05.

Treatment comparisons of CDAI response rate were performed using the Fisher's exact test. The Kaplan-Meier method was used to assess difference in time to relapse between treatment groups. Treatment comparisons of continuous efficacy endpoints were performed using a t-test. As this was a substudy included in an LTE protocol, no multiplicity control was applied for endpoints assessed. All statistical tests were performed at a two-sided significance level of 0.05. Summary statistics were provided for safety data. Non-responder imputation (NRI), which considers rescued or discontinued patients as non-responders, was used for CDAI response analyses (NRI analysis). To determine overall long-term efficacy irrespective of rescue, analyses were also performed including observed data collected after rescue (NRI was still applied for discontinuation). In addition, the effect of reintroduction of baricitinib 4 mg was evaluated in rescued patients.

RESULTS

Patients and disposition

At the time of the 1 September 2016 data cut-off used for these analyses, 2656 patients had been enrolled in the LTE study at 398 sites, with discontinuation <17% (online supplementary figure S2). A total of 975 patients were randomised in the step-down substudy at any time before this cut-off date (online supplementary figures S3 and S4). Of these patients, 559 were randomised ≥48 weeks prior to the cut-off and included in the 48-week analysis population (online supplementary figure S5); most patients completed 48 weeks, with discontinuation rates of 6% for baricitinib 2 mg and 5% for baricitinib 4 mg. At baseline (ie, prior to step-down randomisation), demographics and clinical characteristics, such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), CDAI and simplified disease activity index (SDAI), were well balanced between the randomised groups (table 1).

Efficacy

DMARD-IR patients (RA-BEAM, RA-BUILD and RA-BEACON combined)

Among DMARD-IR patients who achieved sustained disease control with baricitinib 4 mg, dose reduction to 2 mg resulted in statistically significant reduction in LDA rates (CDAI ≤10) at 12, 24 and 48 weeks after randomisation, though the majority of patients retained a state of LDA or remission in both groups (figure 1A). Among patients who were in remission (CDAI ≤2.8) at step-down baseline, the majority were able to maintain remission in both dose groups through 48 weeks (68% baricitinib 4 mg, 56% baricitinib 2 mg; online supplementary figure S6). However, a statistically significantly smaller proportion of patients maintained remission after 24 weeks with dose reduction to baricitinib 2 mg (61%) compared with continued baricitinib 4 mg (76%). Based on exploratory tailoring analyses, there did not appear to be any baseline characteristics or baseline disease activity measures (disease duration, corticosteroid use, CDAI state and Health Assessment Questionnaire-Disability Index (HAQ-DI)) that could be used to define which patients would be better served by continuing baricitinib 4 mg instead of stepping down to baricitinib 2 mg (data not shown).

Dose reduction to 2 mg resulted in modest but statistically significant increases in CDAI, SDAI, Disease Activity Score for 28-joint counts based on the CRP (DAS28-CRP) and DAS28 based on the ESR (DAS28-ESR) compared with maintaining the 4 mg dose (figure 2). Compared with patients who continued on baricitinib 4 mg, statistically significant increases in swollen joint count, tender joint count, Physician's Global Assessment of Disease Activity (figure 3A–C) and high-sensitivity CRP (online supplementary figure S7) were also observed after step-down to baricitinib 2 mg. Statistically significant differences between dose groups were not observed for other composite score components (pain, HAQ-DI, ESR; online supplementary figure S7). The step-down efficacy data at week 48 are included in online supplementary table S3.

Similar results were observed for the larger pools of patients randomised ≥12 and ≥24 weeks before the present data cut-off (online supplementary tables S1 and S2, respectively), and for analyses conducted at the initial August 2015 cut-off when the sponsor was first unblinded (online supplementary table S4).

csDMARD-IR (RA-BEAM and RA-BUILD combined)

CDAI ≤10 and ≤2.8 response rates were similarly reduced after dose reduction for csDMARD-IR patients (online supplementary figure S8A). Findings with respect to continuous measures for composite scores and their components were also consistent with those from the overall DMARD-IR patient group (online supplementary table S5).

bDMARD-IR (RA-BEACON)

A consistent pattern was seen for bDMARD-IR patients, though differences between the two dose groups were not statistically significant (online supplementary figure S9A and table S6), possibly due to the limited number of patients.

Table 1 Baseline characteristics and disease activity at step-down baseline RA-BEGIN, RA-BEAM, RA-BUILD, RA-BEACON analysis set

	Continued baricitinib 4 mg (N=281)	Step-down baricitinib 2 mg (N=278)
Age (year)*	54.5 (12.1)	53.6 (12.1)
Female, n (%)	211 (75.1)	212 (76.3)
Region		
USA and Canada	40 (14.2)	42 (15.1)
European Union	74 (26.3)	74 (26.6)
Central and South America, Mexico	72 (25.6)	71 (25.5)
Asia (excluding Japan)	21 (7.5)	18 (6.5)
Japan	41 (14.6)	44 (15.8)
Rest of world	33 (11.7)	29 (10.4)
Duration of rheumatoid arthritis (year)	9.5 (8.5)	9.3 (8.5)
Anticyclic citrullinated peptide positive†‡, n (%)	231 (82.2)	228 (82.0)
Rheumatoid factor positive§‡, n (%)	230 (81.9)	230 (82.7)
Concomitant glucocorticoid use¶, n (%)	130 (46.3)	112 (40.3)
csDMARDs previously used**, n (%)		
None	32 (11.4)	31 (11.2)
One	110 (39.1)	122 (43.9)
Two	86 (30.6)	71 (25.5)
≥Three	53 (18.9)	54 (19.4)
bDMARDs previously used**, n (%)		
None	246 (87.5)	243 (87.4)
One	23 (8.2)	19 (6.8)
Two	8 (2.8)	9 (3.2)
≥Three	4 (1.4)	7 (2.5)
Concomitant methotrexate use, n (%)	231 (82)	228 (82)
Methotrexate dose (mg/week)	15.2 (5.4)	15.0 (5.5)
Swollen joint count of 66	0.9 (1.7)	0.7 (1.4)
Tender joint count of 68	1.5 (2.1)	1.5 (2.5)
Physician's Global Assessment (0–100 mm)	7.9 (8.8)	7.1 (7.8)
Patient's Global Assessment (0–100 mm)	15.8 (16.4)	16.4 (15.3)
Patient's Assessment of Pain (0–100 mm)	14.5 (15.4)	15.2 (16.4)
HAQ-DI††	0.52 (0.56)	0.53 (0.55)
hsCRP (mg/L)‡‡	4.82 (7.63)	4.19 (7.59)
ESR (mm/hour)	28.0 (21.9)	25.3 (21.3)
DAS28-hsCRP	2.03 (0.65)	2.02 (0.70)
DAS28-ESR	2.73 (0.82)	2.66 (0.93)
CDAI	3.64 (2.77)	3.64 (2.78)
CDAI≤10, n (%)	280 (100)	275 (99.6)
CDAI≤2.8, n (%)	137 (48.9)	127 (46.0)
SDAI	4.12 (2.95)	4.11 (3.04)
SDAI≤11, n (%)	277 (98.9)	266 (97.8)
SDAI≤3.3, n (%)	133 (47.5)	122 (44.9)

*Data reported as mean (SD) patients unless otherwise indicated.
†Anticyclic citrullinated peptide antibody positivity (>ULN=10 U/mL).
‡Anticyclic citrullinated peptide antibody positivity and rheumatoid factor positivity is based on RA-BEYOND baseline.
§Rheumatoid factor positivity (>ULN=14 IU/mL).
¶<10 mg/day of prednisone or equivalent.
**Previous csDMARD and bDMARD use is based on originating study baseline.
††Scores on the HAQ-DI range from 0 to 3, with higher scores indicating greater disability.
‡‡hsCRP (ULN=3.0 mg/L).
bDMARD, biologic disease-modifying antirheumatic drug; CDAI, clinical disease activity index; csDMARD, conventional synthetic disease-modifying antirheumatic drug; DAS28-ESR, Disease Activity Score for 28-joint counts based on the ESR; DAS28-hsCRP, DAS28 based on the hsCRP level; ESR, erythrocyte sedimentation rate; HAQ-DI, Health Assessment Questionnaire-Disability Index; hsCRP, high-sensitivity C-reactive protein; N, number of modified intention-to-treat patients who completed 48 weeks in the step-down substudy, or would have completed 48 weeks if not discontinued; n, number of patients in the specified category; SDAI, simplified disease activity index; ULN, upper limit of normal.

DMARD-naïve (RA-BEGIN)

In the small group of DMARD-naïve patients, where sustained CDAI remission was required for step-down study participation, minimal differences were observed between the continued baricitinib 4 mg and baricitinib 2 mg groups over 48 weeks in the step-down study (online supplementary figure S10A and table S7); in both dose groups, most patients were able to maintain remission status, and most patients who lost remission status were able to retain LDA (online supplementary figure S10A).

Other topics

Maintenance of step-down disease state

When considering the individual patient's CDAI state at the time of randomisation (either LDA or remission), although most patients maintained this in both groups over time, dose reduction resulted in a statistically significantly higher proportion of patients who lost their state of disease control compared with those who remained on the 4 mg dose (29% for baricitinib 4 mg vs 43% for baricitinib 2 mg at week 48, p≤0.01; online supplementary table S8).

Effect of rescue

Rescue rates through week 48 in the overall 48-week mITT analysis set were 10% for those who continued on baricitinib 4 mg and 18% for those who stepped down to baricitinib 2 mg (online supplementary figure S5). Most rescued patients could regain LDA or remission after rescue to baricitinib 4 mg (66.7% for baricitinib 2 mg→4 mg 24 weeks after rescue in the DMARD-IR group; online supplementary table S9). Among the 16 patients who did not recapture their baseline (randomisation) CDAI status 24 weeks after returning from 2 mg to the 4 mg rescue dose, the majority (13/16) were able to do so at a subsequent time point.

Additional analyses were performed to determine overall efficacy irrespective of rescue. Results showed more patients achieved LDA or remission when postrescue data were used in the analyses than when postrescue data were censored and imputed as non-response (figure 1B); online supplementary figure S8B, S9B and S10B).

Durability of treatment effect

The durability of treatment effect was evaluated by examining the kinetics of relapse. Compared with patients who continued on baricitinib 4 mg, dose reduction to 2 mg resulted in significantly more patients having a quicker relapse. This observation was consistent across various definitions of relapse, including loss of step-down eligibility criteria (figure 4A), rescue (figure 4B), loss of step-down eligibility criteria at two consecutive scheduled visits (online supplementary figure S11A) or loss of step-down baseline CDAI status (online supplementary figure S11B).

Safety

Incidence rates from step-down baseline through 48 weeks for step-down-emergent AEs including non-serious infections were numerically higher in patients continuing on baricitinib 4 mg compared with those who stepped down to baricitinib 2 mg (table 2). Incidence rates for serious AEs (including serious infections) and AEs that led to discontinuation through 48 weeks in the step-down study were similar between groups. Findings were generally similar for the subset of patients in CDAI remission (≤2.8) at the step-down baseline, although in this smaller analysis set, the rate of events leading to discontinuation was numerically higher in the continued 4 mg group (online supplementary table S10).

DISCUSSION

RA-BEYOND is an ongoing LTE study designed to assess long-term safety and durability of baricitinib 4 mg or 2 mg administered once a day. The study included a randomised, double-blind evaluation of dose reduction from 4 mg to 2 mg in patients who achieved sustained disease control on the higher dose. In this substudy,

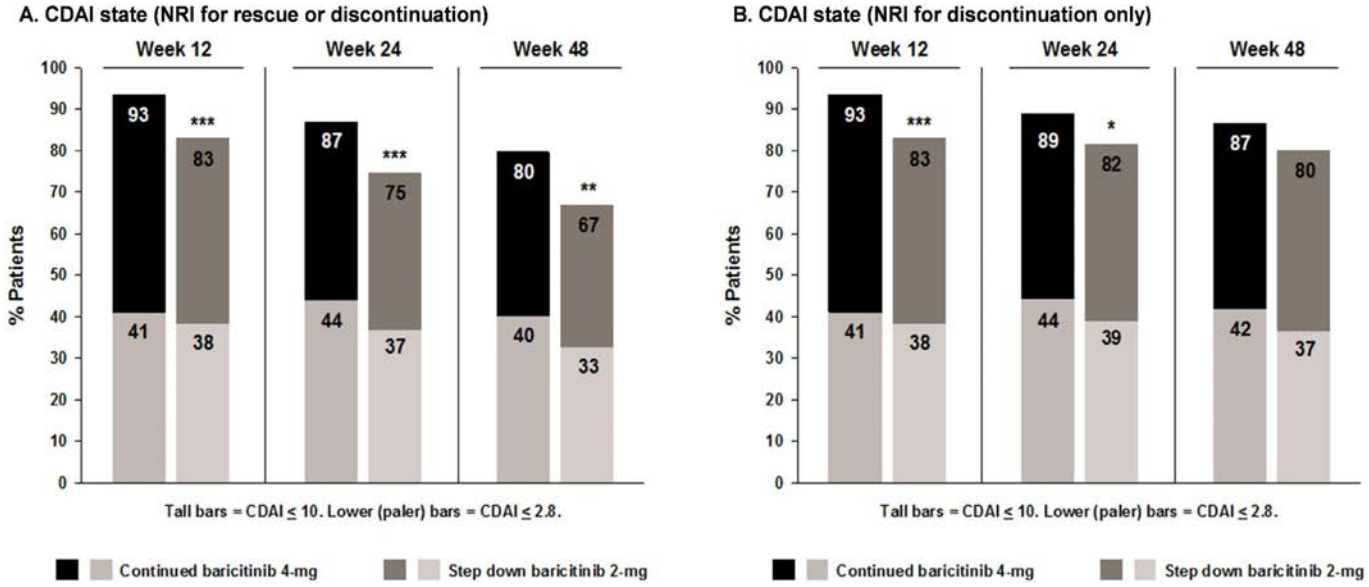

Figure 1 Step-down efficacy through week 48: categorical CDAI state DMARD-IR (RA-BEAM, RA-BUILD, RA-BEACON) analysis set. Patients completed 48 weeks in the step-down substudy, or would have completed 48 weeks if not discontinued. All patients had CDAI≤10 at step-down baseline; a subset could have had CDAI≤2.8. n=245 for each group at each time point. For panel (A), NRI was applied for rescue or discontinuation. For panel (B), observed data were used after rescue; NRI was applied for discontinuation. *P≤0.05; **P≤0.01; ***P≤0.001 versus the continued on baricitinib 4 mg group. CDAI, clinical disease activity index; DMARD-IR, disease-modifying antirheumatic drug-inadequate responder; NRI, non-responder imputation.

dose reduction to 2 mg once a day was associated with statistically significant, if modest, increases in disease activity at subsequent assessments up to 48 weeks. However, most patients in both the continued 4 mg and step-down 2 mg groups retained the state of LDA or remission that led to their randomisation, and a large majority of patients who failed to maintain LDA or remission after stepping down to baricitinib 2 mg were able to recapture control with return to baricitinib 4 mg, if needed.

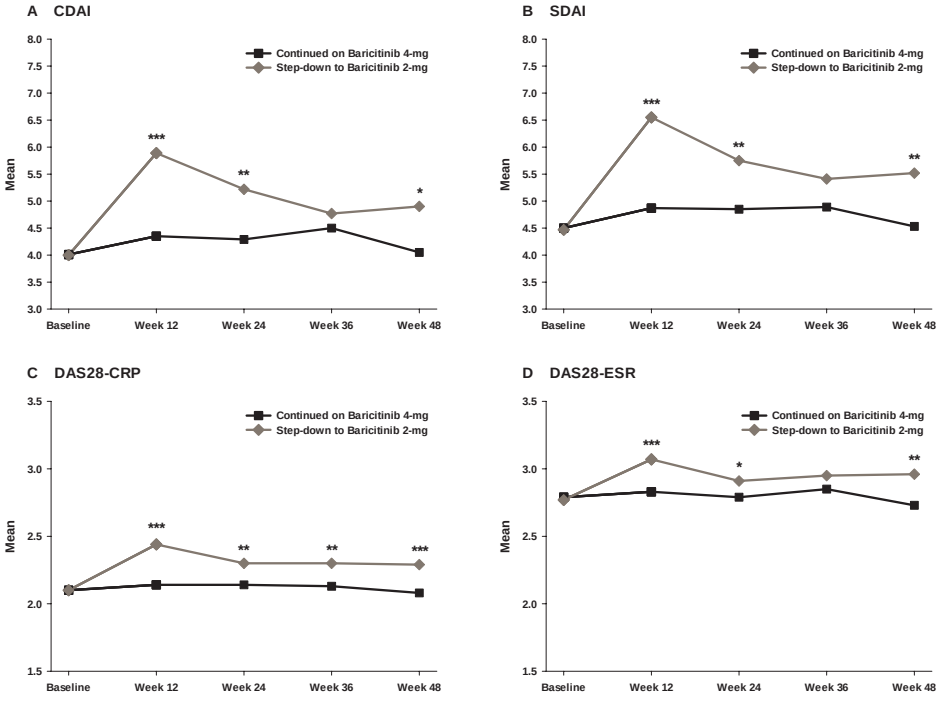

Figure 2 Step-down efficacy through week 48: continuous CDAI (A), SDAI (B), DAS28-CRP (C) and DAS28-ESR (D), DMARD-IR (RA-BEAM, RA-BUILD, RA-BEACON) analysis set. Values are observed means. P value based on difference in change from baseline between groups. Patients completed 48 weeks in the step-down substudy, or would have completed 48 weeks if not discontinued. *P≤0.05; **P≤0.01; ***P≤0.001 versus the continued on baricitinib 4 mg group. CDAI, clinical disease activity index; DAS28-CRP, Disease Activity Score for 28-joint counts based on the C-reactive protein; DAS28-ESR, DAS28 based on the erythrocyte sedimentation rate; DMARD-IR, disease-modifying antirheumatic drug-inadequate responder; SDAI, simplified disease activity index.

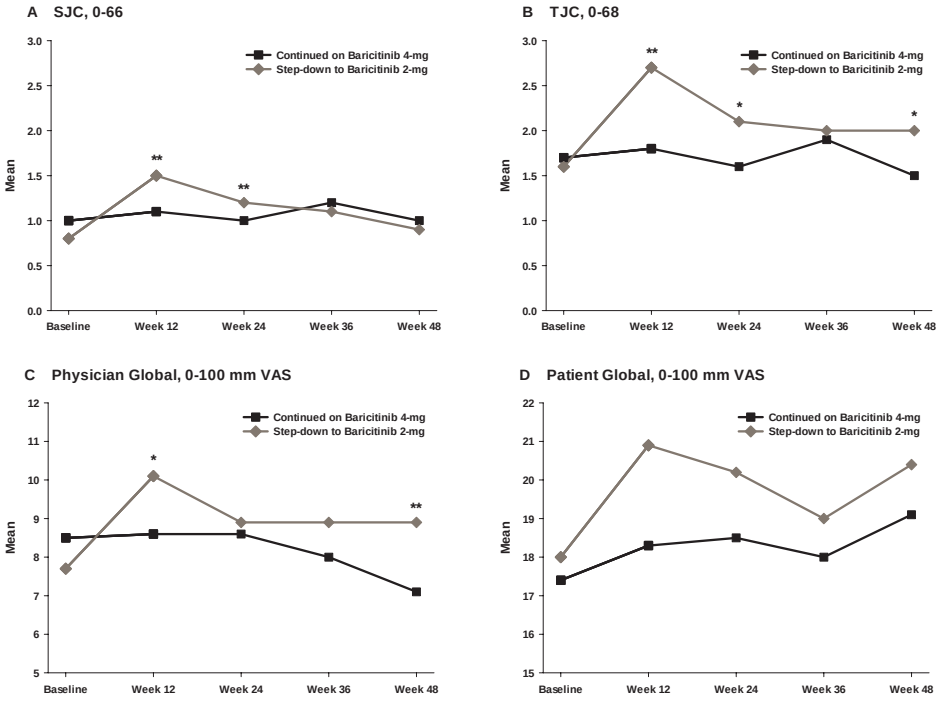

Figure 3 Step-down efficacy through week 48: continuous composite disease activity components SJC (A), TJC (B), Physician Global VAS (C), Patient Global VAS (D) DMARD-IR (RA-BEAM, RA-BUILD, RA-BEACON) analysis set. Values are observed means. P value based on difference in change from baseline between groups. Patients completed 48 weeks in the step-down substudy, or would have completed 48 weeks if not discontinued. *P≤0.05; **P≤0.01; ***P≤0.001 versus the continued on baricitinib 4 mg group. DMARD-IR, disease-modifying antirheumatic drug-inadequate responder; SJC, swollen joint count; TJC, tender joint count; VAS, visual analogue scale.

Timely induction of sustained remission/LDA is a central element of the treat-to-target principles that underlie contemporary standards and professional recommendations in the management of RA.[1] In recent years, guidelines have also recommended that DMARD taper (but not cessation) be considered in patients who have achieved sustained disease control.[2 3] However, to date, few rigorous clinical studies have been conducted to inform such a treatment strategy. Randomised clinical investigation of dose taper following induction of control has been conducted for tumour necrosis factor inhibition; published findings were consistent with those of the present study.[7] To our knowledge, the present study is the first randomised, blinded clinical trial to investigate this treatment strategy with a tsDMARD, and the first for any DMARD to form part of an initial registration programme. Associated data have been reflected in labelling in several regions where baricitinib is approved, including European countries, USA and Japan.

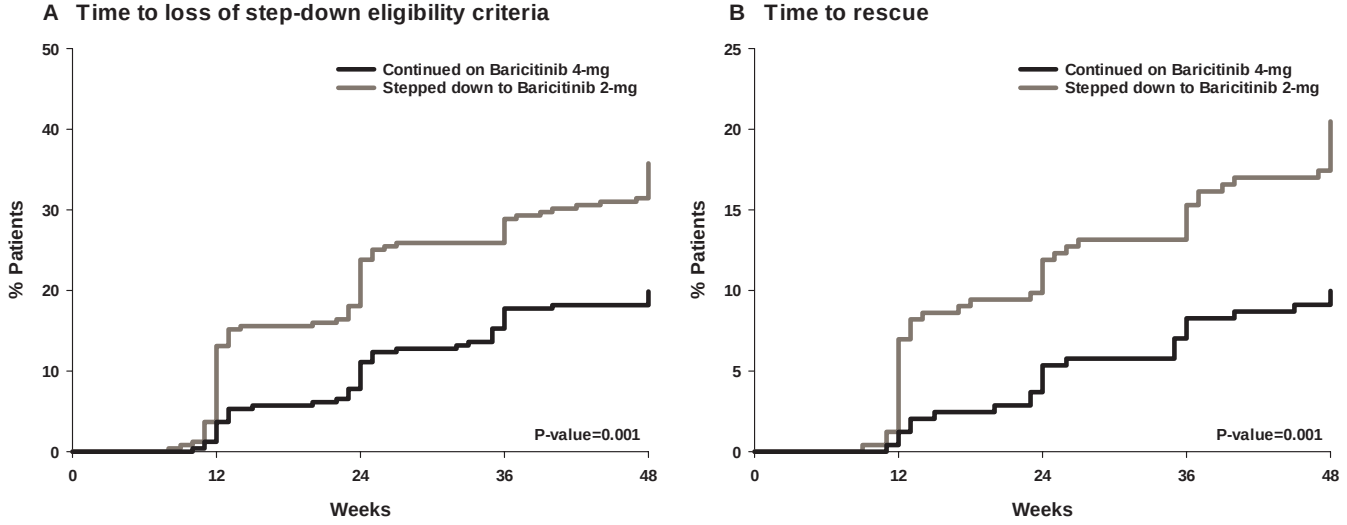

Figure 4 Step-down efficacy through week 48: time to relapse RA-BEAM, RA-BUILD, RA-BEACON analysis set. Patients completed 48 weeks in the step-down substudy, or would have completed 48 weeks if not discontinued. P value is from the Wilcoxon test. For panel (A), relapse was defined as loss of step-down eligibility criteria, or CDAI>10 for DMARD-IR patients originating from RA-BUILD, RA-BEAM or RA-BEACON. For panel (B), relapse was defined as rescue. *P≤0.05; **P≤0.01; ***P≤0.001 versus the continued on baricitinib 4 mg group. CDAI, clinical disease activity index; DMARD-IR, disease-modifying antirheumatic drug-inadequate responder.

Table 2 Step-down safety (weeks 0–48) RA-BEGIN, RA-BEAM, RA-BUILD, RA-BEACON analysis set

n (EAIR/100 PYE)	Continued baricitinib 4 mg (n=281) PYE=254.9	Step-down baricitinib 2 mg (n=278) PYE=236.7
Step-down-emergent adverse event	170 (66.7)	140 (59.2)
Infection	78 (30.6)	59 (24.9)
Serious adverse event	19 (7.5)	15 (6.3)
Serious infection	5 (2.0)	4 (1.7)
Adverse event leading to discontinuation	7 (2.7)	8 (3.4)

Patients completed 48 weeks in the step-down substudy, or would have completed 48 weeks if not discontinued.
EAIR, exposure-adjusted incidence rate; PYE, patient-years of exposure.

The detailed 1-year substudy results reported here complement this information and are informative to clinicians who may wish to consider using a reduced maintenance dose of baricitinib after induction with 4 mg once a day.

Guidelines may recommend consideration of DMARD taper for patients achieving sustained disease control, but in this large, randomised study, increases in disease activity were seen across populations, analyses and outcome measures when the dose was blindly tapered from 4 mg to 2 mg. This is consistent with observations from completed studies supporting 4 mg once a day as the more efficacious dose of baricitinib for patients with RA.[11–14] The question therefore arises as to whether baricitinib dose taper following induction of sustained control is in fact an advisable approach in routine practice, where patients and physicians would be aware of the dose reduction, potentially further accentuating increases in perceived symptoms and signs of RA compared with the current blinded study. A number of observations can be considered supportive. First, 2 mg proved an acceptably efficacious dose for many patients, as evidenced by the fact that fewer than 1 in 5 dose-tapered patients were rescued back to 4 mg by their treating physicians. Of additional importance, for those who did need rescue, prior control of disease activity could be re-established with return to 4 mg. Finally, as a general principle, as long as acceptable efficacy is not sacrificed, use of lower doses may be desirable from a safety perspective, in particular for chronic treatments that are relatively novel. In this regard, although the data are limited, some safety trends appeared to favour the 2 mg dose, including overall AE and infection rates. Therefore, attempted dose taper after induction of sustained RA control appears a reasonable consideration with baricitinib.

This study has a number of limitations. To preserve blinding, no radiographs were taken during the step-down substudy and, therefore, we cannot provide data on potential structural implications of dose reduction. Conclusions pertaining to DMARD-naïve and bDMARD-IR patient subgroups are limited by the small numbers of such patients presently included in the study. Patients with sustained control were randomised only after a minimum of 15 months' treatment with baricitinib 4 mg. This was to provide sufficient stable exposure at this dose during the pivotal programme for intended regulatory registration purposes. It may not reflect the timing at which DMARD dose taper might generally be considered for individual patients in clinical practice,[2 3] according to their individual circumstances, treatment goals and responses. It is also presently not possible to determine which characteristics, if any, might identify patients unsuited to dose reduction, for instance, those who might lose established disease control and then fail to recapture following return to 4 mg. However, it is reassuring to observe, based on present analyses, that if a subset of such patients exists it would appear to be small. Treatment guidelines currently advocate consideration of DMARD taper when sustained remission has been achieved, whereas for patients from studies other than the DMARD-naïve trial RA-BEGIN, LDA or remission was used as the inclusion criterion in this baricitinib dose-taper study. This design element aimed to recognise that a substantial proportion of participants were expected (and transpired) to have failed multiple prior DMARDs, and that for such patients, LDA may be a more feasible treatment target than remission. The fact that observations in the subset of patients in CDAI remission when randomised were generally consistent with the overall is reassuring as to the generalisability of the results. Use of CDAI (rather than SDAI or DAS28) as the principal disease activity instrument for inclusion may raise the question as to whether results could differ if patients were selected for dose step-down only if they achieved sustained LDA/remission using an instrument with an acute phase marker. The observation that LDA/remission rates at baseline were almost identical for SDAI and CDAI would seem to argue against this. No data were generated to investigate dose taper below 2 mg daily; although a reasonable question to consider, the results of earlier phase 2 dose-ranging studies did not further investigate such doses, as they would not offer patients an acceptable probability of efficacy in the current treatment environment.[15–17] Finally, the present analyses are confined to the first 48-week period following randomised dose reduction. Expansion of these observations through evaluations beyond 48 weeks and in larger numbers of patients will be the subject of future analyses and intended disclosures from the ongoing study.

In conclusion, these results from a large, ongoing phase 3 randomised dose-taper study indicate that in patients with RA for whom sustained clinical disease control has been induced with baricitinib 4 mg once a day, dose taper to baricitinib 2 mg results in increased disease activity for some patients. However, most patients can either retain clinical LDA/remission following dose taper, or regain it with return to 4 mg if needed. A slightly lower incidence rate of treatment-emergent AEs (including infections) was observed after step-down in the dose-tapered group compared with patients who continued baricitinib 4 mg.

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CLINICAL SCIENCE

Effects of B-cell directed therapy on the preclinical stage of rheumatoid arthritis: the PRAIRI study

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ABSTRACT

Objectives We explored the effects of B-cell directed therapy in subjects at risk of developing autoantibodypositive rheumatoid arthritis (RA), who never experienced inflammatory arthritis before, and explored biomarkers predictive of arthritis development.

Methods Individuals positive for both anti-citrullinated peptide antibodies and rheumatoid factor but without arthritis were included in a randomised, double-blind, placebo-controlled study to receive a single infusion of 1000 mg rituximab or placebo.

Results Eighty-one individuals received treatment and were followed up for a mean of 29.0 (0–54) months, during which 30/81 (37%) individuals developed arthritis. The observed risk of developing arthritis in the placebo-treated group was 40%, which was decreased by 55% (HR 0.45, 95% CI 0.154 to 1.322) in the rituximab-treated group at 12 months. Rituximab treatment caused a delay in arthritis development of 12 months compared with placebo treatment at the point when 25% of the subjects had developed arthritis (p<0.0001). Erythrocyte sedimentation rate and the presence of anti-citrullinated α-enolase peptide 1 at baseline were significant predictors of arthritis development.

Conclusions A single infusion of 1000 mg rituximab significantly delays the development of arthritis in subjects at risk of developing RA, providing evidence for the pathogenetic role of B cells in the earliest, prearthritis stage of autoantibody positive RA.

Key messages

What is already known about this subject?

- From earlier research reports we learnt that rheumatoid factor (RF) and Anti-Citrullinated Peptide Antibodies (ACPAs) can be found in the peripheral blood of individuals >10 years before the development of autoantibody positive rheumatoid arthritis.
- Research leading to the recognition of this phase of systemic autoimmunity has not only supported the view that the pathogenetic process might not be initiated in the joint but created an opportunity to potentially delay the clinical onset of disease by a targeted intervention in this early phase.
- B-cells play a pivotal role in this process as apart from being predecessors of cells that produce immunoglobulins including RF and ACPAs, B-cells are efficient antigen presenting cells, may activate T cells in the context of co-stimulatory signals, and produce a variety of cytokines.
- Indeed, B-cell targeted therapy is effective in early as well as in late established RA.

Added value of this study

- With a targeted intervention aimed at eliminating a cell key to the underlying pathogenetic process, the B cell, and influencing their function and products, the results of this study support the concept of a preventive window of opportunity.
- In an exploratory randomised, double-blind, placebo controlled clinical trial, we show that a single infusion of 1000 mg of rituximab delays the onset of clinical signs and symptoms of arthritis in subjects who are at a high risk of developing seropositive RA.

Experimental interventions during the earliest stages of immune-mediated inflammatory diseases may provide important insights into their pathogenesis. Autoantibody positive rheumatoid arthritis (RA) is a common and prototypic autoimmune disease. This condition can be preceded by a phase of systemic autoimmunity during which circulating autoantibodies, increased acute phase reactants, proinflammatory cytokines and chemokines are found, even years before the development of clinically evident arthritis.[1–4] Elevated levels of autoantibodies such as IgM rheumatoid factor (IgM-RF), anti-citrullinated peptide antibodies (ACPA) and other RA-specific antibodies against post-translationally modified proteins can be detected in blood samples of individuals later diagnosed with seropositive RA with a median of 5 years before arthritis becomes evident.[3] During this stage, clonal changes in the peripheral blood B-cell receptor (BCR) repertoire can be detected[5] but the synovial tissue is usually completely normal.[6 7] The risk of developing arthritis within 2 years in individuals positive for both ACPA and IgM-RF is ~40%.[8] This risk appears to be higher in individuals with musculoskeletal symptoms,[9] smokers,[10] in people who are obese[10] and in those with decreased vagus nerve tone.[11] The contribution of the HLA-DRB1 alleles encoding the shared epitope to RA development is mainly mediated via the presence of ACPA and does not appear to be a strong predictor of RA

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Key messages

How might this impact on clinical practice or future developments?

- According to the current treatment paradigm, treatment of RA is initiated after the clinical onset of the disease.
- With this approach only a small minority of patients achieve disease remission, which is the treatment goal, and many patients need chronic treatment with biopharmaceuticals or targeted small molecules.
- The results of this study support the view that it may be easier to control the disease process by targeted intervention before signs and symptoms of arthritis have developed, which suggests the existence of a 'preventive window of opportunity'.

development within the ACPA positive pre-RA population.[12] The existence of this preclinical phase offers the opportunity to intervene, and prevent or delay the disease from developing into clinically manifest arthritis.[13 14]

The presence of circulating autoantibodies and changes in BCR repertoire years before the clinical onset of the disease, the specificity of ACPA for the diagnosis of RA and the presence of B cells and plasma cells at the site of inflammation in early established disease[15] highlight the importance of B cells in the pathogenesis of RA. Indeed, treatment of patients with RA with depleting antibodies directed at B cells is effective in late as well as earlier stages of established RA.[16–19] However, there is no experimental evidence for B cells as therapeutic or secondary preventive target during the prearthritis stage of this autoantibody positive immune-mediate inflammatory disease. The purpose of this phase IIb, randomised, double-blind, placebo-controlled study was to test whether B-cell depletion could alter the development of the disease in individuals at high risk of developing RA. We also aimed to identify biomarkers predictive of arthritis development.

METHODS

Participants and study design

One hundred and nine subjects with arthralgia[8 20 21] without any evidence of clinical arthritis (of 66 joints examined) were recruited via rheumatology outpatient clinics of seven participating centres across the Netherlands between January 2010 and December 2013, of which 82 were eligible to be randomised and included in this multicentre, randomised, double-blind, placebo-controlled clinical study (The PRAIRI study: Prevention of clinically manifest rheumatoid arthritis by B-cell directed therapy in the earliest phase of the disease, NTR1969) (figure 1). The original aim of recruiting 90 eligible subjects was amended due to a slow recruitment rate encountered during the third year of the study, still keeping within the original power calculations. Ways of recruiting potential subjects included subjects referred via their general practitioner, engagement of first-degree relatives of known patients with RA via the outpatient clinic (Academic Medical Centre (AMC), Amsterdam) and first-degree relatives recruited at public fairs across the Netherlands: the proband diagnosis of RA was determined by questioning of the first-degree relative by a trained physician who attended the fair. These potential subjects were invited to be screened at the outpatient clinics of the participating centres. To be included into the study, the subjects needed to be between 18 and 80 years old, IgM-RF as well as ACPA (a-CCP2; Immunoscan CCPlus (Euro Diagnostica No RA-96plus) ELISA tests) positive and had to never experienced an inflammatory arthritis nor been treated with a disease-modifying antirheumatic drug

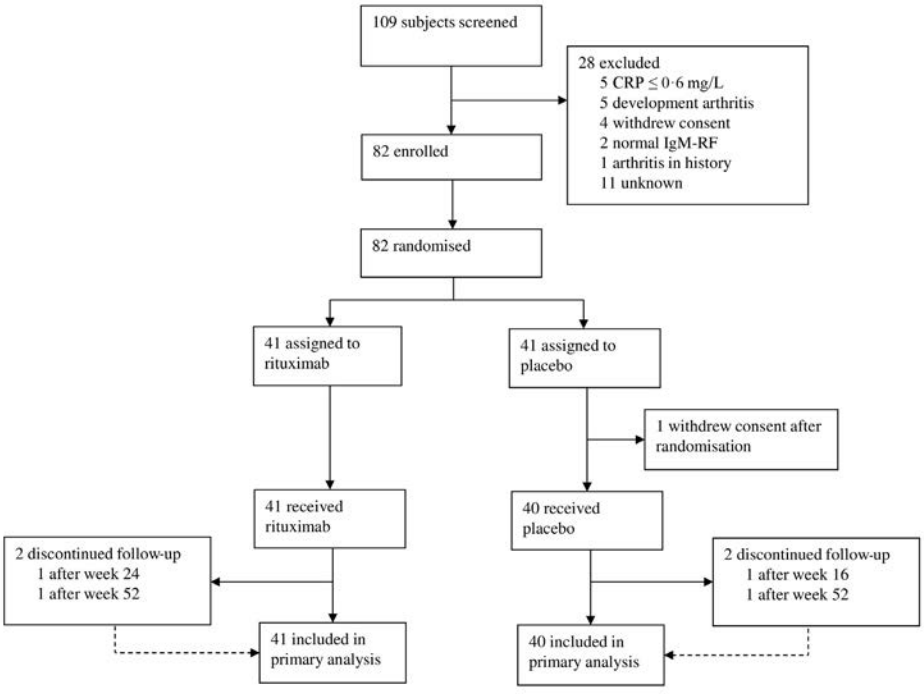

Figure 1 Trial profile. CRP, C-reactive protein; RF, rheumatoid factor.

(DMARD) in the past (phase c+d of the preclinical phase of RA).[22] In addition, the subjects needed to have either C-reactive protein (CRP) levels >0.6 mg/L at screening (the lower limit of detection of the high-sensitivity (hs) CRP assay), or subclinical synovitis as determined by ultrasound or MRI using gadolinium performed in the context of routine clinical care. The cut-off level for serum CRP levels of 3 mg/L in the protocol published on the NTR website was amended into a minimum level of 0.6 mg/L, a change triggered by the advent of the hsCRP assay in routine clinical practice, as the target study population does not have overt inflammation during the preclinical stage of the disease. This amendment was approved by the Medical Ethics Committee of the AMC before enrolment of any subject into the study, who all provided informed consent. The subjects were randomised in a 1:1 ratio to receive either 1000 mg of rituximab (MabThera, Roche Nederland) or placebo (NaCl 0.9%) intravenously, after *all* receiving 100 mg methylprednisolone premedication according to the regular treatment schedule used in patients with RA to prevent potential infusion-related adverse events. Randomisation was stratified for age (<40 years, ≥40 years) as well as gender. One individual withdrew informed consent before receiving study treatment. The primary outcome was time to development of clinical arthritis in subjects in both treatment groups. Clinical arthritis was defined by a swollen and tender joint as observed by two independent, blinded investigators (one rheumatological research physician well trained in assessing joints in clinical trials and one faculty rheumatologist); consensus was reached after assessing the joint together in case of initial discrepancy (for details on the amended in and exclusion criteria compared with the NTR registration information and sample size calculation, visit scc, see online supplementary file). The study physicians, monitors and subjects remained blinded during the study, and all assessments were done by assessors blinded to the treatment allocation. The members of an independent data safety monitoring board and one independent physician overseeing laboratory results for safety reasons were unblinded to the treatment allocation.

Explorative analysis of the effects of study treatment on peripheral blood T and B-cell numbers, their subpopulations using fluorescence-activated cell sorting (FACS) analysis and the presence and levels of disease-specific antibodies were measured in subsets of participants depending on the availability of the samples for the different time points. We measured serum antibodies against various citrullinated peptides and arginine-containing peptides, including anti-alpha citrullinated enolase peptide-1 (CEP-1). The difference between citrullinated and arginine peptides was calculated and the cut-off level defining positivity for each ACPA specificity was determined on the basis of the earlier determined 98th percentile.[7] Absolute levels (arbitrary units, AU) calculated from a calibration sample were used to follow individual and mean changes over time (details on the detection of other autoantibodies against citrullinated peptides can be found in the online supplementary file).

Statistical analysis

All subjects who received treatment were included in the primary and safety analysis. Kaplan-Meier survival analysis was used to determine the effect of rituximab treatment on the development of arthritis. Whether the rituximab treatment effect on the hazard to develop arthritis varied with follow-up time was evaluated using Cox proportional hazards regression by including the interaction between treatment and follow-up time as a continuous time-dependent variable in the model. The Cox model was also used to evaluate the effects of baseline patient characteristics and biomarkers on the hazard to develop arthritis. The change patterns over time during the study of time-dependent biomarkers were analysed using linear mixed-effects regression models with follow-up time, treatment and their interaction as fixed effects and with random intercept and slope(s) of follow-up time per patient as random effects. Joint models were used to evaluate the associations between the changing values over follow-up time of the time-dependent biomarkers and the arthritis hazards. Included in these joint models (a combination of the Cox and mixed-effects models) as a covariate were the predicted values of the time-dependent biomarkers of all individuals at risk for developing arthritis at all time points during follow-up. These joint models were evaluated for each biomarker separately. SPSS V.22 (SPSS), SAS V.9.3 and R V.3.3.2 were used to analyse the data.

Table 1 Baseline characteristics of subjects assigned to the two treatment groups

	Rituximab group (n=41)	Placebo group (n=40)
Sex		
Female	28 (68%)	24 (60%)
Male	13 (32%)	16 (40%)
Age (years)	53.0 (45.0–58.0)	52.5 (43.0–57.0)
C-reactive protein concentration (mg/L), normal <5 mg/L	3.0 (1.5–5.2)	2.9 (1.0–5.0)
Erythrocyte sedimentation rate (mm/hour), range 1–140	10.0 (5.0–15.5)	10.0 (5.0–15.8)
Patient Global Assessment of Disease Activity (mm), range 0–100	31.0 (13.0–52.0)	23.5 (8.0–40.5)
TJC68 (range 0–68, 68=maximum)	2.0 (0–29.0)	0.0 (0–48.0)
SJC66 (range 0–66, 66=maximum)	0.0	0.0
IgM-RF positive*		
Low positive level	15.0 (37%)	16.0 (40%)
High positive level	25.0 (61%)	23.0 (58%)
ACPA positive†		
Low positive level	6.0 (15%)	4.0 (10%)
High positive level	34.0 (83%)	36.0 (90%)
Shared epitope positive‡	21/30 (70.0%)	24/33 (72.7%)
Body mass index (kg/m^2)	28.2 (24.4–31.3)	26.2 (24.4–29.2)
Smoking history ever	32 (84%)	27 (71%)
Current NSAID use	23 (56%)	26 (65%)

Data are n (%), median (IQR). High positive level is defined by >3 times the upper limit of normal; low positive level is defined by ≤3 times the upper limit of normal.
ACPA, anti-citrullinated peptide antibody; IgM-RF, IgM rheumatoid factor; NSAID, non-steroidal anti-inflammatory drug; SJC66, swollen joint count assessing 66 joints; TJC68, tender joint count assessing 68 joints.
*Of two subjects, IgM titres were not determined at baseline; they were elevated in a prebaseline assessment.
†Of one subject, ACPA titres were not determined at baseline; they were elevated in a prebaseline assessment.
‡Of 11 subjects of the rituximab and seven subjects of the placebo group no data on shared epitope are available.

RESULTS

No significant differences in demographic and clinical features such as age, gender, inflammatory markers in the peripheral blood, tender and swollen joint counts, levels of IgM-RF or ACPA, smoking history, body mass index (BMI) and use of non-steroidal anti-inflammatory drugs (NSAID) were observed between the groups at baseline (table 1). None of the subjects had a swollen joint at baseline per protocol. All included subjects met the inclusion criteria based on laboratory parameters and none underwent imaging to ascertain inclusion status. However, in the context of

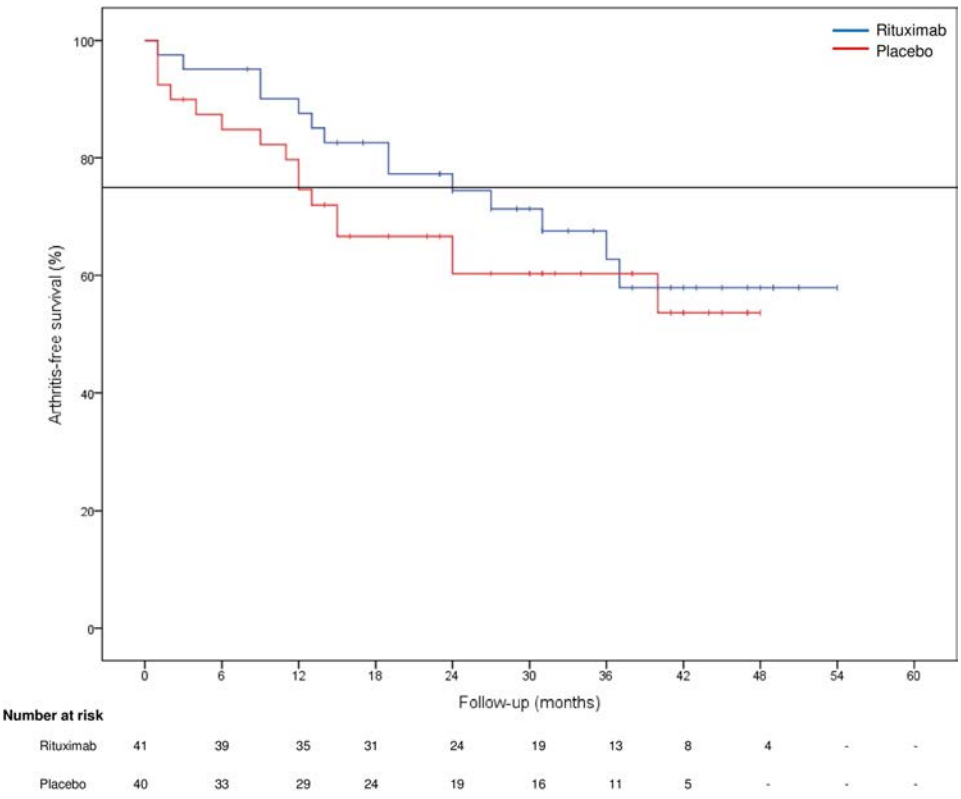

Figure 2 Kaplan-Meier survival plot for primary endpoint of arthritis development. Arthritis-free survival (%) depicted over time in months. At the 25th percentile, a difference of 12 months between the group receiving placebo (blue) versus rituximab (red) was observed (black horizontal line). The number of individuals at risk in each group at every follow-up time point is shown below the graph, follow-up was discontinued after development of arthritis.

routine care, a subset of 48 subjects had undergone imaging of their joints prior to the study of which 46 did not show synovitis in those joints.

Response to treatment

After treatment of 81 subjects (41 received rituximab and 40 placebo), follow-up of a median of 29 months (IQR 14–40; range 0–54 months; one subject developed arthritis 3 weeks after treatment) was available. The risk of development of arthritis over the total follow-up time in the placebo group was 40%. From the routine characteristics measured at baseline, including all baseline characteristics mentioned in table 1: gender, age, CRP, erythrocyte sedimentation rate (ESR), Patient Global Assessment of Disease Activity, tender joint count, IgM-RF presence as well as high and low positive levels, ACPA presence as well as high and low positive levels, BMI, smoking history (ever or never) and current NSAID use, only ESR was correlated with the development of arthritis (p=0.02). Otherwise, no statistically significant differences were found between the subjects who developed arthritis and those who did not. Power calculations were not performed on these baseline characteristics before study start.

Treatment with only one single infusion of rituximab reduced the baseline risk of arthritis development observed in the placebo group with 55% at 12 months (HR 0.45, 95% CI 0.15 to 1.32; p=0.15; see figure 2) and 53% at 18 months (HR 0.48, 95% CI 0.19 to 1.19) of follow-up. The treatment led to a delay of arthritis development of 12.0 months at the point where 25% of the subjects in both treatment groups developed arthritis (the 25th percentile or 75% free of arthritis of the cumulative arthritis-free survival;12 months placebo vs 24 months rituximab). A Cox proportional hazards model was used to analyse the data with treatment and treatment by follow-up time interaction, confirming the statistically significant although temporary preventive effect of rituximab treatment (p<0.0001). The observed effect on delaying arthritis development attenuated over time. Over the complete follow-up time arthritis development was seen in 30 of the 81 subjects: 16/40 (40%) in the placebo group after a median period of 11.5 months (IQR 2.5–15.0, range 1.0–40.0 months) and 14/41 (34%) in the rituximab group after a median period of 16.5 months (IQR 9.0–28.0, range 1.0–37.0 months). The risk of arthritis development over the total follow-up time after a single infusion was not statistically significant (p=0.448) between the two groups.

At the moment of arthritis development, 13 subjects in the rituximab treatment group fulfilled the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010 classification criteria for RA,[23] whereas one patient was classified as having unclassified arthritis.[22] Of the 16 subjects in the placebo group who developed arthritis, 11 fulfilled the ACR/EULAR classification criteria for RA at the time of arthritis development, while five subjects were classified as having unclassified arthritis based on the low number of clinically inflamed joints and low levels of ESR and CRP at the time of arthritis development; three of these were classified as RA after further follow-up. Overall, four subjects without arthritis were lost to follow-up (two in each treatment group).

Exploratory analysis of biomarkers

The association of baseline and repeatedly measured clinical and serological biomarkers with the arthritis hazard was evaluated based on data of all 81 patients. Of these markers, the ESR (mm/hour; HR 1.03, 95% CI 1.01 to 1.06; p=0.016) and the presence of anti-citrullinated α-enolase peptide 1 (anti-CEP-1; HR

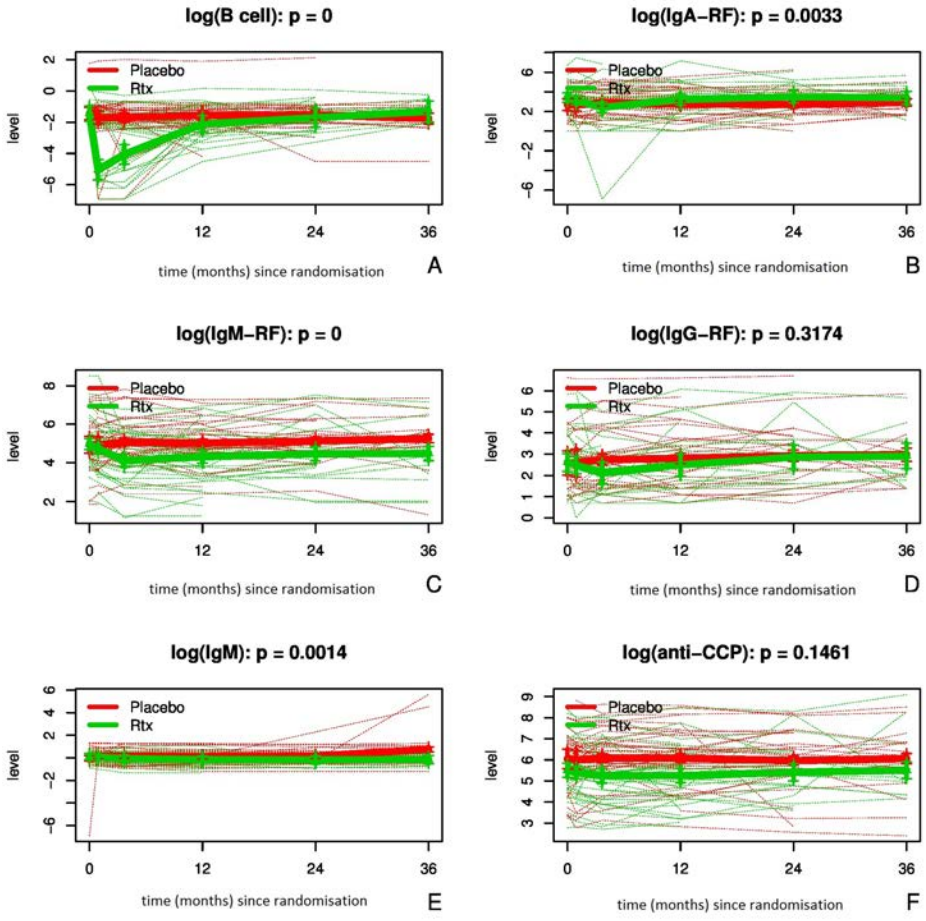

Figure 3 (A–F) Changes of B-cell numbers and B-cell related biomarkers. Total number of B cells (Log_{10} 10^9/L; A), serum IgA rheumatoid factor (RF) (Log kU/L; B), IgM-RF (Log kU/L; C), IgG-RF (Log kU/L; D); IgM (Log g/L; E), and anti-citrullinated cyclic peptide (CCP) test levels (Log kAU/L; F) from baseline to follow-up time points (days) measured in the individuals treated with placebo (red) and rituximab (green). The thin lines represent changes in the individuals and the thick lines represent the mean numbers/levels for each group. Vertical lines represent the 95% CIs. Statistically significant differences (shown p values) between the two treatment groups were found for all values depicted here, except for the serum IgG-RF and anti-CCP levels.

3.71, 95% CI 1.51 to 9.18; p=0.01) in the serum at baseline were positively correlated with the development of arthritis (Supplementary file 1).

Changes in total B-cell numbers ($\times 10^9$/L), their subsets and disease relevant autoantibodies were available in a subgroup of 78 individuals (of whom 40 received rituximab and 38 placebo treatment) from baseline up to 3 years of follow-up. In addition, in a smaller subgroup (n=47; n=19 rituximab and n=28 placebo) serum levels of IgA-RF (kU/L), IgG-RF (kU/L), IgM-RF (kU/L), IgA (g/L), IgG (g/L), total IgM (g/L) as well as anti-citrullinated cyclic peptide (anti-CCP; kAU/L) could be measured at the same time points. A clear and highly significant decrease in the total number of B cells was observed within 4 weeks after treatment in the subjects receiving rituximab (p<0.0001), which was followed by a drop in serum levels of IgA-RF, IgM-RF and IgM, reaching the level of statistical significance (p=0.003, p<0.0001 and p=0.001, respectively) at all time points. Anti-CCP and IgG-RF serum levels dropped as well, but there was no statistical difference between the two treatment groups (p=0.146 and p=0.317, respectively; all values see figure 3A–F). No differences in the levels of total IgA and IgG between the two treatments were found.

The changes in serum levels of IgA-RF, IgM-RF and IgM in the group of individuals treated with rituximab were not associated with the development of arthritis.

A more detailed analysis of subsets of B-cell populations using FACS analysis was performed in 45 subjects (n=18 rituximab, n=27 placebo), based on availability of samples. The results of these analyses can be found in the online supplementary figures S1 and S2.

Safety of the treatment

Study treatment was generally well tolerated with only mild infusion-related symptoms and no serious infections leading to hospitalisation. Although the serious adverse event rate was significantly higher in the rituximab group (13/41 vs 3/40: p=0.014), all events were considered not to be related to the treatment per the independent data safety monitoring board unblinded for the treatment assignment (table 2).

DISCUSSION

In this interventional, proof-of-mechanism study, we show that a single infusion with rituximab is well tolerated and leads to a 12-month delay in the occurrence of clinical arthritis at the

Table 2 Serious adverse events during study follow-up

Rituximab group (n=13)	Placebo (n=3)
Atypical thoracic pain (normal ECG)	Arterial occlusion right foot
Elective total hip replacement for OA	Elective total hip replacement for OA
Elective sigmoid resection after pre-existent recurring diverticulitis	Headache and concentration problems (neurological tests including MRI brain normal)
Elective surgery for nephrolithiasis	
Vertebral fracture after trauma	
Elective herniated disc surgery	
Elective knee arthroplasty for OA	
Hospitalisation for COPD exacerbation (n=2)	
Hospitalisation for depression	
Thrombosis upper extremity; pulmonary embolism	
STEMI caused by left main coronary artery stenosis	
Pre-existent bladder atony	

COPD, chronic obstructive pulmonary disease; OA, osteoarthritis; STEMI, ST elevation myocardial infarction.

moment when 25% of the subjects had developed arthritis, when compared with placebo. The background risk of arthritis development of 40%, which is comparable to earlier reports of observational studies,[3 10] was decreased by 55% at 12 months follow-up after treatment.

RA is one of the most common chronic immune-mediated inflammatory diseases with a significant impact on the individual patient as well as society. Current treatment options are still not sufficiently effective as most patients do not achieve disease remission, which is the treatment goal.[24 25] Early initiation of treatment in patients diagnosed with RA increases the chance of better radiographic outcome and reaching long-term remission, a phenomenon referred to as the 'therapeutic window of opportunity'. We are now capable of identifying individuals during an even earlier stage, when they are at risk of developing seropositive RA[26] but before the onset of arthritis. This makes it possible to study whether there is a 'preventive window of opportunity'.

While a previous, preventive intervention using dexamethasone has been proven unsuccessful,[27] the results of our current study show that a single infusion of rituximab may alter the disease process, although temporarily. There are several possible mechanisms by which B lineage cells may contribute to the disease process, including antigen presentation, activation of T cells by providing costimulatory signals, production of proinflammatory cytokines and production of autoantibodies including RF and ACPA.[28] Immune complexes containing RF or ACPA may directly activate macrophages, resulting in increased production of cytokines and chemokines like tumour necrosis factor and CXCL8 that are associated with the manifestations of clinical signs and symptoms.[29 30]

When the study was designed, we set to explore whether a single infusion of rituximab could reduce the risk of developing RA from the expected 40% to 10% in the studied population, a goal that was set to gratify the introduction of the treatment for this population, which was not achieved as the treatment resulted in markedly delayed onset of disease rather than cure. This result might be explained by persistence of autoreactive B-cell clones in the tissues and subsequent repopulation over time. It is tempting to speculate that repeated treatment, perhaps with a single infusion of rituximab once a year, might be sufficient to control B-cell numbers and prevent clinically manifest disease in a population at high risk of developing RA, whereas a more sophisticated approach would be to specifically target the autoreactive B cells that drive autoimmunity during the preclinical stage of the disease.[5] If relevant autoantigens can be identified, one could envisage the development of therapies that target specifically autoreactive B cells in peripheral blood as well as in tissue niches such as lymph nodes and bone marrow, for example, by using autoantigen-based chimeric immunoreceptors that can direct T cells to kill autoreactive B lymphocytes through the specificity of the BCR.[31] Alternatively, to achieve long-term prevention, polarised proinflammatory innate immune cells might need to be targeted in combination with B-cell depletion to stop reinitiation of the B-cell response, analogous to what has been proposed for the secondary prevention of type 1 diabetes in autoantibody positive subjects without clinically manifest diabetes.[32] Clearly, further studies are needed to prove these concepts aimed at secondary prevention of RA; the current study supports the rationale for such future research. Other studies aimed at prevention of RA by changes of lifestyle-related risk factors, use of DMARDs and statins, or targeted therapies other than rituximab are currently under way.[33]

This study was subject to certain limitations. The biomarker analysis was exploratory in nature, and the relatively small sample size is a limitation. However, the results provide several interesting hypothesis-generating observations based on the changes in B-cell populations and B-cell products in relationship to treatment effects and development of arthritis over time. The results presented here are clearly consistent with the critical role of B cells in the pathogenesis of RA during the earliest stages of the disease and support future studies aimed at secondary prevention of RA, including by the use of targeted treatments.

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Contributors PPT was the principal investigator, and was responsible for the study design, data interpretation and writing of the report. DMG and KIM contributed to the study design, data collection, data analysis, data interpretation and writing of the report. MS contributed to the data collection, data analysis, data interpretation and writing of the report. MWT, SWT, MdH, MJFSK, AvT, MJ, MH and NdV contributed to data collection and writing of the report. AHZ was responsible for data analysis, interpretation of the data and writing of the report.

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Competing interests PPT is a former employee and DMG a current employee of GlaxoSmithKline, UK. GlaxoSmithKline was not involved in the design and/or execution of the study. NdV reports grants from AbbVie, Janssen Biologics, Ergomed Clinical Research, GlaxoSmithKline, Pfizer, Boehringer Ingelheim, and Roche, as well as personal fees from MSD, UCB, Janssen, non-financial support from Roche, personal fees and non-financial support from Pfizer, all outside the submitted work. In addition, NdV has a patent method for determining the risk of developing arthritis pending. MS reports a research grant from AstraZeneca (received in August 2015). AstraZeneca was not involved in this study.

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CLINICAL SCIENCE

Targeting early changes in the synovial microenvironment: a new class of immunomodulatory therapy?

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ABSTRACT

Objectives Controlled immune responses rely on integrated crosstalk between cells and their microenvironment. We investigated whether targeting proinflammatory signals from the extracellular matrix that persist during pathological inflammation provides a viable strategy to treat rheumatoid arthritis (RA).

Methods Monoclonal antibodies recognising the fibrinogen-like globe (FBG) of tenascin-C were generated by phage display. Clones that neutralised FBG activation of toll-like receptor 4 (TLR4), without impacting pathogenic TLR4 activation, were epitope mapped by crystallography. Antibodies stained synovial biopsies of patients at different stages of RA development. Antibody efficacy in preventing RA synovial cell cytokine release, and in modulating collagen-induced arthritis in rats, was assessed.

Results Tenascin-C is expressed early in the development of RA, even before disease diagnosis, with higher levels in the joints of people with synovitis who eventually developed RA than in people whose synovitis spontaneously resolved. Anti-FBG antibodies inhibited cytokine release by RA synovial cells and prevented disease progression and tissue destruction during collagen-induced arthritis.

Conclusions Early changes in the synovial microenvironment contribute to RA progression; blocking proinflammatory signals from the matrix can ameliorate experimental arthritis. These data highlight a new drug class that could offer early, disease-specific immune modulation in RA, without engendering global immune suppression.

Key messages

What is already known about this subject?

► Immunomodulatory signals from the extracellular matrix help to shape immune responses. Activation of toll-like receptor 4 (TLR4) by tenascin-C, a matrix molecule persistently expressed at high levels in people with RA, drives chronic inflammation in models of rheumatoid arthritis (RA).

What does this study add?

► We developed monoclonal antibodies that block the TLR4 binding epitope within the fibrinogen-like globe domain of tenascin-C; these antibodies inhibit cytokine release by RA synovial cells and prevent disease progression and tissue destruction during collagen-induced arthritis.

How might this impact clinical practice?

► This study indicates that antibodies targeting proinflammatory signals from the extracellular matrix should be further explored for use in clinical practice for treating RA.

INTRODUCTION

Environmental signals play a key role in shaping cell identity, imprinting tissue-specific gene expression programmes to enable geographically adapted cell behaviour. This includes, for example, specialisation of gut and brain macrophages, or of synovial and dermal fibroblasts, to fulfil distinct site-specific roles.[1 2] Dynamic tissue remodelling during inflammation creates new microenvironmental niches designed to drive immune responses that restore homeostasis. These temporary structures comprise specialised extracellular matrix molecules that support infiltrating immune cells and proliferating tissue resident cells, pattern soluble effector molecules and signal to cells to orchestrate controlled inflammation.[3 4] Immunomodulatory matrix molecules exhibit restricted expression in healthy tissue, but are persistently expressed at sites of pathological inflammation, leading to their exploitation in the clinic as disease-specific postcodes with which to deliver antibody-linked packages of cytotoxic and anti-inflammatory drugs.[5] Here, we determined whether directly targeting the activity of these matrix molecules could combat pathological inflammation.

Tenascin-C is a large, multimodular extracellular matrix molecule that exhibits limited expression in healthy tissues but is transiently upregulated on cellular stress and tissue injury, where it triggers inflammation by activating toll-like receptor 4 (TLR4). Persistent expression of tenascin-C has been implicated as a driver of chronic inflammation in autoimmune, neurological, metabolic and

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fibrotic diseases, in which expression levels can predict prognosis and reflect treatment outcome.[6] In patients with rheumatoid arthritis (RA), high tenascin-C is associated with more erosive joint disease and predicts poor response to biological treatment.[7] During experimental joint disease, mice lacking tenascin-C are protected from prolonged synovial inflammation and tissue destruction; while inflammation is induced in these animals, it is also swiftly resolved, concomitant with downregulation of key inflammatory cytokines and pathogenic T cell subsets.[8 9]

Mapping the active domain within tenascin-C revealed a unique structural epitope in the fibrinogen-like globe (FBG) that is essential for binding to and activating TLR4.[8 10] Distinct modes of receptor activation and diverse downstream signalling induced by FBG compared with pathogenic TLR4 agonists,[11] revealed an opportunity to ablate pathological 'sterile' inflammation, leaving intact host defence against infection. We reasoned that this makes tenascin-C an attractive candidate for safely modulating inflammatory signals from the microenvironment. However, lack of specific, effective antagonists that block FBG activation of TLR4 have precluded assessment of tenascin-C as a viable therapeutic target.

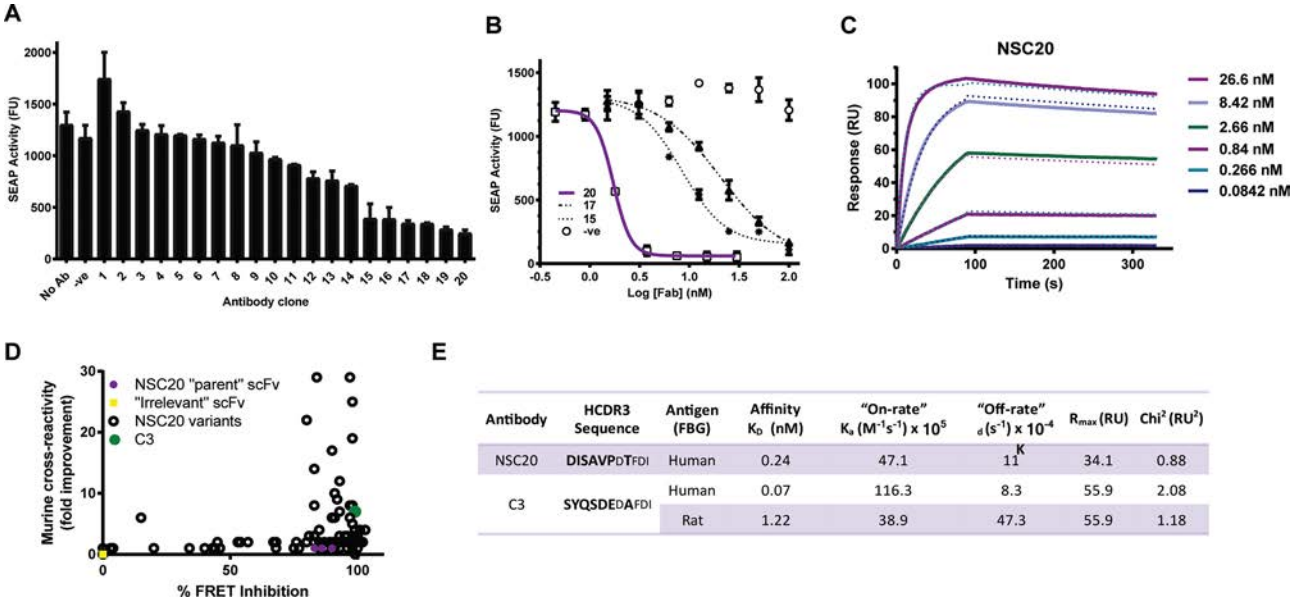
METHODS

All methods are provided in the online supplementary materials section.

RESULTS

Generating neutralising anti-tenascin-C antibodies

We generated monoclonal antibodies against the FBG domain of human tenascin-C using phage display. A panel of 20 sequence unique antibodies that bound to the FBG domain of tenascin-C, but not tenascin-R, the family member possessing the most closely related FBG domain, were selected for conversion into Fab format. Fabs were tested for blockade of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity induced in human monocytic THP1 reporter cells by stimulation with the FBG domain of tenascin-C (figure 1A). Titration of selected antibodies revealed a half maximal inhibitory concentration (IC_{50}) of 1.7 nM for clone NSC20 (figure 1B). NSC20 bound to the FBG domain of human tenascin-C with high affinity (K_D 110 pM at 25°C) (figure 1C) and bound comparably well to canine FBG; however, binding to rodent FBG domains was 117-fold less (data not shown). To generate antibodies whose efficacy could be assessed in human and rodent models of disease, NSC20 was affinity matured. Among 138 individual NSC20 variants whose binding was analysed using homogeneous time resolved fluorescence competition and expression-normalised capture (ENC) assays (figure 1D), clone C3 exhibited a K_D for the FBG domain of human tenascin-C of 70 pM and recognised rat antigen with a K_D of 1.2 nM (figure 1E).



Antibody	HCDR3 Sequence	Antigen (FBG)	Affinity K_D (nM)	"On-rate" K_a ($M^{-1}s^{-1}$) x 10^5	"Off-rate" K_d (s^{-1}) x 10^{-4}	R_{max} (RU)	Chi^2 (RU^2)
NSC20	DISAVPDTFDI	Human	0.24	47.1	11	34.1	0.88
C3	SYQSDEDAFDI	Human	0.07	116.3	8.3	55.9	2.08
		Rat	1.22	38.9	47.3	55.9	1.18

Figure 1 Generation and affinity maturation of anti-tenascin-C antibodies. (A) Fab clones were screened for their ability to inhibit FBG-mediated upregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in THP1-blue cells. (B) The potency of top hits (clones 15, 17 and 20) was further assessed compared with control Fab D1.3 recognising hen egg lysozyme. (A and B) Data are shown as the mean±SD from three experiments. (C) The binding affinity of NSC20 to human FBG was analysed by surface plasmon resonance (SPR) spectroscopy at 25°C. (D) Following affinity maturation, NSC20 variants were screened for improved binding to human and mouse FBG. HTRF measured the ability of affinity matured scFv clones to compete with labelled NCS20 IgG for antigen binding. Data are presented as percentage inhibition of the FRET signal (x-axis). The ENC assay used limiting amounts of immobilised anti-FLAG antibody to capture FLAG-tagged scFv from culture supernatants and thereby normalise the amount of immobilised scFv. After washing to remove excess (unbound) scFv, the ability of immobilised antibody clones to bind biotinylated CD4-his-mTNC-FBG was detected by DELFIA, allowing ranking of clones based on affinity. Parental NSC20 was used as a benchmark, and data are presented as fold-increase in fluorescence signal (y-axis). Open circles represent NSC20 variants, solid green circle indicates lead variant, C3. (E) The heavy chain CDR3 (HCDR3) sequence and binding properties, determined by SPR at 37°C, of C3 are shown compared with the parental antibody NSC20. In contrast to NSC20, no measurable dissociation of the antibody–antigen complex was detectable over a 10 min period at 25°C for C3 (data not shown). Hence, affinity values for C3 were determined at 37°C. ENC, expression-normalised capture; FBG, fibrinogen-like globe; HTRF, homogeneous time resolved fluorescence.

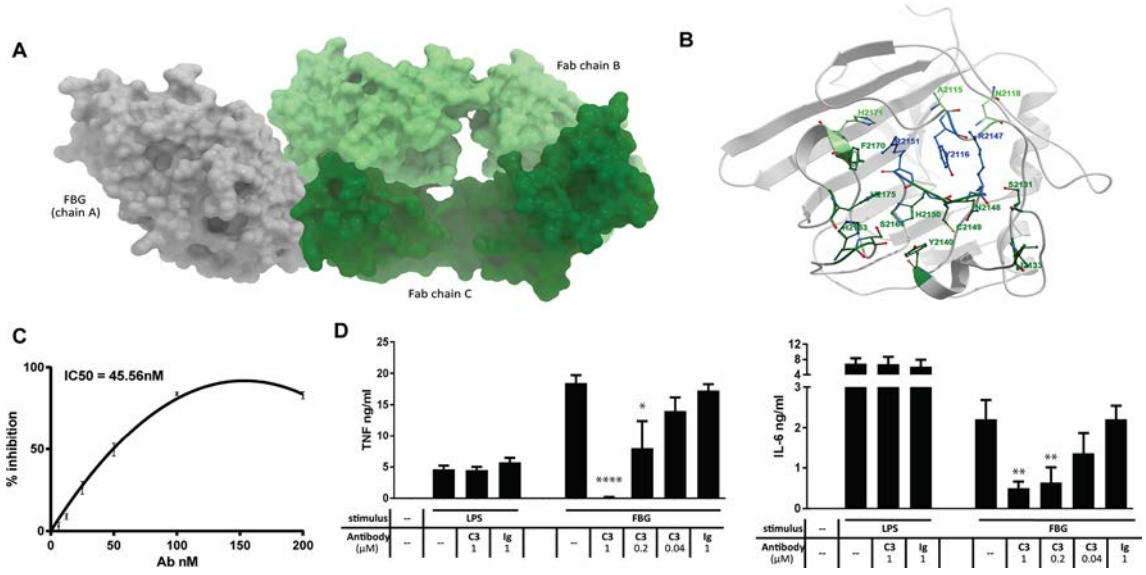


Figure 2 Defining the mode of action of neutralising anti-tenascin-C antibodies. (A and B) The crystal structure of C3 bound to the FBG domain of human tenascin-C. Six FBG residues bind to Fab chain B (heavy chain) (A2115, Y2116, R2147, R2151, N2118 and H2171) and 13 FBG residues with Fab chain C (light chain) (Y2116, S2131, I2133, Y2140, R2147, N2148, C2149, H2150, R2151, H2163, S2164, F2170 and H2175), three of which are shared (Y2116, R2147 and R2151). Light green: FBG residues in contact with Fab chain B; dark green: FBG residues in contact with Fab chain C; blue: FBG residues in contact with Fab chains B and C. This interaction interface is not conserved in the FBG domains of tenascin-R and tenascin–W. Only 9 of these 16 residues are present in FBG-R, and only seven in FBG-W. Of the three residues in FBG-C that interact with both heavy and light chain of the antibody, one residue is substituted in FBG-R, and all three are substituted in FBG-W. These data support experimental evidence that FBG-R does not bind to C3, and the higher sequence divergence of the FBG domain of tenascin-W with that of tenascin-C (54.1%) compared with tenascin-R (61.6%), and the fact that this divergence includes key positions in the C3 binding epitope, predicts that FBG-W will also be unable to bind to C3. (C) Recombinant human TLR4 was coated onto a 96-well plate, and recombinant human tenascin-C FBG, which had been preincubated with C3 or isotype control antibody, was added. Bound FBG was detected, and the percentage inhibition in the C3 preincubated samples was calculated compared with the isotype control samples (IC50=45.5 nM). Data are shown as the mean±SEM from eight experiments. (D) Recombinant human tenascin-C FBG (FBG) (1 μM) or lipopolysaccharide (LPS) (1 ng/mL) were preincubated with the indicated doses of C3 or isotype control antibody (Ig) before being added to primary human macrophages. After 24 hours, supernatants were taken, and cytokine ELISAs were performed. Data are shown as the mean±SEM from four independent donors. One-way analysis of variance was performed to determine significance of C3 inhibition compared with isotype control. *p<0.05, **p<0.01, ****p<0.0001. FBG, fibrinogen-like globe; IL, interleukin; TLR4, toll-like receptor 4; TNF, tumour necrosis factor.

Defining antibody mode of action

Crystallisation of the FBG domain of human tenascin-C in complex with Fab fragments of C3 revealed interactions in the X-ray structure are mediated by hydrogen bonds and three layer pi:pi stacking, predominantly between one of the two Fab chains (figure 2A,B). Comparison of the TLR4 binding epitope[10] with the Fab epitope in FBG predict that antibody binding will abrogate tenascin-C's ability to activate TLR4 by preventing access of the receptor to residues (S2131 and I2133) in FBG that are required for optimal binding to TLR4. This was validated experimentally by demonstration that preincubation of FBG with C3 inhibits binding of FBG to purified recombinant TLR4 in a solid phase binding assay (IC_{50} 45.56 nM)(figure 2C) and that preincubation of FBG with C3 blocks the ability of FBG to induce cytokine synthesis in primary human macrophages, while C3 had no effect on LPS-induced cytokine release (figure 2D).

Assessing antibody efficacy in synovial cells from patients with RA and in experimental arthritis

Staining with anti-FBG antibodies was observed in synovial biopsies from people with joint inflammation; tenascin-C levels were higher in people with early RA, compared with people who had joint inflammation that spontaneously resolved and who did not develop RA, or patients with established RA (figure 3A). FBG staining was predominantly observed in the sublining synovial layer of inflamed tissue where it created a dense matrix surrounding both podoplanin-positive and CD90-positive fibroblasts. FBG staining was also associated with blood vessels, lying underneath and around the CD31+ endothelial cell layer (figure 3B). Costaining of the C-terminal FBG domain with antibodies that recognise the N-terminal epidermal growth factor-like (EGF-L) repeats of tenascin-C revealed largely overlapping localisation and also highlighted areas where anti-FBG staining predominated (online supplementary figure 1). In mixed cell populations isolated from the synovium of patients with RA undergoing joint replacement, monoclonal antibody C3 blocked cytokine release induced by stimulation with FBG, but not LPS (figure 3C). Rats in which joint inflammation was induced by intradermal administration of type II collagen (day 0 and day 7) were treated twice weekly with vehicle (PBS), isotype control (10 mg/kg) or C3 (1, 3 or 10 mg/kg) from day 0 until the end of the experiment at day 28. No significant differences between vehicle and isotype control groups were observed for any parameter measured. However, increasing doses of C3 significantly reduced clinical score (figure 3D) and paw swelling (figure 3E). In addition, C3 treatment reduced the number of affected paws per animal; rats with only one affected paw were restricted to the 3 mg/kg and the 10 mg/kg C3 groups, and rats treated with 10 mg/kg C3 gained significantly more weight throughout the experiment (not shown). Finally, C3 treatment reduced the incidence

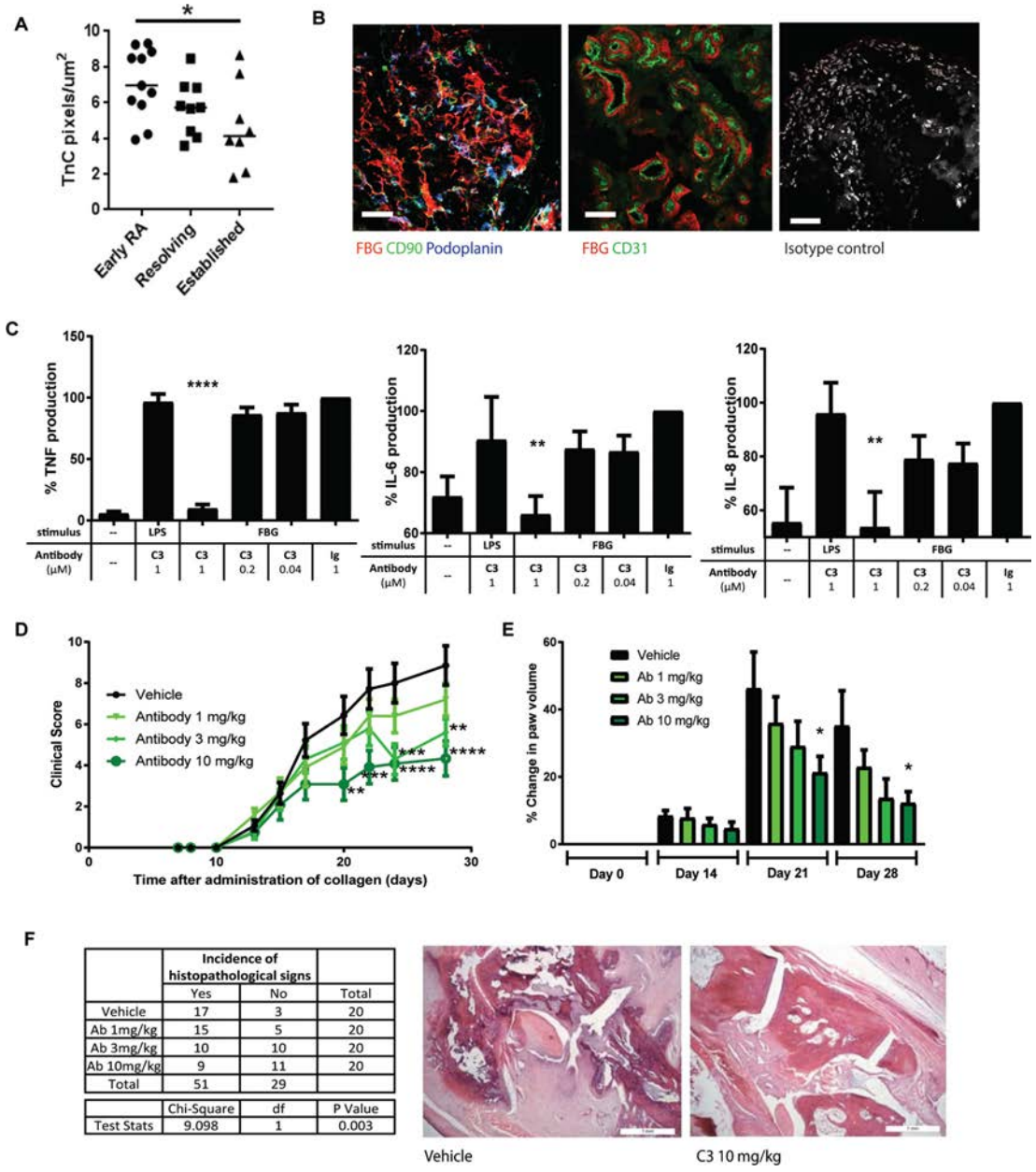


Figure 3 Antibody treatment ameliorates inflammation in experimental arthritis. (A and B) Synovial biopsies from the Birmingham early arthritis cohort were stained with anti-FBG antibodies. Quantification of the number of positive pixels per microgram biopsy was performed in tissue from people with early RA (undiagnosed synovitis of less than 3 months, patients who go on to be diagnosed with RA), people with synovial inflammation that spontaneously resolved (undiagnosed synovitis of less than 3 months that disappears by itself) (resolving) and people with established RA (diagnosed disease, greater than 3 months' duration) (A). Anti-FBG staining was observed in areas of fibrosis in inflamed synovia (left panel: red anti-FBG, green anti-CD90, blue antipodoplanin) and around areas of vascularisation underneath endothelial cells (centre panel: red anti-FBG, green anti-CD31). No staining was observed with isotype controls in place of primary antibodies (right panel). Scale bars 50 μm (B). (C) Mixed populations of cells isolated from RA synovial membranes were stimulated with 1 μM recombinant human tenascin-C FBG or 1 ng/mL LPS, which had been preincubated with either C3 or isotype control antibody. After 24 hours, supernatants were taken and cytokine ELISAs were performed. Data are shown as the mean±SEM from three independent donors. One-way analysis of variance (ANOVA) was performed to determine significance of C3 inhibition compared with isotype control. *p=0.02, ***p<0.0001. (D–F) Rats were injected with bovine type II collagen intradermally on day 0 and day 7. Treatments of PBS (vehicle control) and C3 at 1, 3 or 10 mg/kg were administered twice weekly throughout the experiment by intravenous injection (10 animals per treatment group). Animals were scored for clinical signs of disease three times per week, and the mean±SEM is shown (D). Paw volumes were measured using a plethysmometer on days 0, 14, 21 and 28, and the mean change±SEM normalised to the day 0 measurements is shown (E). Two-way was carried out to test for significance of changes between vehicle and treatment groups. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. (F) At termination on day 28, hind limbs were assessed for histological signs of inflammation, articular cartilage damage and damage to the underlying metaphyseal bone. A χ^2 test for trend was used to confirm that the presence or absence of histopathological signs is associated with antibody treatment (χ^2=9.098, p=0.003), with only 3 of 20 paws were free of any sign of histopathology in the vehicle treated group, whereas 11 of 20 paws were disease free in the 10 mg/kg treated group (table). Representative histological images of destructive arthritis (vehicle treated) and a normal joint (10 mg/kg) are shown (right panels). FBG, fibrinogen-like globe; IL, interleukin; RA, rheumatoid arthritis; TNF, tumour necrosis factor.

of histopathological changes in the joint (χ^2=9.098, p=0.003) (figure 3F).

DISCUSSION

This study describes the production of monoclonal antibodies that prevent the FBG domain of tenascin-C from binding to and activating TLR4. Staining biopsies of inflamed synovia with these antibodies revealed protein expression very early in RA and at higher levels than in people with established disease. Prophylactic administration of anti-FBG antibodies to rats with collagen-induced arthritis did not affect the induction of joint inflammation but inhibited disease progression and prevented joint damage. These data highlight that early changes in the synovial microenvironment contribute to the development of RA and that blocking inflammatory signals from the extracellular matrix could offer a new therapeutic strategy for treating this disease.

Development of anti-FBG antibodies provides evidence of a non-redundant role for tenascin-C activation of TLR4 in experimental models of joint inflammation. These antibodies also constitute a useful tool with which to learn more about how endogenous inflammatory stimuli shape immune responses. Both stromal and immune cells express TLR4 in the RA joint; identification of biological processes and effector molecules that are modulated by FBG blockade in each of these different cell types during disease amelioration may reveal new opportunities for suppressing inflammation. This will also inform preclinical benchmarking studies, for example, if anti-FBG treated animals phenocopy tenascin-C null animals, antibody treatment would block persistent synthesis of several cytokines from different cellular sources, including tumour necrosis factor, interleukin (IL)-6 and IL-17,[9] raising the possibility that this approach could be more effective than single cytokine blockade.

Current approaches to targeting TLR4 in RA focus on antibodies that prevent receptor dimerisation, offering blockade of TLR4 activation by a broad range of pathogenic and endogenous ligands.[12 13] These antibodies are well tolerated in healthy adults and are currently in phase 2a trials in patients with RA,[14] for treatment of TLR4-driven disease defined by serum autoantibody signature.[15] Here, we show that targeting a single endogenous TLR4 agonist is sufficient to offer therapeutic benefit in arthritis models. This strategy could enable a move away from blocking TLR4-mediated inflammation at the receptor level, hitting only disease-specific stimuli, without engendering global immune suppression. Tenascin-C is dispensable for the induction of joint inflammation but required for its persistence(figure 3),[8] indicating that its blockade can be used simply to restore the resolution of inflammation, without hindering immune defence. This premise can now be interrogated by assessing the susceptibility of anti-FBG treated animals to infection, and by comparison of the efficacy and safety profiles of anti-FBG and anti-TLR4 antibodies. Reducing the risk of opportunistic or recurrent latent infection[16–19] would be a significant step forward in the management of RA.

Treating RA early provides significant clinical benefit to patients.[20] However, while a myriad of dysregulated signalling pathways and cytokine networks contribute to persistent inflammation in well-established disease, events that dictate progression from early synovitis to chronic joint inflammation and tissue destruction remain incompletely understood. This study reveals discreet, tenascin-C-rich niches around blood vessels and at sites of fibrosis in inflamed synovia, arising early in disease development. These data implicate changes in the synovial microenvironment in the onset of disease, warranting further investigation of the therapeutic window within which anti-FBG treatment can achieve efficacy and if this offers a realistic avenue for treating people with early disease.

Finally, while antibodies that specifically target FBG highlight this domain of tenascin-C as a critical driver of chronic synovial inflammation, the capacity of tenascin-C to exert both beneficial and deleterious effects across different joint tissues[21] illustrates a fascinating context specificity for this molecule that remains poorly understood. For example, administration of exogeneous full-length tenascin-C prevents cartilage degeneration during murine models of osteoarthritis[22] and promotes cartilage repair when applied to osteochondral defects in rabbits.[23] It is not yet clear whether activation of TLR4 by the FBG domain occurs in isolation from, or in synergy with, signalling by other tenascin-C domains, how signals from this multidomain molecule are integrated within complex tissue networks and how tissue-specific responses to tenascin-C are mediated. While distribution of the EGF-L repeats and the FBG domain of tenascin-C overlap in inflamed synovia, areas of single antibody positivity may indicate locally elevated availability of the TLR4-activating epitope, for example, via tenascin-C conformations that expose the FBG domain and conceal the EGF-L repeats, or generation of FBG-containing proteolytic fragments. Better understanding how different forms of tenascin-C are distributed across different tissues, as well as in discreet niches within tissues, will provide further mechanistic insight into how this matrix molecule influences cell behaviour in situ.

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CLINICAL SCIENCE

To switch or not to switch: results of a nationwide guideline of mandatory switching from originator to biosimilar etanercept. One-year treatment outcomes in 2061 patients with inflammatory arthritis from the DANBIO registry

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ABSTRACT

Objectives Real-world evidence on effectiveness of switching to biosimila r etanercept is scarce. In Denmark, a nationwide guideline of mandatory switch from 50 mg originator (ETA) to biosimilar (SB4) etanercept was issued for patients with rheumatoid arthritis (RA), psoriatic arthritis (PsA) and axial spondyloarthritis (AxSpA) in 2016. Clinical characteristics and treatment outcomes were studied in ETA-treated patients, who switched to SB4 (switchers) or maintained ETA (non-switchers). Retention rates were compared with that of a historic cohort of ETA-treated patients. Switchers who resumed ETA treatment (back-switchers) were characterised.

Methods Observational cohort study based on the DANBIO registry. Treatment retention was explored by Kaplan-Meier plots and Cox regression (crude, adjusted).

Results 1621 (79%) of 2061 ETA-treated patients switched to SB4. Disease activity was unchanged 3 months' preswitch/postswitch. Non-switchers often received 25 mg ETA (ETA 25 mg pens/syringes and powder solution were still available). One-year adjusted retention rates were: non-switchers: 77% (95% CI: 72% to 82%)/switchers: 83% (79% to 87%)/historic cohort: 90% (88% to 92%). Patients not in remission had lower retention rates than patients in remission, both in switchers (crude HR 1.7 (1.3 to 2.2)) and non-switchers (2.4 (1.7 to 3.6)). During follow-up, 120 patients (7% of switchers) back-switched to ETA. Back-switchers' clinical characteristics were similar to switchers, and reasons for SB4 withdrawal were mainly subjective.

Conclusion Seventy-nine per cent of patients switched from ETA to SB4. After 1 year, adjusted treatment retention rates were lower in switchers versus the historic ETA cohort, but higher than in non-switchers. Withdrawal was more common in patients not in remission. The results suggest that switch outcomes in routine care are affected by patient-related factors and non-specific drug effects.

Key messages

What is already known about this subject?

- Real-world evidence on effectiveness of switching from originator to biosimilar etanercept in inflammatory arthritis is scarce.

What does this study add?

- Despite national mandatory guidelines, ≈20% of Danish patients treated with originator etanercept did not switch to biosimilar SB4.
- Baseline characteristics differed among patients who switched (switchers) and patients who maintained treatment (non-switchers).
- Adjusted treatment retention rates were lower in switchers than in a historic cohort, but higher than in non-switchers.
- Withdrawal was more common in patients not in remission.

How might this impact on clinical practice?

- Switch outcomes in routine care seem affected by patient-related factors and non-specific drug effects.

With the marketing of the first biosimilar disease-modifying antirheumatic drugs (bDMARDs) a new era has started, in which effective treatment of inflammatory arthritis at lower costs can be expected.[1 2] A biosimilar must have equivalent efficacy and comparable safety to its reference product, and an immunogenicity not greater than that of its reference product.[3]

In 2015, the first biosimilar etanercept (SB4), was approved in Europe.[3–5] At the time of marketing, SB4 had only been tested in patients with rheumatoid arthritis (RA).[6] However, SB4 is also prescribed in for example, psoriatic arthritis (PsA) and axial spondyloarthritis (AxSpA), corresponding to the

approved indications of the originator drug.[7 8] This is theoretically of importance since age, genetics, comedication with conventional synthetic DMARDs and drug dose differ across diseases and may affect immunogenicity, pharmacokinetics and dynamics.[5 9–11] Furthermore, patients included in randomised controlled trials (RCTs) differ from patients treated in routine care who are often older, have more comorbidities or atypical disease presentation.[12] Thus, real-world evidence through post-marketing monitoring of safety across indications and long-term effectiveness outcomes in nationwide registries with prospective follow-up in routine care is a valuable supplement to RCTs.[13–15]

A Danish national guideline issued in April 2016 stated that all patients with inflammatory arthritis treated with originator etanercept (ETA) (Enbrel) must switch to SB4 (Benepali, 50 mg subcutaneous) for economic reasons.[16] On marketing in Denmark, 50 mg SB4 costed 49% less than ETA. Based on data from the nationwide DANBIO registry, we have previously reported outcomes after a similar non-medical switch from originator to biosimilar infliximab (CT-P13) performed the previous year in 802 patients with arthritis.[17] Switch outcomes for the two biosimilars might differ due to different active substances (monoclonal antibodies vs receptor fusion protein) and increasing experience with, and confidence in, the use of biosimilars in patients and community over time. Furthermore, different administration routes (intravenous vs subcutaneous) might affect pharmacokinetics and healthcare behaviour (treatment given in hospital vs at home, close vs scarce contact to healthcare personnel), and for a subcutaneously administered biosimilar, the injection device might differ from the reference product. Finally, at the time of marketing of the biosimilars in Denmark, originator ETA was still available (25 mg syringes/pens, 50 mg powder solution), whereas originator infliximab was unavailable. Knowledge on real-world switching from ETA to SB4 is scarce.[18–20]

The aims of this nationwide, observational study were to investigate in ETA-treated patients (1) the proportions of patients who switched to SB4 (switchers) or maintained ETA treatment (non-switchers). Furthermore to investigate in switchers: (2) 3 months' disease activity before/after switching, (3) reasons for withdrawal, safety events and patient characteristics associated with withdrawal, (4) frequency, characteristics and outcomes of switch patients who resumed ETA (back-switchers) and to compare in switchers and non-switchers: (5) the 1-year retention rates with that of a historic cohort of ETA-treated patients. Finally, we aimed to characterise non-switchers including reasons for withdrawal.

METHODS

DANBIO covers >95% of adults with rheumatic diseases treated in routine care with bDMARDs.[21 22] According to national treatment guidelines, disease activity and outcomes are monitored 1–2 times annually, and when medication is changed.[21] The current study was approved by the Data Protection Agency (RH-2015–209, I-Suite 04145). In Denmark, registry research neither requires patient consent nor ethical approval.

Patients with RA, PsA and AxSpA treated with ETA by 1 April 2016 were identified in DANBIO. The following cohorts were defined: switchers*:* patients who switched from ETA to SB4 between 1 April 2016 and 1 January 2017. A time gap of 0–90 days between stop of ETA and start of SB4 was allowed to comply with differences in registration practice. Non-switchers*:* the group of ETA-treated patients who did not switch to SB4 during follow-up. Back-switchers: switchers, who stopped SB4 and resumed treatment with ETA during follow-up. Furthermore, a historic comparison cohort of ETA-treated patients by 1 January 2015 was identified in DANBIO.

Eighteen of 23 departments of rheumatology in Denmark accepted to validate DANBIO data regarding switch date, disease activity and reasons for SB4 withdrawal. Thus, 84% of included treatment series were validated. Data were censored by 28 August 2017. The data collection in DANBIO has been described previously.[21] For switchers, the index date (baseline) was the date of switch to SB4 from ETA. For non-switchers, the index date was 1 April 2016 and for the historic cohort 1 January first 2015.

Through linkage by social security numbers, comorbidities (Charlson Comorbidity Index, excluding musculoskeletal comorbidity)[23] from index date and 10 years back were identified in the Danish National Patient Registry, which has complete data regarding hospitalisations and outpatient care.[24] Vital status was obtained from the Danish Civil Registry.

Statistics

Descriptive data are presented by medians (IQR) or as numbers (percentages) for discrete data. Non-parametric statistics were used for comparisons of patient characteristics (X^2 or Mann-Whitney tests as appropriate). Unless otherwise stated, analyses were based on available data with no imputation of missing data.

In switchers, disease activity 3 months before switch (preswitch), at the time of switch, after 3 months (postswitch) and changes over time (Δpreswitch and Δpostswitch) were calculated in each patient. Predefined time windows were applied for measures of disease activity. Missing data at the 3 months' visit was imputed with the 6 months' visit. For patients who withdrew ≤3 months postswitch (n=105), data from the latest registered visit after baseline were carried forward. Disease flare in patients with RA and PsA was defined as (1) changes in 28-joint Disease Activity Score (DAS28) ≥0.6 and (2) ΔDAS28 ≥1.2. In AxSpA, Δ(Ankylosing Spondylitis)ASAS Disease Activity Score (ASDAS) ≥1.1 was considered a flare. Remission was defined as DAS28 <2.6 and ASDAS <1.3, respectively.

Retention rate was the proportion of patients who maintained the same drug in a given time period. Retention rates (=drug survival) in switchers, non-switchers, and the historic cohort was explored with Kaplan-Meier plots and log rank tests. Multiple Cox proportional hazards regression analyses and HRs stratified by indication (RA/PsA/AxSpA) were conducted to estimate withdrawal rates adjusted for clinically relevant baseline variables. Comparisons were performed as two sets of analyses: switchers versus the historic cohort and switchers versus non-switchers. The following baseline variables were included: age, gender, methotrexate (MTX) (yes/no), comorbidities (0/≥1), remission (yes/no) and ETA start year (1998–2010/2011–2016). Similarly, adjusted 1-year retention rates with 95% CI were calculated. In the comparison of switchers versus the historic cohort, robust variance calculation was applied to account for repeated subjects with left truncation of events (1 January 2015), and all observations were censored after 1 year. Baseline data were complete for all covariates except remission status, which was available in 79% of switchers, 92% of non-switchers and 91% of patients in the historic cohort.

Since remission status was closely associated with patient's global score (PGS), additional multiple Cox regression analyses were performed for sensitivity, in which remission status (yes/no) was replaced by PGS (categorical: ≤30 mm/>30 mm).

Table 1 Baseline demographics in patients who switched from originator etanercept (ETA) to biosimilar etanercept (SB4) and in patients who maintained ETA treatment (non-switchers) stratified by indication. One-year treatment retention and reasons for withdrawal are also shown

	RA, N=1219		PsA, N=407		AxSpA, n=435	
	Switchers N=933 (77%)	Non-switchers N=286 (23%)	Switchers N=351 (86%)	Non-switchers N=56 (14%)	Switchers N=337 (77%)	Non-switchers N=98 (23%)
Baseline characteristics*						
Female, n (%)	689 (74%)	217 (76%)	160 (46%)	31 (55%)	115 (34%)	34 (35%)
Age, years	61 (49 to 70)	62 (48 to 70)	52 (43 to 61)	52 (43 to 58)	48 (39 to 57)	48 (40 to 57)
Concomitant MTX, n (%)	556 (60%)	140 (49%)	168 (48%)	17 (30%)	51 (15%)	18 (18%)
In remission, %†	65%	55%	70%	73%	28%	21%
PGS, mm	29 (13 to 55)	34 (16 to 64)	30 (12 to 54)	36 (19 to 63)	30 (12 to 53)	37 (17 to 70)
PGS <30 mm,%	52%	45%	51%	43%	51%	42%
DAS28	2.1 (1.6 to 3.0)	2.5 (1.8 to 3.3)	2.0 (1.6 to 2.8)	2.0 (1.8 to 2.8)	–	–
PASS yes, %	81%	67%	77%	68%	80%	77%
ASDAS	–	–	–	–	1.9 (1.2 to 2.6)	2.1 (1.4 to 3.1)
CRP, mg/L	3 (1 to 6)	3 (2 to 9)	2 (1 to 4)	3 (1 to 7)	3 (1 to 4)	3 (1 to 7)
HAQ	0.8 (0.3 to 1.3)	0.9 (0.4 to 1.5)	0.5 (0.0 to 1.0)	0.8 (0.6 to 1.3)	0.4 (0.0 to 0.8)	0.4 (0 to 0.9)
bDMARD treatment no, ETA, n (%)						
1	491 (53%)	116 (41%)	181 (52%)	23 (41%)	123 (36%)	42 (43%)
2	280 (30%)	104 (36%)	123 (35%)	18 (32%)	130 (39%)	33 (34%)
≥3	162 (17%)	66 (19%)	47 (13%)	15 (27%)	84 (25%)	23 (23%)
ETA dose, mg/dose, n (%)						
25	10 (1%)	124 (43%)	3 (1%)	10 (18%)	3 (1%)	35 (36%)
50	887 (95%)	142 (50%)	339 (96%)	39 (70%)	319 (95%)	52 (53%)
0ther/unknown	36 (4%)	20 (7%)	9 (3%)	7 (13%)	15 (4%)	11 (11%)
ETA interval, days. n (%)						
3.5	7 (1%)	76 (27%)	4 (1%)	6 (11%)	4 (1%)	21 (21%)
7	751 (80%)	181 (63%)	303 (86%)	44 (79%)	273 (81%)	61 (62%)
Other/unknown	175 (19%)	29 (10%)	44 (13%)	6 (11%)	60 (18%)	16 (16%)
Prior ETA treatment duration, years	6.0 (3.6 to 8.6)	5.3 (2.4 to 8.6)	4.3 (2.9 to 7.3)	3.4 (1.6 to 6.0)	4.6 (2.8 to 6.8)	4.7 (2.9 to 9.0)
≥1 Comorbidities, %	29%	31%	26%	18%	22%	23%
ETA start year, n (%)						
1998–2004	72 (3%)	26 (9%)	16 (5%)	1 (2%)	9 (3%)	9 (9%)
2005–2009	344 (37%)	94 (33%)	92 (26%)	14 (25%)	84 (25%)	34 (35%)
2010–2016	517 (55%)	166 (58%)	243 (69%)	41 (73%)	244 (72%)	55 (56%)
1-year treatment retention‡						
Withdrawal during follow-up, n (%)	194 (21%)	96 (33%)	53 (15%)	25 (45%)	52 (15%)	24 (23%)
Prior ETA duration in withdrawers, years	5.6 (2.9 to 8.8)	4.4 (2.3 to 8.0)	3.6 (2.5 to 6.1)	3.3 (0.9 to 5.5)	3.4 (1.7 to 5.3)	3.7 (2.3 to 7.1)

Numbers are medians (interquartile ranges) unless otherwise stated.
*Baseline is according to first SB4 dose (−90 to +6 days) for switchers and according to 1 April 2016 (±180 days) for non-switchers.
†DAS28 <2.6 (RA, PsA), ASDAS <1.3 (AxSpA).
‡Median follow-up switchers: 383 (314–414) days, non-switchers: 483 (222–483) days.
ASDAS, the Ankylosing Spondylitis Disease Activity Score; AxSpA, axial spondyloarthritis; CRP, C reactive protein; DAS, Disease Activity Score; HAQ, Health Assessment Questionnaire; MTX, methotrexate; PASS, patient acceptable symptom state; PGS, patient's global score; PsA, psoriatic arthritis; RA, rheumatoid arthritis; bDMARDs, biosimilar disease-modifying antirheumatic drugs.

For back-switchers, disease activity at the SB4 index date and at the time of back-switching to ETA were compared, and changes (=delta values) were calculated in each patient. Delta values were reported as medians (IQR) stratified by indication (RA/PsA/AxSpA). Baseline characteristics of back-switchers (gender, age, PGS, swollen joint count (RA, PsA), C reactive protein) were compared with the rest of the switch population.

Statistical analyses were performed by SPSS (V.22) and SAS (V.9.4). P values <0.05 were considered statistically significant.

RESULTS

Among 2183 ETA-treated patients identified in DANBIO, 2061 patients were included of which 1621 (79%) switched to SB4 (see online supplementary figure 1, table 1). In 49% of switchers, ETA was the first bDMARD, and prior to switching 99% received 50 mg and 1% 25 mg ETA doses. In non-switchers, ETA was the first bDMARD in 41%, and prior to the index date 34% received 25 mg ETA doses (55% twice weekly, 31% once weekly, 14% unknown or other intervals).

Baseline characteristics of switchers and non-switchers

Among patients with RA, AxSpA and PsA, 77%, 77% and 86%, respectively switched to SB4 (table 1). Switchers more frequently received concomitant MTX than non-switchers (in RA and PsA), whereas gender and age distributions stratified by indication were similar (table 1). Switchers had longer previous ETA treatment duration and fewer previous bDMARDs compared with non-switchers. At baseline, switchers with RA had lower disease

Table 2 Disease activity 3 months prior to vs 3 months after the switch from ETA to SB4 stratified by indication

	Disease activity			Changes over time	
	3 months preswitch	Switch	3 months postswitch	ΔPreswitch	ΔPostswitch
RA, n=933					
Patients with available data, n (%)*	639 (68)	745 (80)	568 (61)	485 (52)	436 (47)
DAS28	1.9 (1.3 to 2.8)	2.1 (1.6 to 3.0)	2.1 (1.7 to 3.1)	0.0 (0.0 to 0.0)	0.0 (−0.4 to 0.5)
HAQ (0–3)	0.8 (0.3 to 1.3)	0.8 (0.3 to 1.3)	0.8 (0.3 to 1.3)	0 (−1 to 1)	0 (−1 to 1)
CRP, mg/L	3 (1 to 7)	3 (1 to 6)	3 (1 to 6)	0 (−2 to 1)	0 (−1 to 1)
PGS, mm	30 (14 to 57)	29 (13 to 55)	32 (12 to 62)	0 (−11 to 8)	1 (−8 to 11)
PsA, n=351					
Patients with available data, n (%)*	223 (64)	253 (72)	197 (56)	158 (45)	152 (43)
DAS28	1.8 (1.1 to 2.4)	2.0 (1.6 to 2.8)	2.1 (1.5 to 2.8)	0.0 (0.0 to 0.0)	0.1 (−0.4 to 0.5)
HAQ (0–3)	0.5 (0.1 to 1.0)	0.5 (0.0 to 1.0)	0.5 (0.1 to 1.0)	0.0 (−0.1 to 0.1)	0.0 (−0.1 to 0.1)
CRP, mg/L	2 (1 to 4)	2 (1 to 4)	2 (1 to 4)	0 (−2 to 1)	0 (−1 to 1)
PGS, mm	33 (13 to 58)	30 (12 to 54)	31 (12 to 58)	0 (−9 to 6)	0 (−7 to 10)
AxSpA, n=337					
Patients with available data, n (%)*	187 (55)	217 (64)	243 (72)	117 (35)	168 (50)
BASDAI, mm	33 (15 to 52)	27 (12 to 47)	31 (18 to 52)	0 (−8 to 6)	1 (−3 to 10)
CRP, mg/L	3 (1 to 6)	3 (1 to 5)	3 (1 to 5)	0 (−2 to 1)	0 (−1 to 1)
PGS, mm	32 (15 to 59)	30 (12 to 53)	34 (17 to 60)	−1 (−13 to 6)	3 (−5 to 14)
ASDAS	1.9 (1.3 to 2.8)	1.9 (1.2 to 2.6)	1.9 (1.3 to 2.7)	−0.1 (−0.4 to 0.3)	0.1 (−0.2 to 0.6)
3 months' flare rates preswitch vs postswitch†					
RA (ΔDAS28 ≥0.6), %				22	24
PsA (ΔDAS28 ≥0.6), %				21	23
RA (ΔDAS28 ≥1.2), %				8	13
PsA (ΔDAS28 ≥1.2), %				8	11
AxSpA (ΔASDAS >1.1), %				4	5

Numbers are medians (interquartile ranges) unless otherwise stated (%).
Missing data at the 3 months' visit were imputed with the 6 months' visit according to the following time windows:
Time windows preswitch: 3 months' window: 0 to 25 weeks, 6 months' window: 25 to 32 weeks before start of SB4.
Time window switch: 12 weeks before until 1 week after start of SB4.
Time window postswitch: 3 months' window: 9 to 17 weeks, 6 months' window: 17 to 32 weeks after start of SB4. Overlapping time windows at baseline were allowed to reduce missing data. Any visit was only used once, and the registration closest to the given time point was selected. If a patient had no registrations, data were registered as missing for that visit.
*Number of patients with available data varied slightly across measures of disease activity. Exact numbers are shown for DAS28 and ASDAS. Individual patients might not have complete data for all variables at a certain time point. Comparisons of before vs after the switch were done in the patients who had complete data for that variable.
†There was no overlap between the patients who had a flare preswitch vs postswitch.
‡Comparison of Δpreswitch vs Δpostswitch byWilcoxon matched-pair signed rank test.
ASDAS, the Ankylosing Spondylitis Disease Activity Score; AxSpA, axial spondyloarthritis; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; CRP, C reactive protein; DAS28, 28-joint Disease Activity Score (four variables, CRP-based); ETA, originator etanercept; HAQ, Health Assessment Questionnaire; PGS, patient's global score; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SB4, biosimilar etanercept.

activity than non-switchers. A similar pattern was observed for PsA (mainly for the subjective measures PGS, PASS) and AxSpA (table 1). Available data are shown in online supplementary table S1. The percentage of patients with available data regarding baseline remission status was 71% for switchers and 90% for non-switchers.

Outcomes in switchers and non-switchers

In switchers, disease activity and flare rates 3 months preswitch versus postswitch were similar with no clinically relevant differences (table 2). For RA and PsA, two different definitions of disease flare were applied (table 2).

During follow-up (median 401 days (IQR: 336 to 443 days), 299 switchers (18%) and 145 non-switchers (33%) withdrew from treatment with SB4 and ETA, respectively. In both patient groups, lack of effect was the most common reason for withdrawal (table 3). In switchers, adverse events were mainly unspecific, and no major safety signals were observed (table 3).

Among switchers, the SB4 retention rate was lower in patients with RA (figure 1A), in patients who had started ETA treatment during the later years (overall, figure 1B, and stratified by indication, not shown) and in patients not in remission at the time of switching (overall, figure 1C, HR 1.7 (95% CI 1.3 to 2.2) and stratified by indication, not shown). Similarly, in the cohort of non-switchers, retention rate was lower in patients not in remission (HR 2.4 (1.7 to 3.6)) and in patients who started treatment during the later years, and withdrawal was more frequent in PsA (all $p<0.01$, not shown).

When comparing retention rates in switchers with non-switchers stratified by indication, switchers were less likely to withdraw from treatment than non-switchers (crude HR for withdrawal ranging from 0.42 to 0.89, most pronounced in RA and PsA) (table 4). In adjusted analyses, switch status was no longer statistically significant (table 4). Similar results were found in sensitivity analyses replacing remission status with PGS (≤30 mm/>30 mm) as baseline covariate (not shown).

Switchers versus historic cohort, one-year retention rates

A historic cohort of patients treated with ETA by 1 January 2015 was identified in DANBIO (n=2363). The percentage of

Table 3 Reason for withdrawal in switchers and non-switchers

	Switchers N=1621	Non-switchers N=440
Reason, n (% of withdrawals)		
Lack of effect	137 (46)	48 (34)
Adverse events	77 (26)*	14 (10)
Several reasons	9 (3)	1 (1)
Cancer	6 (2)	11 (8)
Remission	8 (3)	10 (7)
Pregnancy	4 (1)	3 (2)
Death	9 (3)	15 (10)
Infection	3 (1)	8 (6)
Loss to follow-up	1 (2)	9 (6)
Surgery	2 (1)	1 (1)
Other	14 (5)	18 (13)
Not stated	29 (10)	7 (5)
Withdrawals, total, n (%)	299 (100)	145 (100)

*Adverse events during biosimilar etanercept (SB4) treatment in switchers (77 patients): anxiety 1 patient, arthralgia 1, bladder dysfunction 1, blurred vision 1, chest pain 2, diarrhoea 4, dizziness 2, dyspnoea 2, erectile dysfunction 1, fatigue 1, fever 2, hair loss 1, headache/migraine 9, hyperhidrosis 2, hypertension 1, hypotension 1, infections 2, leg cramps 2, leucopenia 3, local injection problems 3, myalgia 2, nausea 4, neuropathies 1, psoriasis worsening or pustulosis 2, rash/itching 11, not stated 39 (total=101 events).

patients from the cohorts of switchers, non-switchers and back-switchers that were also included in the historic cohort were 94%, 86% and 100%, respectively. Furthermore, 376 patients were only in the historic cohort and were not included in the switch/non-switch cohorts. The baseline demographics of the historic and the switch cohort were similar (see online supplementary table S2). The 1-year crude retention rate was lower in switchers (82% (95%CI: 79% to 83%)) than in the historic cohort (88% (87% to 90%)) but better than in non-switchers (70% (66% to 74%)) (figure 1D). The corresponding 1-year adjusted retention rates were 83% (79% to 87%)) in switchers, 90% (88% to 92%)) in the historic cohort and 77% (72% to 82%)) in non-switchers. In adjusted analysis of treatment withdrawal in switchers compared with the historic cohort, switch status remained significant (table 4). Similar results were found in sensitivity analyses replacing PGS with remission status as baseline covariate (not shown).

Frequency and outcomes of back-switching

During follow-up, the 299 switchers, who had withdrawn SB4 therapy, either commenced treatment with another bDMARD (n=104), switched back to ETA (n=120), died (n=9), were lost to follow-up (n=1) or did not restart bDMARDs (n=65) (see online supplementary table S2).

Among the 120/1641 switchers (7%) who withdrew from treatment with SB4 and switched back to ETA, the main reason for SB4 withdrawal was lack of effect (table 5). Baseline characteristics were similar in back-switchers and the rest of the switch population (all p>0.05). Changes in disease activity at the time of ETA restart compared with SB4 index date were mainly observed for PGS whereas changes in CRP and swollen joint counts were close to zero (table 5). The SB4 treatment duration before back-switching to ETA was median 120 (IQR 73 to 193) days, and the time interval between stop of SB4 and restart of ETA was 1 (1–1) days. At the time of censoring, 104 of 120 back-switchers (87%) were still treated with ETA with median treatment duration of 236 (155 to 302) days.

DISCUSSION

In the current study, treatment outcomes of a nationwide guideline with mandatory switching from ETA to SB4 were investigated in 2061 patients of whom 79% switched to biosimilar SB4. The 21% non-switchers less frequently had PsA and tended to have higher disease activity than the switchers and received concomitant MTX less frequently (in patients with PsA and RA). Some non-switchers received the 25 mg ETA dose, which was still available. Regarding treatment outcomes, this study showed mixed results. On one hand, the disease activity among switchers was stable 3 months before and after the switch. On the other hand, the 1-year SB4 retention rate was lower than that of a historic ETA cohort. However, the non-switch cohort had even higher withdrawal rate. Our study indicates that patient-related factors, for example, being in remission or not, rather than drug (originator or biosimilar) were important for the decision to withdraw treatment. A subgroup of SB4-treated patients switched back to ETA. They had no distinct clinical or disease characteristics at the start of SB4, and reasons for back-switching appeared to be of a more subjective rather than objective nature.

According to recent European League Against Rheumatism (EULAR) recommendations, biosimilars should be included in the treatment algorithm on equal terms as the originator drugs.[25] However, regarding non-medical switching (ie, switching for economic reasons in patients who are receiving treatment with the originator drug), recommendations are less clear.[1] A recent task force concluded that a single switch from a bio-originator to one of its biosimilars is safe and effective[8]—a recommendation that has been debated by others.[15] Currently, the use of biosimilars and switch procedures in routine care vary substantially across countries.[26–28]

Experience regarding real-world use of biosimilar drugs is needed as a supplement to RCTs.[8 15 28 29] Thus, RCTs mainly report outcomes in highly selected and often bDMARD naïve patients with short follow-up,[6] whereas observational studies provide data in large unselected patient -groups that may be switchers from other bDMARDs and with the opportunity of long follow-up. To our knowledge, this study is the largest to explore outcomes of a non-medical switch from originator ETA to SB4 in routine care.[18–20 30] We observed no new major safety events for SB4. The efficacy and safety profile of switching from ETA to SB4 has been demonstrated in one RCT of patients with moderate–severe RA despite previous MTX treatment[6 31] where a subgroup of patients initially randomised to ETA treatment (n=119) after 1 year switched to SB4 in an open label design.[4] The authors reported no excess risk and comparable efficacy, safety and immunogenicity in the switch group compared with patients who continued treatment with SB4.[4] Previous observational studies (abstracts only) have, similar to our findings, reported stable disease activity 6 months after switching[19] (147 patients) and a 6 months' SB4 withdrawal rate of 9%–10% (in two cohorts of 92 and 642 patients, respectively).[18 20]

Although the Danish guideline that preceded the current study stated that the switch was mandatory, 21% remained on ETA treatment in contrast to a previous mandatory switch to biosimilar infliximab.[17] The originator drug was still available (as 25 mg syringe/pen or as 50 mg powder solution) which may partly explain why one in five patients did not switch. Most non-switchers received 50 mg ETA. Patient-related factors, for example, more comorbidities (indicated by more deaths, infections and cancers during follow-up), and

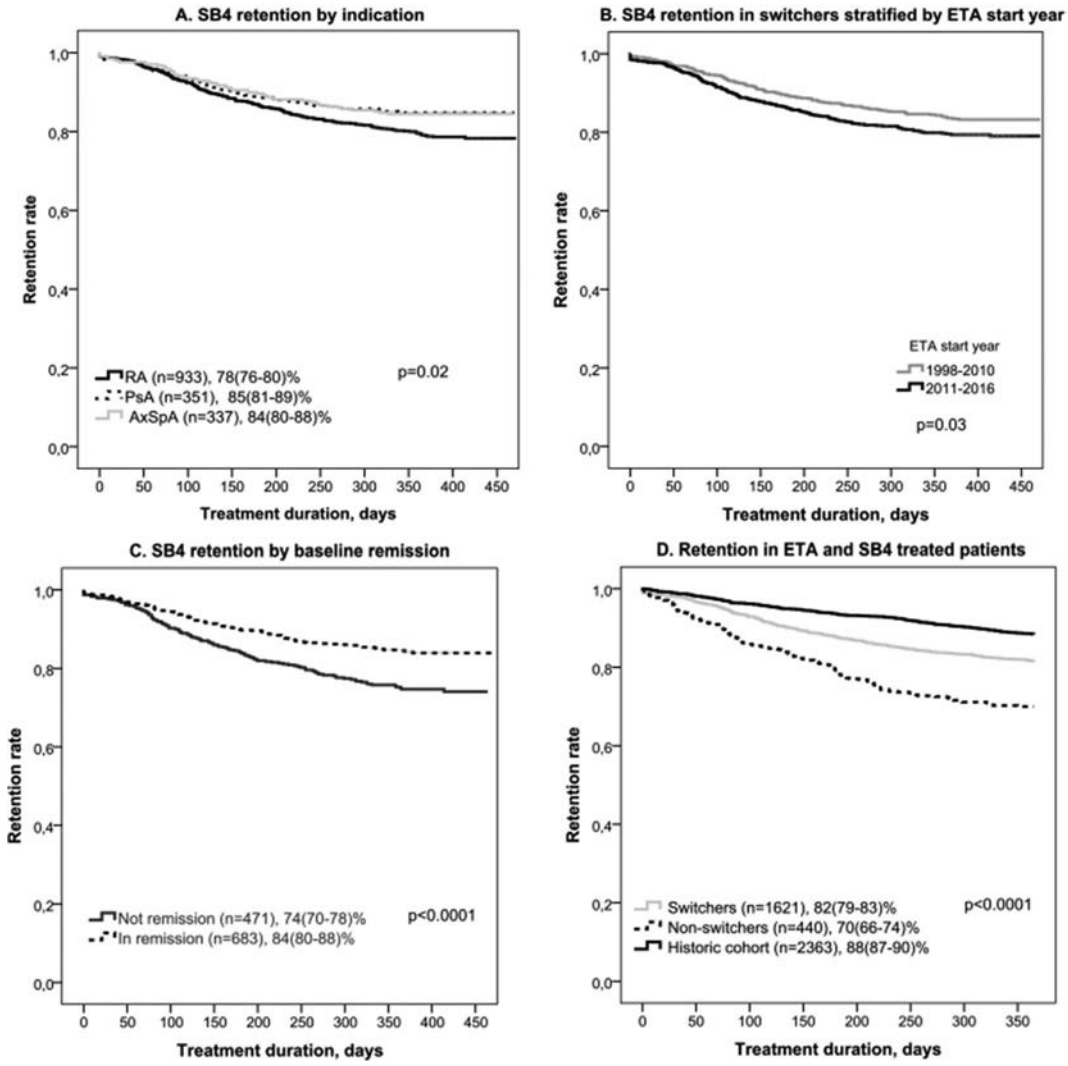

Figure 1 Kaplan-Meier plots of crude treatment retention rates among SB4 switch patients. (A) Stratified by indication. (B) Stratified by start year of ETA treatment (all indications) (. (C) Stratified by baseline remission (all indications). (D) Compared with non-switchers and a historic ETA cohort (all indications). Percentages are 1-year treatment retention (95% CI). AxSpA, axial spondyloarthritis; ETA, originator etanercept; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SB4, biosimilar etanercept.

higher disease activity in non-switchers might have contributed to non-switch. Thus, the study results are likely biassed by the fact that the final outcomes (drug retention, remission status, etc) were influenced by the patient's and physician's choice to comply with the guideline (and agreed in switching) or refused to do so (and did not switch). An indication for the presence of such bias can be found in the baseline differences between switchers and non-switchers. In that regard, the

Table 4 Treatment retention in (A) switchers vs non-switchers and (B) switchers vs historic cohort. Results from univariable and multivariable Cox regression analysis (HR, with 95% CI) stratified by indication

	RA		PsA		AxSpA	
	HR	P values	HR	P values	HR	P values
A. Switchers vs non-switchers*						
Crude	0.68 (0.51 to 0.91)	0.0005	0.42 (0.24 to 0.73)	0.0019	0.89 (0.49 to 1.60)	0.70
Adjusted†	0.81 (0.59 to 1.11)	0.18	0.55 (0.28 to 1.07)	0.079	0.92 (0.50 to 1.73)	0.82
B. Switchers vs historic comparison cohort*						
Crude	1.73 (1.36 to 2.19)	<0.0001	1.93 (1.26 to 2.96)	0.0024	2.29 (1.45 to 3.61)	0.0003
Adjusted†	1.76 (1.39 to 2.23)	<0.0001	2.15 (1.42 to 3.25)	0.0003	2.37 (1.51 to 3.73)	0.0002

*Number of patients included in cohorts: RA (switchers 684 patients/non-switchers 264/historic cohort 1239), PsA (253/49/364), AxSpA (217/81/412), patients with missing data regarding remission status excluded.
†Adjusted for gender, age, methotrexate use (yes/no), remission (yes/no), comorbidities (≥1/0), ETA start year (1998-2010/2011-2016). Remission defined as DAS28 <2.6 (RA, PsA), ASDAS <1.3 (AxSpA).
ASDAS, the Ankylosing Spondylitis Disease Activity Score; AxSpA, axial spondyloarthritis; CRP, C reactive protein; DAS28, 28-joint Disease Activity Score (four variables, CRP-based); ETA, originator etanercept; PGS, Patient's globalscore; PsA, psoriatic arthritis; RA, rheumatoid arthritis.

Table 5 ETA-SB4-ETA back-switchers (n=120). Characteristics at the start of SB4, reasons for SB4 withdrawal and changes in disease activity among withdrawals due to LOE

	RA	PsA	AxSpA
Patient number, n	80	20	20
Characteristics at the start of SB4			
Female, n (%)	58 (73)	11 (55)	7 (35)
Age, years	59 (52 to 70)	45 (36 to 56)	43 (38 to 56)
Concomitant MTX, n (%)	39 (49)	7 (35)	1 (5)
Patients with available data, n*	64	17	18
In remission, %	61	82	19
PGS, mm*	27 (12 to 54)	25 (13 to 63)	23 (13 to 44)
DAS28	2.2 (1.6 to 3.2)	1.8 (1.4 to 2.2)	–
CRP, mg/L	3 (1 to 8)	1 (1 to 5)	3 (1 to 6)
Swollen joint count	0 (0 to 1)	0 (0 to 0)	–
ASDAS	–	–	1.7 (1.4 to 2.4)
PASS yes, %	81	82	88
Reason for SB4 withdrawal, n (%)			
AE	34 (42)	7 (35)	6 (30)
LOE	38 (48)	11 (55)	13 (65)
Other/several/not stated	8 (10)	2 (10)	1 (5)
Characteristics at the restart of ETA in patients who stopped due to LOE and back-switched, n=62			
Patient number, n	38	11	13
Swollen joint count	2 (0 to 5)	0 (0 to 2)	–
CRP, mg/L	3 (2 to 11)	3 (2 to 7)	4 (1 to 6)
PGS, mm	64 (50 to 76)	78 (18 to 90)	42 (35 to 63)
Delta values† in patients who stopped due to LOE and back-switched			
Patients with available data, n†	31	8	11
Delta-swollen joint count	1 (0 to 4)	0 (0 to 0)	–
Delta-CRP, mg/L	0 (-1 to 5)	1 (0 to 2)	0 (0 to 4)
Delta-PGS, mm	30 (12 to 52)	15 (7 to 77)	25 (19 to 35)

Numbers are medians (interquartile ranges) unless otherwise stated.
Patients stopped due to adverse events, n=47: arthralgia 1 patient, bladder dysfunction 1,blurred vision 1, diarrhoea 4, dizziness 2, dyspnoea 2, erectile dysfunction 1, hair loss 1,headache/migraine 4, hyperhidrosis 2, hypertension 1, hypotension 1, infections 2, leg cramps 1, local injection problems 3, myalgia 1, nausea 2, neuropathies 1, psoriasis worsening or pustulosis 1, rash/itching 9, not stated 21 (total=62 events, this is a subgroup of the eventsshown in table 3).
*Available data varied according to variable, numbers are shown for PGS.
†Calculated as disease activity at time of restart ETA minus at the time of SB4 start for each patient.
AE, adverse event; ASDAS, the Ankylosing Spondylitis Disease Activity Score; AxSpA, axial spondyloarthritis; CRP, C reactive protein; DAS28, 28-joint Disease Activity Score (four variables, CRP-based); LOE, lack of effect; MTX, methotrexate; PASS, patient acceptable symptom state; PGS, patient's global score; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SB4, biosimilar etanercept.

study results do not represent an unbiased comparison of the effects of switching versus non-switching, and other (patient related) factors than the switching alone may have influenced the outcomes.

Many ETA-treated patients were not in disease remission when they were switched to SB4 and they withdrew more often than patients who were in remission. We have previously reported similar results in patients who switched from originator to biosimilar infliximab.[17] Interestingly, the same pattern was observed in patients who maintained ETA treatment (non-switchers). This suggests that a switching-to-biosimilar guideline facilitated clinical decision making and withdrawal of ineffective therapy independent of switch status.

The knowledge regarding biosimilar drugs in the general population and patients with chronic diseases is still low.[32] Both physicians and patients may be reluctant to use biosimilars.[2 33] The nocebo effect (ie, negative expectations towards a given treatment), patient-related factors and subjective health experiences may have influenced the perception of treatment outcomes and adverse events.[34–39] The majority of the 120 back-switchers were still treated with ETA on data censoring. However, changes in disease activity and the reported AEs tended to be subjective rather than objective.

The study has strengths and weaknesses. We report nationwide, prospectively collected data in a large cohort of ETA-treated patients treated in routine care which strengthens external validity. Patients acted as their own controls in the evaluation of disease activity before/after switch, and outcomes could be compared with those of both a historic cohort and of a non-switch cohort. However, due to the observational study design, we report associations, not definitive causal relationships. Furthermore, residual confounding may affect results. Data were collected as part of routine care and missing data might bias results. Approximately half of patients contributed consecutive data on changes in disease activity 3 months prior to versus after the switch. Patients in remission are potentially monitored less frequently and the same might apply to frail patients with comorbidities. Data completeness was lower than in a previous publication from DANBIO describing switch from originator to biosimilar infliximab[17] and may reflect less frequent monitoring of patients treated with subcutaneous (ie, self-administered) bDMARDs.

In conclusion, we found that a nationwide switch from originator to biosimilar ETA in 1621 patients with inflammatory arthritis had no negative impact on 3 months' disease activities, and no major safety events were observed. Despite mandatory switch recommendations, one in five ETA-treated patients did not switch. In both patient groups, withdrawal was most common in patients not in remission. These real-world data indicate that switch outcomes in routine care are affected by non-specific drug effects and patient-related factors.

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CLINICAL SCIENCE

Comparing patient-perceived and physician-perceived remission and low disease activity in psoriatic arthritis: an analysis of 410 patients from 14 countries

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ABSTRACT

Background The objective was to compare different definitions of remission and low disease activity (LDA) in patients with psoriatic arthritis (PsA), based on both patients' and physicians' perspectives.

Methods In ReFlap (Remission/Flare in PsA; NCT03119805), adults with physician-confirmed PsA and >2 years of disease duration in 14 countries were included. Remission was defined as very low disease activity (VLDA), Disease Activity index for PSoriatic Arthritis (DAPSA) ≤4, and physician-perceived and patient-perceived remission (specific question yes/no), and LDA as minimal disease activity (MDA), DAPSA <14, and physician-perceived and patient-perceived LDA. Frequencies of these definitions, their agreement (prevalence-adjusted kappa), and sensitivity and specificity versus patient-defined status were assessed cross-sectionally.

Results Of 410 patients, the mean age (SD) was 53.9 (12.5) years, 50.7% were male, disease duration was 11.2 (8.2) years, 56.8% were on biologics, and remission/LDA was frequently attained: respectively, for remission from 12.4% (VLDA) to 36.1% (physician-perceived remission), and for LDA from 25.4% (MDA) to 43.9% (patient-perceived LDA). Thus, patient-perceived remission/LDA was frequent (65.4%). Agreement between patient-perceived remission/LDA and composite scores was moderate to good (kappa range, 0.12–0.65). When patient-perceived remission or LDA status is used as reference, DAPSA-defined remission/LDA and VLDA/MDA had a sensitivity of 73.1% and 51.5%, respectively, and a specificity of 76.8% and 88.0%, respectively. Physician-perceived remission/LDA using a single question was frequent (67.6%) but performed poorly against other definitions.

Conclusion In this unselected population, remission/LDA was frequently attained. VLDA/MDA was a more stringent definition than DAPSA-based remission/LDA. DAPSA-based remission/LDA performed better than VLDA/MDA to detect patient-defined remission or remission/LDA. Further studies of long-term outcomes are needed.

Key messages

What is already known about this subject?

- In Psoriartic arthritis (PsA), remission or alternatively, low disease activity (LDA) is the treatment ojective.
- Remission/LDA can be assessed using 2 main composite scores in PsA: very low disease activity (VLDA)/minimal disease activity (MDA) or Disease Activity iIndex for PSoriatic Arthritis (DAPSA).

What does this study add?

- Investigating an unselected, standard of care population of 410 patients with psoriatic arthritis, both remission and low disease activity (LDA) were frequently attained: from 12.4% to 36.1% for remission and from 25.4% to 46.8% for LDA.
- Patient-perceived remission/LDA was frequent (65.4%), indicating patients often reported themselves in a low level of disease activity.
- Patient-perceived remission was as frequent as remission based on composite scores (very low disease activity (VLDA)/minimal disease activity (MDA) or Disease Activity index for PSoriatic Arthritis (DAPSA)); both were less frequent than physician-reported remission using a single question.
- VLDA/MDA showed a lower sensitivity than DAPSA versus patient perspective (52% vs 73%) but had a higher specificity (88 vs 77%).

How might this impact on clinical practice or future developments?

- DAPSA-based status had both sensitivity and specificity of around 75%, indicating that this score appears to better reflect patient-perceived LDA.

INTRODUCTION

Psoriatic arthritis (PsA) is a complex inflammatory disease that spans a wide spectrum to include the peripheral joints, skin, entheses, spine and other adjacent tissues.

Recent management recommendations state that remission (REM), or in some cases low disease activity (LDA), is the treatment goal in PsA.[1–4] Several composite disease activity measures have been

developed, and the currently discussed treatment target definitions for REM/LDA are VLDA (very low disease activity)/MDA (minimal disease activity)[5–7] and DAPSA (Disease Activity index for PSoriatic Arthritis) cut-offs of ≤4/≤14 (or clinical DAPSA, cDAPSA).[8–10] These definitions each has strengths and weaknesses which hamper achieving consensus on one definition.[11 12] To briefly summarise some of the issues, on the one hand, VLDA/MDA includes a measure of function (Health Assessment Questionnaire, HAQ) which can be influenced by factors other than disease activity—this may be a methodological issue. On the other hand, DAPSA only assesses joints and not directly any other domain of PsA, such as entheses or skin, and MDA does not assess dactylitis, and both do not assess all patient-important domains. While the Outcome Measures in Rheumatology Core Set states that all domains mentioned are of importance,[13] the various multidimensional composite measures have major differences in their components and none uses all components. The question of unidimensional versus monodimensional scores has been widely addressed; however, there is currently no consensus in this respect. To this end we have used a unidimensional (DAPSA) and a multidimensional (MDA) instrument. Three recent studies have compared the VLDA/MDA outcomes with DAPSA outcomes in terms of frequency but did not assess the patient's perspective in parallel.[14–16]

REM/LDA from the patient's perspective has not been defined. The above composite measures factor in patient-reported outcomes including pain and patient global assessment.[5–10] However, they were developed with little patient involvement, and cut-offs for REM/LDA were not patient-driven.[17 18] This may be important since disagreements in the assessment of disease activity have a potential impact on treatment decisions and shared decision-making.[19–21] The only data available regarding the patient's assessment of REM/LDA are issued from studies on aspects of disease impact.[22 23] However patient-perceived LDA or REM can be approached by specific designated questions, by the 'patient acceptable symptom state' or using low values of patient global assessment (PGA).[24–26] REM/LDA can also be defined, from the physician's perspective, as achieving an REM/LDA based on a global assessment of the physician (yes/no). Such single questions may have clinical relevance, although they have not yet been assessed formally.

Since alignment between patients and health professionals in terms of treatment targets is thought to be a key component for shared decision-making,[27 28] it is of great interest to compare physician-perceived REM/LDA and composite scores with patient-perceived REM/LDA in the assessment of PsA.

The objectives of the present study were to assess the frequency of REM/LDA using different definitions according to the patient's and physician's perspective, and to assess agreement between these definitions.

METHODS

Study population and study design

The ReFlaP (Remission/Flare in PsA) study was a prospective, multicentre, international, longitudinal, observational study which took place in 21 centres in 14 countries (including 7 countries across Europe, the UK, Russia, Canada, the USA, Brazil, Turkey and Singapore) between June 2017 and August 2018 (NCT03119805). The objective of the study was to assess REM/LDA in PsA. Patients were seen twice; here, baseline data were used.

Adult patients with a diagnosis of PsA as defined by their rheumatologist and more than 2 years of disease duration were recruited. Investigators were advised to consider the Classification Criteria for Psoriatic Arthritis (CASPAR) criteria for classification of PsA. Patients with no definite PsA or less than 2 years of disease duration, patients who did not speak or read the local language, or were not comfortable filling in a paper form in the local language were excluded. The inclusion of patients was performed consecutively.

Data collection

Medical data

The collected data included patient demographic variables (age, gender, work status, level of education) and the following disease characteristics: disease duration, predominant type of PsA (peripheral, axial or entheseal), current treatment (conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) and/or biologic disease-modifying antirheumatic drugs (bDMARDs)). The Functional Comorbidity Index and the last available result (<4 weeks) for C reactive protein (CRP) were collected.[29] Physical examination included assessment with 66 swollen joint count, 68 tender joint count, tender entheseal points (by the Leeds Enthesitis Index), body surface area of psoriasis and physician global assessment (on a scale of 0–10).[30]

Patient-reported outcomes

PGA with a wording focused on disease activity was collected on a 0–10 numeric rating scale, as follows—'*How active was your rheumatic disease on average during the last week?*' (from 'Not active' to 'Very active')—and was used to calculate the composite scores.[31] This wording refers to the concept of disease activity and has been used in other rheumatic diseases.[31] As sensitivity analyses, this wording was replaced in the composite scores by wordings referring to global joint and global skin assessments.[32] Also collected were the HAQ disability index and Patient Acceptable Symptom State (PASS) (in the absence of a standardised PASS question, the following wording was used: 'If you were to remain for the next few months as you were during the last 48 hours, would this be acceptable or unacceptable for you?' yes/no).[33 34] The PsA Impact of Disease assesses the impact of PsA on 12 aspects, with a final result between 0 and 10 (higher results indicate a worse condition).[35]

The patient data collection form was translated by two persons into each local language according to usual procedures.

REM and LDA definitions

Composite scores

VLDA/MDA, DAPSA and cDAPSA were used to define REM and LDA (table 1).

Physician perspective

Physicians were asked two separate single questions for REM/LDA, formulated by the steering committee as 'At this time, is the psoriatic arthritis in remission, if this means: the absence of clinical and laboratory evidence of significant inflammatory disease activity?' and 'At this time, is the psoriatic arthritis in low or minimal disease activity?'

Of note the physicians answered these questions unblinded to other results (eg, they could consult the patient questionnaires and CRP results if they wished as in their routine clinical practice). No instructions were given as to which aspects of the disease should be considered when answering these questions, but the rheumatologists including patients into this study were all experienced in treating PsA and the question was related to PsA rather than to skin involvement, which was addressed in a separate question.

Table 1 Composite indices used to define REM and LDA in PsA

Index	Components	Cut-off for REM	Cut-off for LDA
VLDA/MDA	Tender joints (≤1). Swollen joints (≤1). Skin psoriasis (PASI ≤1 or BSA ≤3%). Entheses (≤1). Pain (≤15). Patient global for joints and skin (≤20). HAQ (≤0.5).	VLDA: 7/7 of the criteria	MDA: 5/7 of the criteria
DAPSA	Tender joints. Swollen joints. Pain. Patient global assessment. CRP.	DAPSA remission ≤4	DAPSA LDA: 5 to ≤14
cDAPSA	Tender joints. Swollen joints. Pain. Patient global assessment.	cDAPSA remission ≤4	cDAPSA LDA: 5 to ≤13

BSA, body surface area; CRP, Creactive protein; DAPSA, Disease Activity Index for PSoriatic Arthritis; HAQ, Health Assessment Questionnaire; LDA, Low Disease Activity; MDA, minimal disease activity; PASI, Psoriasis Activity And Severity Index; PsA, psoriatic arthritis; REM, Remission; VLDA, very low disease activity; tender joint count on 68 joints; swollen joint count on 66 joints; cDAPSA, clinical DAPSA.

Patients' perspective

REM/LDA separate questions for patients were developed with input from four patient research partners with PsA and were based on previous work in the field of rheumatoid arthritis.[36 37] The phrasing was the following: 'At this time, is your psoriatic arthritis in remission, if this means: you feel your disease is as good as gone?' (for REM) and 'At this time, are you in low disease activity, if this means: your disease is in low activity but it's not as good as gone?' (for LDA).

From patients' perspective, two potential definitions for REM were used: patient-perceived remission (single question as above) and PGA ≤1. Also, two definitions for LDA were used: patient-perceived LDA (single question) and PGA ≤3. The PGA cut-offs were informed, for REM, by the rheumatoid arthritis international REM criteria since no cut-off has been defined in PsA.[38] For LDA, the cut-off of PGA ≤3 was selected by the steering committee. Such cut-offs are arbitrary, and given issues around circularity between PGA and the composite scores, the PGA external criterion should be considered as indicative only.

As a comparison outcome, the PASS was compared with a state of LDA.

Statistical analysis

All patients with items available to calculate REM/LDA with all definitions were analysed. Demographic, clinical and biologic variables were expressed as mean±SD for continuous variables and as frequencies (percentages) for categorical variables. No imputation of missing data was performed; data were analysed on complete cases. To obtain an overview of the meaning of patient-defined disease states, patient characteristics in each self-defined disease state were described. Proportions achieving each REM/LDA criterion were calculated, and for the composite score definitions REM and LDA groups were analysed separately and then also combined. Venn diagrams were used to represent the number of patients meeting different REM/LDA criteria. To assess performances of the composite scores, their sensitivity and specificity were calculated versus the reference definition, which was here patient-perceived status (ie, REM or REM/LDA). Thus, sensitivity was the percentage of patients in self-reported good status who was found in good status using the composite score, and specificity was the percentage of patients in self-reported lack of good status who were found in lack of good status using the score.

The agreement between the tested definitions was established using 2×2 tables and calculation of Cohen's kappa and prevalence-adjusted bias-adjusted kappa (PABAK) where necessary, using Bennett's method.[39 40] In cases of discrepancy between Cohen's kappa and PABAK, the paradox of the kappa may apply and PABAK should be analysed preferentially. Usual cut-offs to interpret kappas were used, namely 0.00–0.20 slight agreement, 0.21–0.40 fair, 0.41–0.60 moderate and 0.61–0.80 good agreement. R V.3.4.3 software was used for all statistical analyses.

RESULTS

Demographic and clinical characteristics

A total of 466 patients were included: 56 were ineligible (no confirmation of diagnosis, n=11; age below 18, n=1) or had missing data (mainly CRP, n=27; entheseal assessment, n=6; or HAQ, n=2; other criteria were missing in 9 patients). Thus, 410 with complete data were analysed (table 2). Of these, 50.7% were male and the mean disease duration was 11.2 years. Disease activity was moderate and the majority were receiving csDMARDs (59.3%) and/or bDMARDs (56.8%). Disease activity was lower in patients in self-defined REM or LDA, supporting validity of the questions applied in the present study (table 2).

Prevalence of REM/LDA according to the different definitions

Remission

The most frequent REM status was obtained using physician single question: 148 (36.1%) patients. cDAPSA (25.6% REM) and both of the patient-defined REM (single question, 21.5%; or PGA ≤1, 24.4%) were of similar frequency. DAPSA (19.0% REM) and especially VLDA (12.4%) were more stringent.

Low disease activity

This status was frequent, in particular when using the patient single question (43.9%; figure 1). The definition leading least frequently to this status was MDA (25.4%).

Remission + low disease activity

VLDA/MDA was difficult to reach with only 37.8% in REM/LDA; DAPSA was less limiting with 58.5% of patients. Patient-perceived

Table 2 Characteristics of 410 patients with PsA

	All (N=410)	Patients in self-defined REM (n=88)	Patients in self-defined LDA (n=180)	Patients in other disease states (n=142)
Male, n (%)	208 (50.7)	58 (65.9)	95 (52.8)	55 (38.7)
Mean age, years (SD)	53.9 (12.5)	53.7 (13.5)	54.3 (12.3)	53.4 (12.1)
Mean PsA duration, years (SD)	11.2 (8.2)	11.9 (8.7)	11.3 (8.3)	10.8 (7.9)
Mean level of schooling, years (SD)	12.9 (3.4)	13.4 (3.4)	12.8 (3.5)	12.6 (3.5)
Paid work, n (%)	233 (56.8)	53 (60.2)	106 (58.9)	74 (52.1)
Current smoking, n (%)	68 (16.6)	9 (10.2)	25 (13.9)	34 (23.9)
Elevated acute phase reactants (CRP >5 mg/L), n (%)	156 (38.0)	23 (26.1)	60 (33.3)	73 (51.4)
Radiographic lesions according to CASPAR criteria, n (%)	124 (30.2)	26 (29.5)	51 (28.3)	47 (33.1)
Conventional synthetic DMARD intake, n (%)	243 (59.3)	54 (61.4)	112 (62.2)	77 (54.2)
Biologic DMARD intake, n (%)	233 (56.8)	53 (60.2)	108 (60.0)	72 (50.7)
Oral glucocorticoids, n (%)	67 (16.3)	10 (11.4)	26 (14.4)	31 (21.8)
Number of comorbidities, mean (SD)	1.3 (1.0)	1.4 (1.1)	1.2 (1.0)	1.3 (1.0)
No current psoriasis skin lesions, n (%)	142 (34.6)	45 (51.1)	63 (35.0)	34 (23.9)
Body surface area of psoriasis ≥5%, n (%)	38 (9.3)	3 (3.4)	14 (7.7)	19 (13.3)
Tender entheseal points, LEI mean (SD)	0.6 (1.4)	0.4 (1.3)	0.3 (0.9)	1.1 (1.8)
Tender joint count (0–68), mean (SD)	4.9 (9.8)	3.4 (10.6)	2.9 (6.8)	8.4 (11.5)
Swollen joint count (0–66), mean (SD)	2.4 (7.3)	0.9 (3.6)	1.6 (5.6)	4.3 (10.0)
Physician's global assessment of PsA, mean (SD)	3.1 (2.5)	1.7 (2.0)	2.6 (2.1)	4.7 (2.4)
Patient's assessment of pain (0–10), mean (SD)	4.1 (2.8)	2.2 (2.4)	3.5 (2.3)	6.2 (2.2)
Patient's global assessment of PsA (0–10), mean (SD)	4.2 (2.8)	2.4 (2.5)	3.5 (2.2)	6.2 (2.3)
DAPSA, mean (SD)	17.0 (17.7)	9.4 (15.5)	12.8 (15.0)	27.0 (17.8)
DAPSA<28, n (%)	344 (83.9)	84 (95.5)	167 (92.8)	49 (65.5)
HAQ (0–3), mean (SD)	0.68 (0.68)	0.36 (0.53)	0.54 (0.58)	1.06 (0.70)
PsAID 12, mean (SD)	3.4 (2.5)	1.8 (1.9)	2.8 (2.1)	5.2 (2.1)

CASPAR, Classification Criteria for Psoriatic Arthritis; CRP, Creactive protein; DAPSA, Disease Activity Index for PSoriatic Arthritis; DMARD, disease-modifying antirheumatic drug; HAQ, Health Assessment Questionnaire; LDA, low disease activity; LEI, Leeds Enthesitis Index; PsA, psoriatic arthritis; PsAID, PsA Impact of Disease; REM, remission.

REM/LDA and physician-perceived REM/LDA were also less limiting than VLDA/MDA and had similar frequencies (65.4% and 67.6%, respectively)

Of note, 269 (65.6%) patients were in PASS.

Agreement between REM/LDA definitions

Agreements between definitions are shown in table 3.

Remission

There was a very high agreement between DAPSA and cDAPSA REM, reflecting the similarity of the two definitions.[12 13] The agreement between DAPSA/cDAPSA and VLDA and between PGA≤1 and VLDA, cDAPSA and DAPSA was high; however, the latter may reflect some circularity since PGA is a component of these measures.[4–10] The agreement between VLDA/cDAPSA/DAPSA and patient-perceived REM was moderate to good and comparable (table 3).

Low disease activity

Excluding expected high agreement between DAPSA and cDAPSA LDA, agreements were lower for LDA than for REM (table 3).

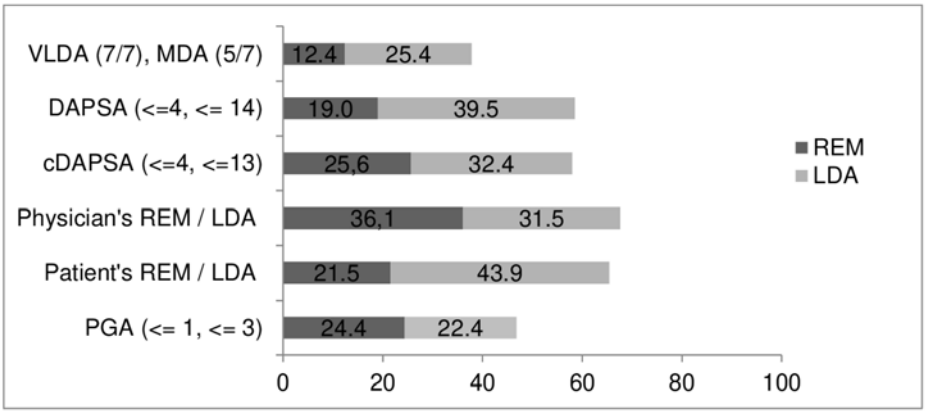

Figure 1 Prevalence of REM/LDA according to different definitions in 410 patients with PsA. Results are presented for REM and LDA separately (without overlap of definitions). cDAPSA, clinical DAPSA; DAPSA, Disease Activity index for PSoriatic Arthritis; LDA, low disease activity; MDA, minimal disease activity; patient's REM/LDA, patient's single question for REM/LDA; physician's REM/LDA, physician's single question for REM/LDA; PGA, patient global assessment; PsA, psoriatic arthritis; REM, remission; VLDA, very low disease activity.

Table 3 Agreement between different definitions of REM/LDA in 410 patients with PsA

REM					
	cDAPSA REM	**VLDA**	**Physician-perceived REM**	**Patient -perceived REM**	**PGA ≤1**
DAPSA REM	0.81 (0.87)	0.64 (0.81)	0.39 (0.49)	0.38 (0.60)	0.64 (0.76)
cDAPSA REM		0.58 (0.74)	0.44 (0.52)	0.40 (0.57)	0.73 (0.80)
VLDA			0.32 (0.46)	0.39 (0.65)	0.61 (0.76)
Physician-perceived REM				0.30 (0.41)	0.32 (0.41)
Patient-perceived REM					0.43 (0.60)
LDA					
	cDAPSA LDA	**MDA**	**Physician-perceived LDA**	**Patient-perceived LDA**	**PGA>1 to 3**
DAPSA LDA	0.77 (0.79)	0.31 (0.81)	0.24 (0.30)	0.30 (0.32)	0.28 (0.36)
cDAPSA LDA		0.23 (0.36)	0.24 (0.34)	0.25 (0.28)	0.33 (0.46)
MDA			0.12 (0.28)	0.17 (0.22)	0.14 (0.38)
Physician-perceived LDA				0.17 (0.20)	0.06 (0.25)

Results are presented as Cohen's kappa (prevalence-adjusted and bias-adjusted kappa). In cases of discrepancy the prevalence-adjusted bias-adjusted measures should be interpreted.
Patient-perceived and physician-perceived statuses are based on the single question for each status.
DAPSA, Disease Activity Index for PSoriatic Arthritis; LDA, low disease activity; MDA, minimal disease activity; PGA, patient global assessment; PsA, psoriatic arthritis; REM, remission; VLDA, very low disease activity; cDAPSA, clinical DAPSA.

Agreement between PASS and composite scores was moderate (kappa 0.56 and 0.59 and PABAK 0.33 and 0.58 for VLDA or MDA and DAPSA REM or LDA, respectively; data not shown).

Sensitivity/specificity of different REM/LDA definitions versus the patient's assessment of status

Performances of different definitions are shown in table 4, with detailed Venn diagrams in online supplementary figures 1–3.

Remission

When patient-perceived REM is used as a reference, the sensitivity of DAPSA-defined REM and VLDA was, respectively, 47.7% and 38.6%, and specificity was, respectively, 88.8% and 94.7% (table 4). Physician-perceived REM was less stringent, thus leading to higher sensitivity but with lower specificity (table 4).

Low disease activity

There were 180 patients in patient-perceived LDA. Of these, 62 (sensitivity, 34.4%) met the MDA criteria, 101 (56.1%) were in DAPSA-LDA and 60 (33.3) were not in LDA according to any composite score (table 4).

When analysing as outcome, either patient-perceived REM or LDA (ie, the sum of patients in these outcomes), the sensitivity of DAPSA-defined REM/LDA and VLDA/MDA versus patient-perceived status was, respectively, 73.1% and 51.5% (figure 2). Conversely, the specificity for DAPSA-defined REM/LDA and VLDA/MDA was, respectively, 76.8% and 88.0%.

When replacing in the composite scores, the PGA phrasing by phrasings referring to global assessment of joints and of skin psoriasis, [32] results were very similar (online supplementary table 1).

DISCUSSION

This unique cohort of unselected patients with PsA brings important information on REM/LDA concepts and adds a dimension related to the patient's perspective. Defining a specific target for REM/LDA is of importance because a treat-to-target approach with either REM or LDA as the target is now recommended in standard care by guidelines for patients with PsA.[1 8] We were able to explore patient-perceived and physician-perceived REM/LDA using novel questions. We found that patient-perceived REM/LDA was frequent (65.4%); thus, patient-perceived REM/LDA was similar in terms of prevalence to physician-perceived REM/LDA (67.6%) and to DAPSA-based REM/LDA (58.5%) compared with a lower frequency of MDA/VLDA (37.8%). When comparing patient-perceived status and composite scores, we found neither DAPSA-REM nor VLDA could detect all patients in self-reported REM, although DAPSA performed better (sensitivity 47.7% and 38.6%, respectively). When analysing the status of pooled REM/LDA , agreement with composite scores was moderate to good; sensitivity was low

Table 4 Assessment of sensitivity and specificity of different definitions of REM/LDA against the anchor of patient-perceived REM/LDA

Definition tested	Property	Anchor: patient-perceived REM	Anchor: patient-perceived LDA	Anchor: patient-perceived REM or LDA
VLDA/MDA	Sensitivity (n/n)	38.6% (34/88)	34.4% (62/180)	51.5% (138/268)
	Specificity (n/n)	94.7% (305/322)	81.7% (188/230)	88.0% (125/142)
DAPSA REM/LDA	Sensitivity (n/n)	47.7% (42/88)	56.1% (101/180)	73.1% (196/268)
	Specificity (n/n)	88.8% (286/322)	73.5% (169/230)	76.8% (109/142)
Physician-perceived REM/LDA	Sensitivity (n/n)	65.9% (58/88)	40.6% (73/180)	81.0% (217/268)
	Specificity (n/n)	72.0% (232/322)	75.7% (174/230)	57.7% (82/142)

Sensitivity n/n: n patients perceived as in the status by the score/n patients in the status according to the patient-defined anchor status.
Specificity n/n: n patients perceived as NOT in the status by the score/n patients NOT in the status according to the patient-defined anchor status.
DAPSA, Disease Activity Index for PSoriatic Arthritis; LDA, low disease activity; MDA, minimal disease activity; REM, remission.

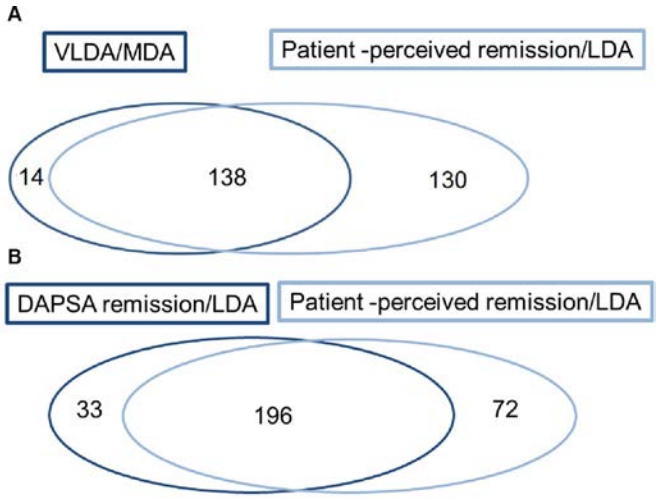

Figure 2 Venn diagram representing the number of patients meeting REM/LDA when comparing patient-perceived status and composite scores, among 410 patients with PsA (of whom, 268 in patient-defined REM/LDA). (A) VLDA/MDA versus patient perspective (sensitivity, 51.5%; specificity 88.0%). (B) DAPSA versus patient perspective (sensitivity, 73.1%; specificity, 76.8%). DAPSA, Disease Activity index for PSoriatic Arthritis; LDA, low disease activity; MDA, minimal disease activity; PsA, psoriatic arthritis; VLDA, very low disease activity.

for VLDA/MDA (51.5%) and higher for DAPSA-based cut-offs (73.1%), whereas specificity was high for both scores, although higher using VLDA/MDA (88.0% and 76.8%, respectively). Physician-perceived status appeared too lenient when using a single question, with low agreements with other definitions of REM. Finally, agreements between definitions were moderate for LDA (when analysed alone), indicating the concept of LDA may need further exploration.

This study had strengths and weaknesses. Recruitment occurred in tertiary care centres as reflected by a high percentage of patients under biologics, which may limit external validity. Nevertheless, it is generalisable due to the international large-scale recruitment strategy of consecutive patients with PsA. Furthermore, frequencies of REM/LDA were similar to other studies, which supports the validity of the present findings.[14–16] Another difficulty was to choose among many possible definitions of REM/LDA since no consensus exists. The instruments investigated in this study, DAPSA and MDA, are the ones recommended by an international task force to be applied when measuring disease activity in PsA.[3] This study brings new information on these instruments. Other possible definitions of REM/LDA provided by other measures[41 42] were not assessed, since they did not obtain a majority vote in the treat-to-target recommendations which were developed by a large international task force.[3] However, further research may explore such other instruments.

The scores were calculated using a wording for PGA, referring to disease activity and referring more to joints than skin; however, the results were overall similar when performing the analyses with patient global questions referring to either joints or skin. It is noteworthy that missing data were low (<15%) even though no queries were sent to the investigators, which supports the feasibility of these scores in clinical practice. A potential weakness is the use of non-validated single questions to explore patient-perceived and physician-perceived REM/LDA. It was not possible to use consensual questions since none exist. Thus, questions were developed for the purpose of this study. Of note, great attention was paid to their elaboration process by involving patient research partners to ensure face and content validity, while physician-perceived REM/LDA questions were developed by the steering committee. Thus, these questions were developed with relevant input and support the REM/LDA concepts. However, they reflect more PsA concepts than skin psoriasis concepts—this ought to be taken into account when interpreting the study. It should also be recognised that the present population had limited skin involvement, as is often the case in patients with PsA seen in rheumatology clinics.[43] The results may differ in patients with more severe skin disease, for example, patients with PsA seen predominantly in dermatology offices or in patients with less well-controlled disease.

This study focused on patient-perceived REM/LDA. Patients defined themselves as in REM/LDA in around 65% of cases (figure 1). This is encouraging in terms of the overall disease burden of PsA[44] and should be interpreted in the context that many of our study patients were receiving biologics. These results are in line with recent efforts to identify patients' priorities.[13 45] Interestingly, similar frequencies of low activity were found using REM/LDA questions and the PASS single question; this does not mean we suggest a PASS should be used as treatment target though; this criterion was used as grounding element only. Patient-perceived status refers to the disease process but also to patient expectations.[23] Considering recruitment occurred in 14 countries for the present study, it is interesting to note that patient status was self-reported as satisfactory so often, since recent data have indicated high patient expectations in countries with higher gross domestic product.[46] Such notions should be further explored.

When considering REM as the treatment target, we found composite scores to be only moderately in agreement with the patient perspective. In particular, 48.8% of patients in self-reported REM were not in VLDA or DAPSA-based REM, and 33.3% of those in self-reported LDA were not considered in LDA by composite measures. These figures lead to low sensitivities of composite scores to detect patient-defined REM, although DAPSA performed better than VLDA in this respect. Concordance was higher when pooling REM and LDA concepts. This may indicate limits of the composite scores to perfectly distinguish REM from LDA, and/or difficulties for patients to distinguish these states. LDA may be a personal concept and is more likely to carry different meanings for different people depending on their disease phenotype. Another explanation is that patients' and physicians' opinions on REM/LDA may differ and that composite measures may not entirely consider patients' priorities.[13 47] Patients probably do not only refer to disease activity when considering the concept of REM; thus, some discordance is expected. It would be interesting to further investigate the connection between achieving different disease activity states and long-term prognosis.

In the present study, physician-perceived REM/LDA was explored using designated specific questions. We found that physicians defined patients as in REM much more often than composite scores or patients themselves. This indicates physicians' expectations for REM may be low, as has been previously suggested.[19–21 23 47]

Cross-tabulation of patient-perceived and physician-perceived REM/LDA is a novel contribution of our work. Agreement between patient-perceived and physician-perceived REM was not high, and as stated physicians were more lenient to define REM. However, the tendency was reversed for LDA: the frequency of patient-perceived LDA was 43.9% vs 31.5% for physician-perceived LDA. Perhaps the concept of LDA needs to

be further defined with both patients and physicians. Considerably higher agreement and concordance of patient-perceived REM/LDA with composite REM/LDA definitions versus physician perceived REM/LDA confirms that physicians should not base medical decisions or their global assessment/gestalt (as this may underestimate disease activity) but use validated scores instead.[48]

In the present study, we confirmed that the frequency of REM and LDA was very variable according to the definition used, and in particular REM and LDA were more difficult to reach using VLDA/MDA than DAPSA-based cut-offs, as has been previously reported.[14–16] This may be because of the inclusion of diverse domains of PsA (and in particular skin involvement), or because of low cut-offs for each measure. The psychometric properties of VLDA/MDA with Boolean features also make them more strict.[38 49] Concerning agreements between these scores, kappas were also similar to the literature, with moderate agreement for REM but fair for LDA whatever the definition used.[14 15]

An original feature of our study was to cross-tabulate these composite measures with the patient's perspective as an external anchor. To provide data on using one measure over another is of great importance since there is no consensus on what measure should be used in PsA. Kappa agreements were moderate to good for both of the scores and did not allow us to conclude. However, the comparison of these scores against patient-defined status, performed here for the first time, was very informative. We found that more patients in patient-perceived good status were also in DAPSA-based good status, both for REM, LDA and the combination. Of note, we advocate that REM should be the treatment goal, in accordance with recommendations; however, the exploration of REM/LDA was also valuable.[1–4] In our study, patient-perceived REM/LDA occurred slightly more frequently as DAPSA-based definitions, with VLDA/MDA being rarer. DAPSA-based REM or REM/LDA had much higher sensitivity than VLDA/MDA against the reference of the patient-defined status, with only a slight loss of specificity. This means that DAPSA-based definitions correctly 'detected' much more patients in patient-defined REM or REM/LDA than VLDA/MDA. However, there were slightly more patients in DAPSA-based good status who did not report themselves in good status than among patients in VLDA/MDA (as illustrated for REM/LDA in figure 2). Thus each of these scores has different strengths depending on if the objective is sensitivity (ie, to detect patient-defined good status: here DAPSA performed better) or specificity (ie, to avoid overdetecting patients who did not self-report as doing well: here, VLDA/MDA performed better). However overall DAPSA-based cut-offs seemed to align better with the patient's perspective. These results suggest that DAPSA-based status is closer to patients' expectations than VLDA/MDA.

In conclusion, this international study of PsA disease activity highlights several important concepts regarding REM and LDA, including the aspect of truthfulness of the measures evaluated. Further studies of patients' expectations and studies demonstrating the prognostic value of different disease states/definitions for long-term outcomes are needed to inform treatment targets.

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Contributors
All authors except MdW were responsible for acquisition of data. CG, DP-Z, A-MO, LCC, JSS, MdW and LG contributed to study conception and design and data analysis. All authors contributed to data interpretation. CG and LG take responsibility for the integrity of the data and the accuracy of the data analysis. All authors were involved in drafting the article or revising it critically for important intellectual content, and approved the final version to be submitted for publication.

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CLINICAL SCIENCE

Immunological and clinical effects of low-dose interleukin-2 across 11 autoimmune diseases in a single, open clinical trial

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ABSTRACT

Objective Regulatory T cells (Tregs) prevent autoimmunity and control inflammation. Consequently, any autoimmune or inflammatory disease reveals a Treg insufficiency. As low-dose interleukin-2 (ld-IL2) expands and activates Tregs, it has a broad therapeutic potential.

Aim We aimed to assess this potential and select diseases for further clinical development by cross-investigating the effects of ld-IL2 in a single clinical trial treating patients with 1 of 11 autoimmune diseases.

Methods We performed a prospective, open-label, phase I–IIa study in 46 patients with a mild to moderate form of either rheumatoid arthritis, ankylosing spondylitis, systemic lupus erythematosus, psoriasis, Behcet's disease, granulomatosis with polyangiitis, Takayasu's disease, Crohn's disease, ulcerative colitis, autoimmune hepatitis and sclerosing cholangitis. They all received ld-IL2 (1 million IU/day) for 5 days, followed by fortnightly injections for 6 months. Patients were evaluated by deep immunomonitoring and clinical evaluation.

Results ld-IL2 was well tolerated whatever the disease and the concomitant treatments. Thorough supervised and unsupervised immunomonitoring demonstrated specific Treg expansion and activation in all patients, without effector T cell activation. Indication of potential clinical efficacy was observed.

Conclusion The dose of IL-2 and treatment scheme used selectively activate and expand Tregs and are safe across different diseases and concomitant treatments. This and preliminary indications of clinical efficacy should licence the launch of phase II efficacy trial of ld-IL2 in various autoimmune and inflammatory diseases.

Trial registration number NCT01988506.

Key messages

What is already known about this subject?

- Whereas high dose interleukin-2 (IL-2) activates effector T cells, low-dose interleukin-2 (ld-IL2) expands and activates regulatory T cells (Tregs), and likewise has a broad therapeutic potential for many autoimmune and inflammatory diseases.
- Proof-of-concept clinical trials have already reported the safety and indication of ld-IL2 efficacy in hepatitis C-related vasculitis, systemic lupus erythematosus and graft versus host disease.

What does this study add?

- This is the first prospective, phase IIa clinical trial that cross-analyses the safety, biological and clinical effects of ld-IL2 across 11 individual diseases chosen to represent the whole spectrum of autoimmune/inflammatory chronic diseases.
- We report that ld-IL2 selectively activates and expands Tregs without activating effector T cells whatever the disease.
- We report signals of efficacy without safety issues of ld-IL2 across diseases by using a unique global evaluation scale, validated in the assessment of psychiatric disease therapies but not yet used in autoimmune/inflammatory diseases.

INTRODUCTION

Regulatory T cells (Tregs) prevent autoimmunity and control inflammation.[1] Consequently, any autoimmune or inflammatory disease denotes a Treg insufficiency. Low-dose interleukin-2 (ld-IL2) expands and activates Tregs, and so has a broad therapeutic potential.[2] This potential is further supported by the central role of IL-2/IL-2 receptor in autoimmune diseases (AIDs), as recently highlighted in a genetic meta-analysis,[3] and by IL-2 pleiotropic functions.[2] Indeed, robust data demonstrate that IL-2 expands Tregs and blocks the differentiation of CD4 naïve T cells into follicular helper or proinflammatory helper 17 (Th17) T cells.[4 5] Therefore, ld-IL2 can act on three distinct arms of the immune response involved in AID pathophysiology: cellular and humoral immune responses and inflammation. Likewise, ld-IL2 is now being investigated in various clinical settings.[6–12] Results of

Key messages

How might this impact on clinical practice or future developments?

- Our study highlights a 'universal' biological efficacy and a potential clinical efficacy and safety of ld-IL2 across a wide range of patients suffering from autoimmune/inflammatory conditions.
- These results should licence the initiation of randomised controlled trials in numerous indications in order to confirm these promising preliminary results.

open trials have already yielded promising signs of efficacy, such as in systemic lupus erythematosus (SLE).[13 14]

However, ld-IL2 efficacy may be affected by different factors: (1) Tregs from all patients may not respond similarly to ld-IL2, as some diseases may carry some intrinsic deficit of the IL-2 activation pathway[15–18]; (2) Treg efficacy might be limited by high levels of inflammation such as during flares; (3) the existence of Tregs with appropriate T cell receptor antigen specificity that could be mobilised for therapy for each disease context is unknown; and (4) the global effect of ld-IL2 may be affected by the fact that Treg-dependent suppression of immune responses and inflammation depends on numerous cells, molecules and pathways that are likely to be affected differently in various AIDs. Finally, although ld-IL2 activates Tregs at doses at least 20-fold lower than for activating other cell types,[12 19 20] IL2 can affect effector T cells (Teffs), natural killer cells, type 2 innate lymphoid cells and eosinophils in a (high) dose-dependent manner.[21–23] Thus, it remains to be seen whether a common appropriate dose/scheme of administration of ld-IL2 can be applied to various AIDs.

To address these questions, we designed a clinical trial in which we treated similarly ld-IL2 patients with 1 of 11 selected AIDs chosen to represent diverse pathophysiological processes. All patients received the same treatment and were monitored similarly. The aim was to cross-analyse the biological and clinical effects of ld-IL2 in heterogeneous patients, such as to appreciate the universality of ld-IL2 effects, and select diseases for conducting further phase II trials. We report here the results of the cross-analysis of 46 treated patients.

METHODS

Study design and participants

TRANSREG is a multicentre, interventional open study comparing biochemical and clinical responses to the administration of ld-IL2 across 11 selected diseases (ClinicalTrials.gov trial registration number, NCT01988506). The selected diseases were rheumatoid arthritis (RA), ankylosing spondylitis (AS), SLE, psoriasis, Behçet's disease, granulomatosis with polyangiitis, Takayasu's disease, Crohn's disease (CD), ulcerative colitis (UC), autoimmune hepatitis and sclerosing cholangitis. Patients were selected based on common and disease-specific exclusion and inclusion criteria (online supplementary table S1A and S1B). The main inclusion criteria were a documented diagnosis of at least one of the selected diseases of mild to moderate activity, and being on stable standard therapy for ≥2 months at the time of inclusion. The main exclusion criteria were having another severe or progressive autoimmune/inflammatory disease, haematological disorders, vital organ failure, cancer, and active HIV, Hepatitis B Virus (HBV) or Epstein-Barr Virus (EBV) infections (online supplementary table S1A).

For homogeneity and proper cross-analyses, we report here the results of the first 46 patients who have been treated with IL-2 as Aldesleukin (Proleukin 18 MIU, Novartis Pharma SAS, Rueil-Malmaison, France), before we switched to a different formulation of IL-2 (ILT-101, ILTOO Pharma, Paris, France). Indeed, the use of Proleukin necessitates a cumbersome preparation by a pharmacist to dilute the product and prepare syringes that have limited time span.

Treatment

We previously showed the dose relation between IL-2 and Treg activation/expansion.[8 12] We selected the dose and scheme of administration of IL-2 used in TRANSREG from these results and a mathematical modelling of the long-term effects of IL-2 administration.[24] Likewise, patients received 1 Million International Units (MIU)/day of IL-2 from day 1 to day 5 (the induction period), and then every 2 weeks from day 15 to day 180 (the maintenance period). A follow-up visit was made 2 months after the end of the IL-2 treatment (day 240). Patients continued to receive their standard background therapy.

Immunomonitoring

All the immunomonitoring procedures are described in the online supplementary methods.

Endpoints

The primary endpoint was the change in the relative concentration of peripheral blood Tregs on day 8 compared with baseline.

Biological secondary endpoints were the area under the curve (AUC) of the changes from baseline in relative concentration of Tregs during the maintenance period from day 30 to day 183 and the changes in inflammation markers from baseline to the end of treatment.

Clinical secondary endpoints were Clinical Global Impression (CGI),[25] disease-specific and Five-level EuroQol Five-dimensional (EuroQL-5D-5L) scores. Chronic fatigue and arthralgia were also evaluated by physicians at baseline, month 3 and month 6 as they are the most common symptoms shared by patients across all pathologies included in this trial. All clinical evaluation procedures are described in the online supplementary methods.

Statistical analysis

Changes in Tregs, immunological parameters and inflammation biomarkers between day 1 and day 8 were analysed using analysis of variance for ranked data (Conover's method) considering factor time and disease. The global effect of treatment at the initiation of treatment and its persistence during the maintenance phase was evidenced by demonstrating that the AUCs (calculated by the trapezoidal method) of the changes from baseline between day 1 and day 15 ($iAUC_{D1–D15}$) and between day 30 and day 180 ($mAUC_{D30–D180}$) were significantly different from zero using Wilcoxon test.

Changes in specific clinical scores and EuroQL-5D-5L were analysed by means of the Wilcoxon test. Changes in CGI severity and activity were analysed by t-test. Fatigue and arthralgia at months 3 and 6 were compared with baseline using Fisher's test.

RESULTS

Fifty-one patients were included between January 2014 and May 2016. Five patients were not eligible, three because of a viral load of EBV greater than that permitted and two for intercurrent diseases. Thus, 46 patients were treated (figure 1). Patients

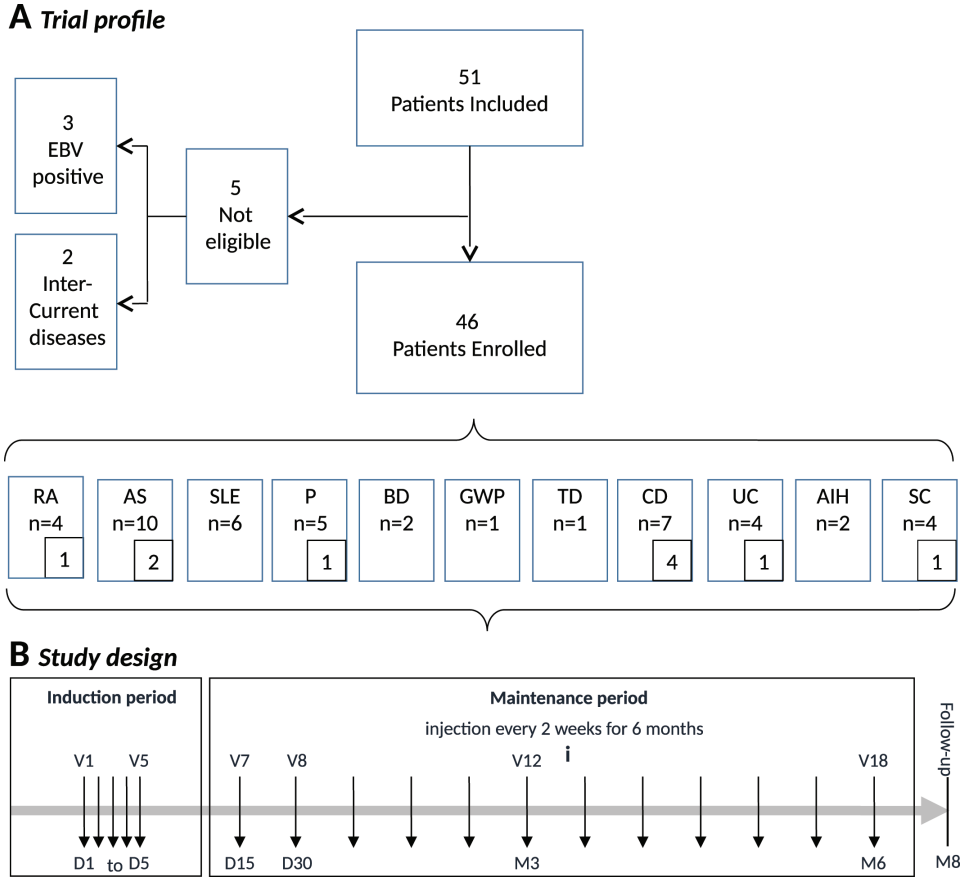

Figure 1 (A) Trial profile. We included 51 patients suffering from 11 autoimmune diseases: rheumatoid arthritis (RA), ankylosing spondylitis (AS), systemic lupus erythematosus (SLE), psoriasis (P), Behcet's disease (BD), granulomatosis with polyangiitis (GWP), Takayasu's disease (TD), Crohn's disease (CD), ulcerative colitis (UC), autoimmune hepatitis (AIH) and sclerosing cholangitis (SC). Five patients were not eligible: three patients for an EBV viral load >1000 copies/mL and two patients for intercurrent diseases; 10 patients dropped out of the study (n in small inserts). (B) Study design. Patients received 1 MIU/day of interleukin-2 from D1 to D5 (the induction period), and then every 2 weeks from D15 to D180 (the maintenance period). D1, day 1; D5, day 5; D15, day 15; D30, day 30; EBV, Epstein-Barr Virus; M3, month 3; M6, month 6; M8, month 8; MIU, Million Intenational Units; V, visit.

were heterogeneous in terms of age (23–75 years), disease duration (10–536 months), body mass index (18.3–40.8), per cent of Tregs at baseline (2.2%–12.8%) and background therapy (online supplementary table S2). Several patients had other concomitant autoimmune or allergic diseases (online supplementary table S2). Concomitant Sjogren's syndrome (n=3), antiphospholipid antibody syndrome (n=3), morphea (n=1), Raynaud's phenomenon (n=1) or psoriasis (n=1) were observed in patients with SLE and RA. Concomitant allergic rhinitis (n=1), allergic asthma (n=1), multiple food allergy (n=1) and cutaneous contact hypersensitivity (n=1) were observed in patients with UC and psoriasis. In agreement with the inclusion criteria, the value of CGI activity and severity score ranged from 0 to 4 at baseline, with mild to moderate specific clinical scores for each disease. There were no other noticeable aspects of baseline demographic and laboratory characteristics of patients.

No major deviations were observed during the study. The most common minor protocol deviations were out of windows visits (n=11) or drug administration not performed because of intercurrent diseases (n=7) in the maintenance period. Ten patients dropped out of the study (online supplementary table S3).

The mean±SD baseline percentage of Tregs in patients was 5.8%±2.3% of $CD4^+$ T cells (online supplementary table S2). On day 8, the primary efficacy endpoint was reached with an increase of Tregs to a mean of 11.1%±4.6%, corresponding to a 2.0±0.6-fold increase ($p<0.0001$) (figure 2A and online supplementary table S4A). Treg expansion on day 8 appeared similar across the various diseases for which a minimum of four patients were treated (figure 2B), and cross-comparisons of these responses between diseases showed no significant differences (online supplementary table S4B). Moreover, we did not observe difference in Treg increase between patients receiving antiproliferative drugs and patients receiving non-steroid anti-inflammatory treatments or corticosteroids (online supplementary figure S1). On day 15, that is, 10 days after the last ld-IL2 administration of the induction phase, Treg increase was still significant ($p=0.02$) (figure 2A and online supplementary table S4A). Treg expansion persisted during the maintenance period. From months 1–6, the mean AUC value of Treg changes from baseline for all patients was significantly different from 0 ($AUC_{M1-M6}=35.1\pm13.1$, $p<0.001$). It is noteworthy that the recorded results may underestimate the true effect of ld-IL2 because Treg measurements were performed just before the IL-2 injections and thus capture only the residual increase from the previous injection 14 days earlier. The significant changes in percentages of Tregs were also observed for absolute numbers of Tregs (online supplementary table S4A).

There were no detectable effects of ld-IL2 on Teffs (defined as all $Foxp3^-CD4^+$ and $CD8^+$ cells), or on activated $CD4^+CD25^{lo/+}Foxp3^-$ Teffs (online supplementary figure S2). This led

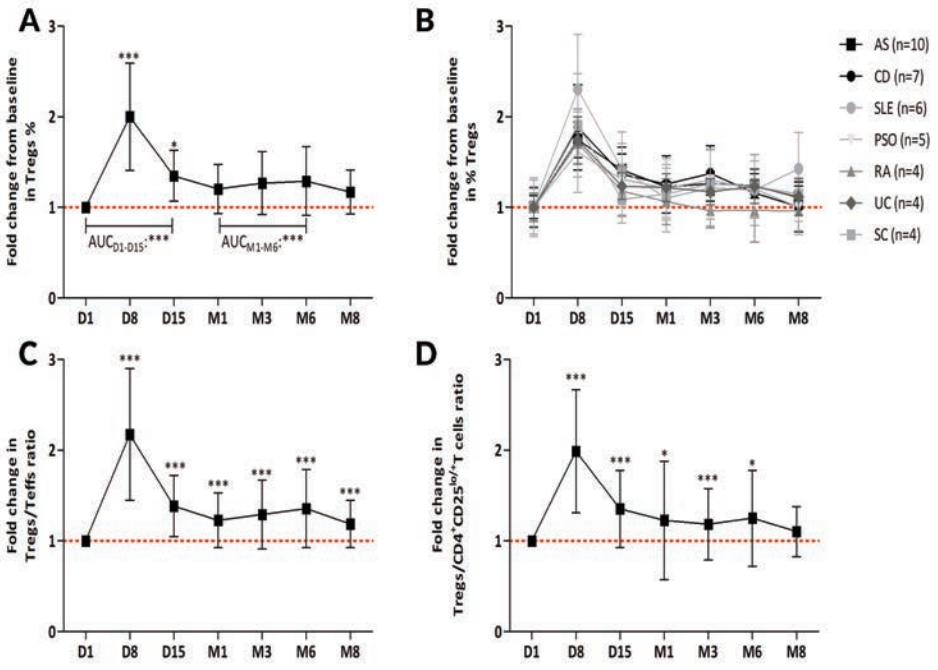

Figure 2 Changes in Treg cells and Teffs cells in patients treated with ld-IL2. Treg cells were gated in $CD4^+$ T cells and identified as $CD25^{hi}CD127^{lo/-}Foxp3^+$cells. (A) Data represent changes in Tregs as percentages among $CD4^+$ T cells for all patients from day 1 to month 8. (B) Data represent changes in Tregs in patients for diseases with n≥4. (C) Changes in Treg:Teff ratio defined as the percentage of Tregs divided by the percentage of the non-Treg $CD4^+$ and $CD8^+$ T cells. (D) Changes in Treg:activated $CD4^+$ T cells ratio as the percentage of Tregs divided by the percentage of $CD4^+CD25^{lo/+}Foxp3^-$ T cells. (A–D) Data are represented as mean±SD. Data were normalised by baseline values for each patient at the different time points and are represented as fold change, but all statistics were made on raw data. Comparison between day 8 and baseline (main endpoint) was made by Wilcoxon signed-rank test. For each type of cell, global effect of the treatment at its initiation and its persistence during the maintenance phase was evidenced by showing that the AUC of the changes from baseline between day 1 and day 15 ($iAUC_{D1–D15}$) and between day 30 and day 180 ($mAUC_{D30–D180}$) was significantly different from 0 using Wilcoxon test. *P<0.05, **p<0.01, ***p<0.001. AS, ankylosing spondylitis; AUC, area under the curve; CD, Crohn's disease; D1, day 1; D8, day 8; D15, day 15; ld-IL2, low-dose interleukin-2; M1, month 1; M3, month 3; M6, month 6; M8, month 8; PSO, psoriasis; RA, rheumatoid arthritis; SC, sclerosing cholangitis; SLE, systemic lupus erythematosus; Teffs, effector T cells; Tregs, regulatory T cells; UC, ulcerative colitis.

to a 2.17±0.72-fold increase of the Treg:Teff ratio (p<0.0001) (figure 2C), as well as to a 1.98±0.42-fold increase of Tregs/activated non-Treg $CD4^+$ cells (p<0.0001) at the end of the induction course (figure 2D). It is worth noting that every single patient responded to 1 MIU/day of IL-2 by expanding their percentage of Tregs in the peripheral blood by at least 25%.

Because unwanted stimulation of Teff could be deleterious in the treatment of AIDs, the demonstration of a specific effect of ld-IL2 on Tregs is of utmost importance for the treatment of AIDs caused by Teffs. Although classic immunophenotyping did not show any expansion of non-Treg T cells, we further assessed that the effects of IL-2 are Treg-specific in a group of nine patients in whom an extensive immunophenotyping was performed[26] and using unsupervised analyses. Using t-distributed stochastic neighbor embedding (t-SNE) and flowMeans R packages,[27–29] clusters were defined automatically based on Foxp3 and CD127 expression (figure 3A). Cluster 6 corresponded to $Foxp3^+CD127^{-/low}$ cells and thus defines the Treg cluster, while the Teff cells corresponded to all the other clusters. We repeated the procedure to automatically recluster separately Tregs and Teffs based on the other markers of staining (CD25, Helios, CD45RA and CCR5). This generated six and eight clusters for Tregs and Teffs, respectively (figure 3B,D). For each cluster, comparison of the different values per marker on day 8 versus baseline showed statistically different values only for Tregs (p=0.03), and among them those with the more activated phenotypes (figure 3C). There were no differences for any of the Teff subsets (figure 3E), with p value not even close to significance. The same analysis strategy was then applied to another panel of antibodies (online supplementary figure S3). Here again, only two Treg subsets corresponding to those with the phenotype of either resting/naïve $CD45RA^+CD95^+CCR7^+ICOS^{+/-}HLA\text{-}DR^-$) or activated/memory Tregs ($CD45RA^-CD95^+CCR7^{+/-}ICOS^{+/-}HLA\text{-}DR^+$) were significantly expanded on day 8 while other T cell subsets were not affected.

Analysis of T, B and natural killer (NK) cell subsets showed little change during the induction period (online supplementary table S4A). As previously described,[12] we observed an increased frequency of the regulatory $CD56^{bright}$ NK cell subset (online supplementary figure S4A-C). Eosinophils levels were heterogeneous at baseline. For the 43 patients with normal counts, we observed a slight increase on day 8 that stayed under the normal value limit, or slightly above in two patients. For the three patients with eosinophilia at baseline, eosinophil counts approximately doubled after the induction period for two of them and remained stable for the other. All values returned to baseline during the maintenance treatment (online supplementary figure S5).

Plasma levels of Th1/Th2/Th17/Treg cytokines were unchanged all along treatment (online supplementary figure S6). In agreement with the mild to moderate activity of the diseases, C-reactive protein (CRP) was detected (>5 mg/dL) in only 10

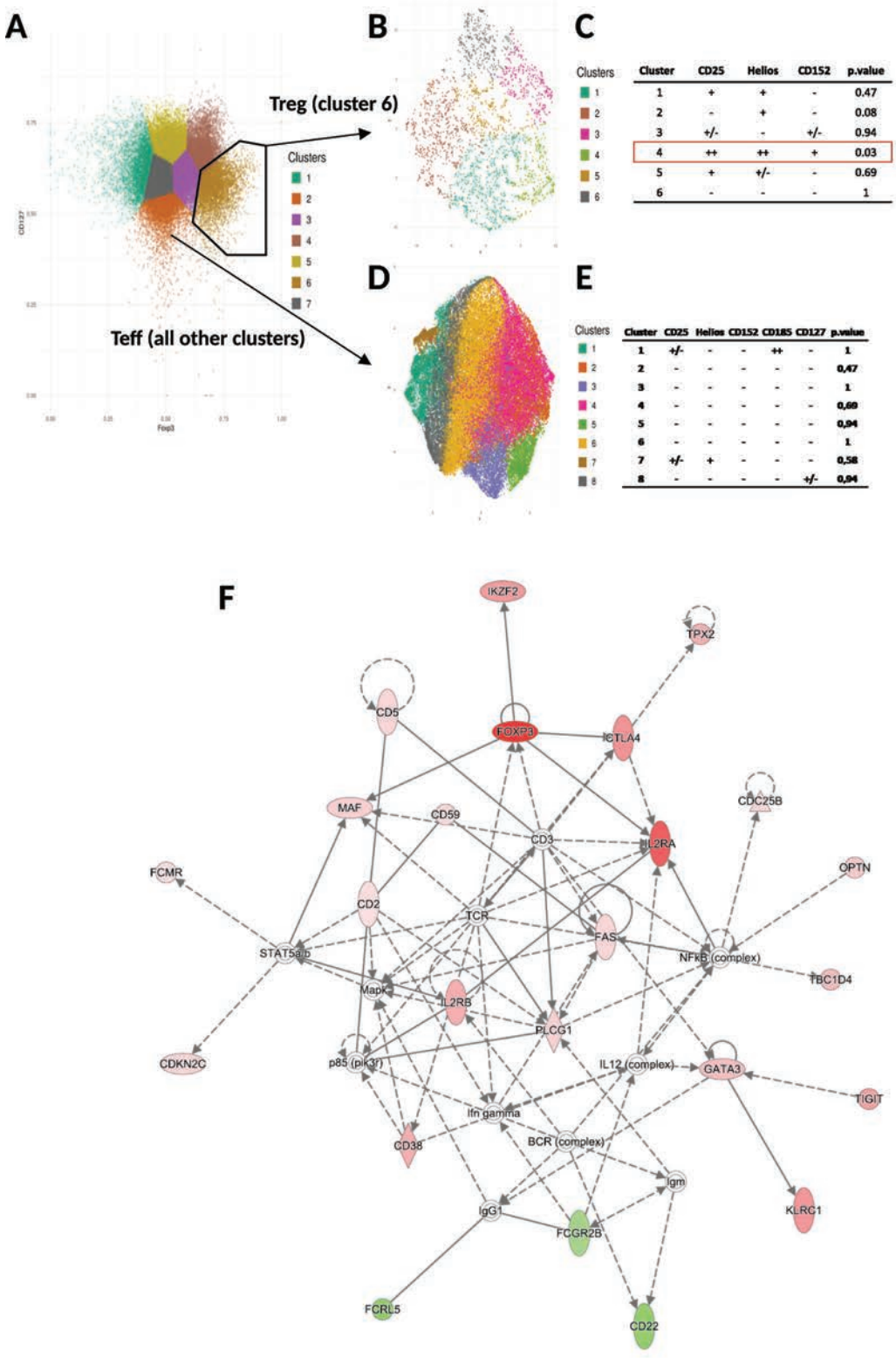

Figure 3 Unsupervised analysis of Treg phenotype and transcriptome. (A–E) Cells were stained for Helios, CD25, CXCR5, Ki67, CTLA-4, FOXP3, CD8, CD127, CD4 and CD8. A first step of automatic clustering based on the expression of FOXP3 and CD127 in $CD3^{+}CD4^{+}$cells was performed on a merge of samples from baseline and day 8 for the nine patients studied. (A) The algorithm generated seven clusters that are represented in different colours on a FoxP3/CD127 biplot. (B and D) A second step of automatic clustering was performed separately on Tregs (cells from cluster 6 in A) and Teffs (cells from all other clusters in A) based on $FoxP3^{-}CD3^{+}CD4^{+}$ cells. (C and E) P values of the statistical analysis of the difference between baseline and day 8 for each cluster identified in B and D, respectively (statistical test: Wilcoxon pairwise test, p values <0.05 were considered as significant). (C) Also shows the phenotype of each cluster of Treg cells. (F) Transcriptomic analysis of significantly regulated genes from PBMCs on day 8 compared with baseline using Ingenuity Pathway Analysis reveals a Treg-related pathway (upregulated genes in red and downregulated genes in green; direct and indirect interactions between molecules are depicted by solid and dotted lines, respectively). PBMCs, peripheral blood mononuclear cells; Teffs, effector T cells; Tregs, regulatory T cells.

patients at baseline and thus could not be studied as an endpoint (online supplementary figure S7).

We reported that ld-IL2 has a global anti-inflammatory effect based on a transcriptome analysis of peripheral blood mononuclear cells (PBMCs).[6] Here, we similarly analysed the global transcriptome of PBMCs before and after ld-IL2. We found 91 differentially expressed genes with a Benjamini-Hochberg corrected p value <0.05 on day 8 versus baseline (online supplementary table S5). Ingenuity Pathway Analysis showed significant enrichment of four pathways/signatures all directly or indirectly related to Treg. The most significantly modulated pathway (-log(p value)>3) is organised around upregulated genes such as Foxp3, IL-2Ra and Rb and CTLA4 that are essential for Treg function (figure 3F). We also assessed the modulation of a recent Treg signature defined from single cell transcriptomic.[30] This signature was significantly upregulated on day 8 versus baseline (p<1e-04) as well as at month 3 versus baseline (p<0.03), indicating that ld-IL2 effects on Tregs were maintained over time across diseases (online supplementary figure S8).

As previously reported, we did not observe anti-IL-2 antibodies in patients' plasma after ld-IL2 treatment (online supplementary figure S9).[12 31]

Low-dose recombinant human (rh)IL-2 was well tolerated. Six patients displayed seven serious adverse events, none of which was considered related to IL-2 (online supplementary table S6A). Most non-serious adverse events (NSAEs) were injection site reactions, which occurred in approximately a quarter of the injections. The frequency of seasonal upper or lower respiratory tract infections (n=28), with associated fever of over 38°C in 17 of them, was as expected. The investigators did not report any unforeseen outcome of these infections (online supplementary table S6A). Finally, the analysis of the NSAEs according to background therapy did not show any significant difference (online supplementary table S6B).

Clinical secondary endpoints were CGI, disease-specific and EuroQL-5D-5L scores. CGI was selected as a clinical evaluation method that could work across our heterogeneous group of diseases. Indeed, CGI was originally developed for use in clinical trials to provide a brief, stand-alone assessment of the clinician's view of the patient's global functioning prior to and after initiating a study medication.[25] CGI is commonly used in psychiatry but has not yet been validated in AIDs. Compared with baseline, a statistically significant improvement of the CGI scored by the physician was found at months 3 and 6 for CGI activity (p<0.001) (figure 4A) and at month 6 for CGI severity (p<0.001) (figure 4B). Significant changes were also found at the follow-up visit 2 months after discontinuation of the treatment (CGI activity p=0.02 and CGI severity p=0.04). Among the 46 treated patients, there were 26 with documented arthralgia and 26 with chronic fatigue at baseline. At month 3, there was a significant decrease of the percentage of patients with fatigue (p=0.002) and with arthralgia (p=0.00015) (figure 4C), and this trend continued at month 6. Evaluation of the impact of ld-IL2 on quality of life using EuroQL-5D-5L showed a non-significant improvement. We also assessed the disease-specific score for diseases with at least four patients treated. There was an improvement for AS (figure 5A), UC (figure 5B), SLE (figure 5C and online supplementary figure S9) and psoriasis (figure 5D,E), but not for CD (online supplementary table S7).

DISCUSSION

We previously reported a double-blind, placebo-controlled dose-finding study of ld-IL2 in type 1 diabetes.[8] This identified a dose

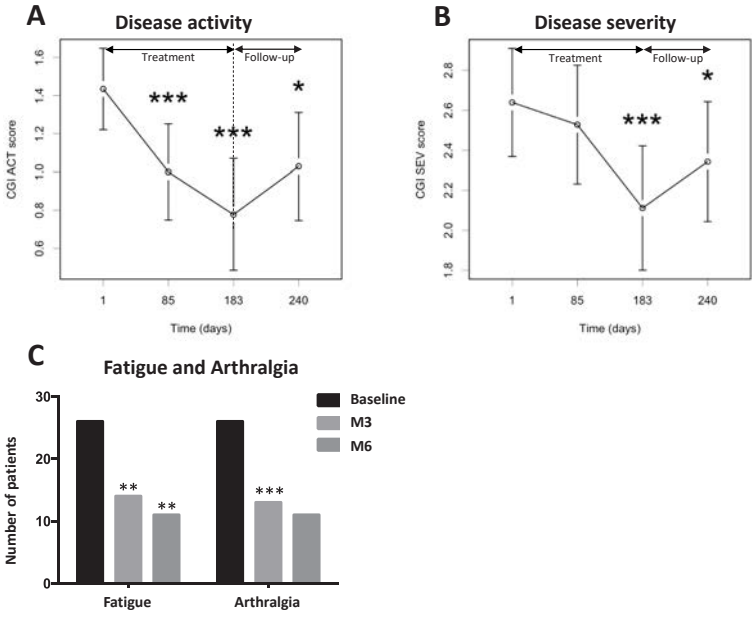

Figure 4 Clinical effects of ld-IL2 across the 11 autoimmune diseases. Clinical Global Impression (CGI) for (A) activity and (B) severity was scored by the physician at baseline (day 1), month 3 (day 85), month 6 (day 183) and at a follow-up visit at month 8 (day 240). Data are represented as mean±SD. Data were compared with baseline using t-test. (C) Arthralgia pain intensity and fatigue level were assessed by the physician at baseline, month 3 and month 6. Data are represented as the number of patients presenting arthralgia or fatigue. Data were compared with baseline using Fisher's test. *P<0.05, **p<0.01, ***p<0.001. ACT, activity; ld-IL2, low-dose interleukin-2; SEV, severity; M3, month 3; M6, month 6.

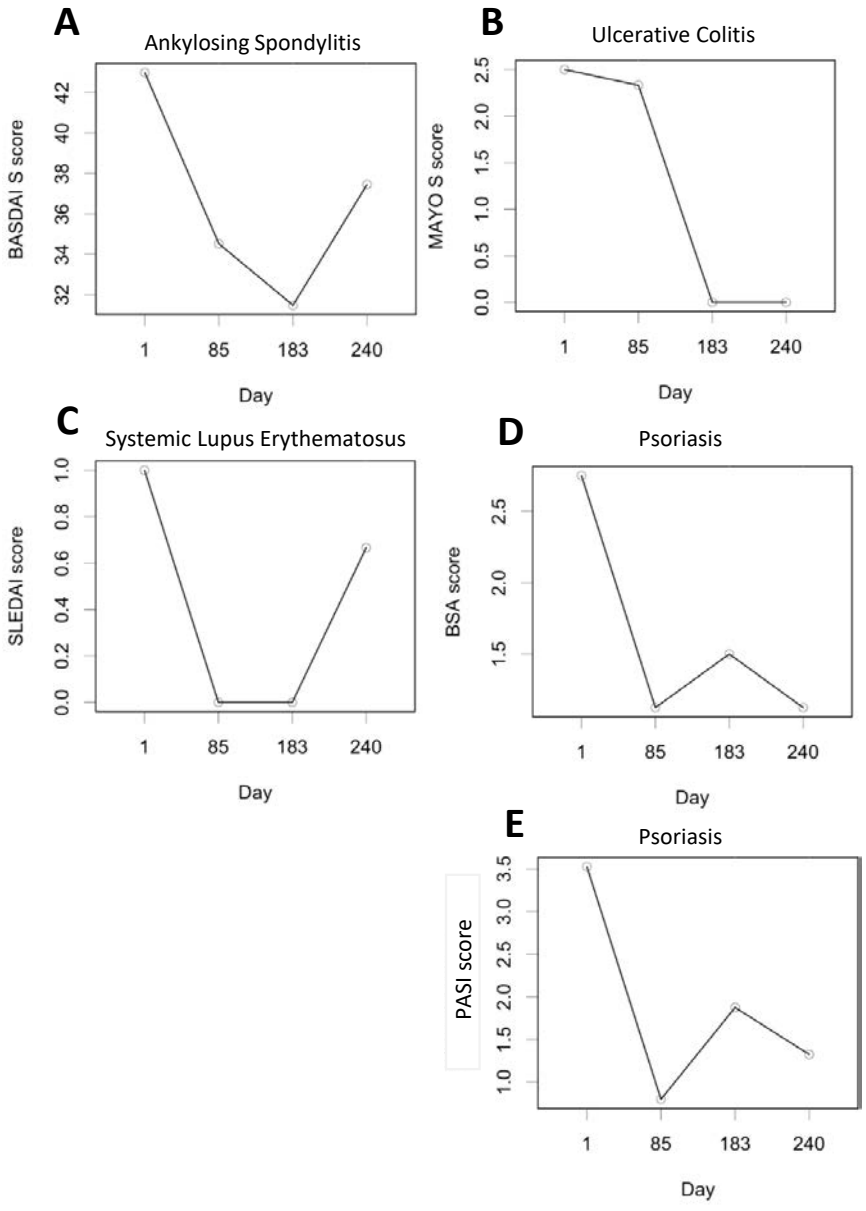

Figure 5 Clinical effects of low-dose interleukin-2 in specific diseases. Specific clinical scores were measured at baseline (day 1), month 3 (day 85), month 6 (day 183) and follow-up visit at month 8 (day 240): (A) BASDAI for patients with ankylosing spondylitis (n=10); (B) Mayo for patients with ulcerative colitis (n=4); (C) SLEDAI for patients with systemic lupus erythematosus (n=6); (D) BSA and (E) PASI for patients with psoriasis (n=4). Data are represented as means. BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BSA, body surface area; PASI,Psoriasis Area Severity Index; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

of 1 MIU/injection as well tolerated and boosting Tregs without effects on Teffs.[8 12] Given the heterogeneity of ld-IL2 target diseases and of their pathophysiological background, it remained to investigate how 'universal' would be the effects of this dosage. We thus initiated a trial aimed at cross-evaluating ld-IL2 in 11 different AIDs chosen to cover diseases that are organ-specific and systemic, T cell-mediated or antibody-mediated, and with high or little inflammation.

Our supervised analyses clearly demonstrated that 1 MIU/injection, with the scheme used, selectively activates and expands Tregs without activating Teffs, whatever the disease. This translates in a significant increase of both the Treg/Teff as well as the Treg/activated CD4+ T cell ratios. A similar response was observed for patients with low or high Treg counts at baseline. Both naïve and activated/memory Tregs expanded after ld-IL2, while we did not detect expansion of CD4 effector memory cells. As previously reported, at the dose used, we did not observe an effect on the overall NK cell population, but only on the non-cytotoxic $CD56^{hi}$ NK cell subset, also called regulatory NK cells. Given the importance of the specificity of the effect on Tregs, we also evaluated it using unsupervised analyses that can be considered as less biased. This fully confirmed the specificity of the ld-IL2 effects for Tregs, further showing that the only cells responding to ld-IL2 were the resting/naive and activated/memory Tregs. Thus, supervised and unsupervised cellular and molecular analyses indicate that, with the dose/scheme used, ld-IL2 triggers a 'universal' specific effect for Tregs across a group of very heterogeneous patients.

Although this trial was powered only to evaluate the effects of IL-2 on Tregs, we also monitored secondary efficacy criteria relating to clinical status. With the idea of a cross-analysis of

diseases with various symptoms and scores, we chose CGI as our main per protocol clinical endpoint for cross-evaluation. Despite the fact that patients had mild or moderate disease forms, thus low CGI scores at baseline, and were heterogeneous, we observed an overall significant improvement of CGI scores. Improvements in CGI scores were already noted at month 3 and continued to increase at month 6. A potential clinical benefit was also evaluated across diseases by specifically monitoring arthralgia and chronic fatigue, which were the most shared symptoms of our patients. There was a significant improvement of these symptoms, already noted at month 3. Two months after treatment discontinuation, CGI scores had a tendency to increase but were still significantly improved compared with baseline. No flare was observed during this period. Finally, we evaluated the disease-specific scores for diseases with such available scores and with at least four patients included. As previously reported, we saw improvement in SLE. We also saw improvements for patients with UC, AS and psoriasis but not in those with CD. Altogether, these evaluations converge to suggest a broad potential of ld-IL2 and contributed to the selection of SLE as the target disease of an ongoing phase II trial (NCT02955615). They also suggest to further evaluate CGI in the field of AIDs. Indeed, as many drugs being developed target pathways involved in multiple AIDs, they thus have a broad therapeutic potential. Our trial design and methods could represent an early clinical cross-evaluation step that would help select diseases for further evaluation.

The safety profile of ld-IL2 across the different diseases and across various background treatments was very good. There has been no serious adverse event related to treatment. The most frequent adverse events were reaction at the injection sites, which are common for biologics, and are of unknown mechanisms. As in other trials of ld-IL2, we did not observe induction of anti-IL-2 antibodies under treatment.[12 31] Based on safety data from our previous clinical trials and our modelling of the effects of IL-2 on Tregs,[24] we used in this trial the dose of 1 MIU/injection, with once-a-fortnight injections during the maintenance course. Should a more pronounced effect on Treg during the maintenance phase be desired, one could use weekly injections as we do in our current LUPIL-2 trial (NCT02955615).

Altogether, our study highlights the 'universal' safety, biological efficacy and possible clinical efficacy of ld-IL2 across a group of very heterogeneous patients. It also highlights that the therapeutic window of plain IL-2 is satisfactory and thus licences the initiation of phase II efficacy trials, which are now necessary to ascertain the therapeutic potential of ld-IL2.

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Contributors MR participated in the design of the study, supervised immunomonitoring, analysed the results and helped write the article. RL participated in the design of the study, was the principal clinical investigator, analysed the results and helped write the article. PC participated in the design of the study and was a clinical investigator of the study. FP, KES and HPP analysed the immunological and transcriptomic results. SA, BB, LB, FB, BF, JC, OC, CC, CF, AM, ER, DS, PS, JS and J-ES were clinical investigators of the study. CR was an assistant clinical investigator and supervised the logistics. CB and AD-N were in charge of treatment management. VD and JM contributed to the study performance and regulatory affair follow-up. EV participated in the design of the study, wrote the statistical report, analysed the results and helped write the article. DK conceived and supervised the study, was the principal investigator, analysed the results and wrote the first draft of the article. All authors edited and approved the final draft of the article.

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Competing interests MR, RL, PC, FB, BF, PC, JS, DS, CB and DK are inventors for patent applications related to the therapeutic use of ld-IL2, which belongs to their academic institutions and has been licensed to ILTOO Pharma. MR, VD, JM and DK hold shares in ILTOO Pharma. HPP, VD and JM are employees of ILTOO Pharma. No other potential conflicts of interest relevant to this article were reported.

Patient consent Obtained.

Ethics approval The study was approved by the institutional review board of Pitié-Salpêtrière Hospital (CPP-IdFVI) and performed in accordance with the Declaration of Helsinki and good clinical practice.

Provenance and peer review Not commissioned; externally peer reviewed.

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CLINICAL SCIENCE

Visualisation of interstitial lung disease by molecular imaging of integrin αvβ3 and somatostatin receptor 2

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ABSTRACT

Objective To evaluate integrin αvβ3 (alpha-v-beta-3)-targeted and somatostatin receptor 2 (SSTR2)-targeted nuclear imaging for the visualisation of interstitial lung disease (ILD).

Methods The pulmonary expression of integrin αvβ3 and SSTR2 was analysed in patients with different forms of ILD as well as in bleomycin (BLM)-treated mice and respective controls using immunohistochemistry. Single photon emission CT/CT (SPECT/CT) was performed on days 3, 7 and 14 after BLM instillation using the integrin αvβ3-targeting ^{177}Lu-DOTA-RGD and the SSTR2-targeting ^{177}Lu-DOTA-NOC radiotracer. The specific pulmonary accumulation of the radiotracers over time was assessed by in vivo and ex vivo SPECT/CT scans and by biodistribution studies.

Results Expression of integrin αvβ3 and SSTR2 was substantially increased in human ILD regardless of the subtype. Similarly, in lungs of BLM-challenged mice, but not of controls, both imaging targets were stage-specifically overexpressed. While integrin αvβ3 was most abundantly upregulated on day 7, the inflammatory stage of BLM-induced lung fibrosis, SSTR2 expression peaked on day 14, the established fibrotic stage. In agreement with the findings on tissue level, targeted nuclear imaging using SPECT/CT specifically detected both imaging targets ex vivo and in vivo, and thus visualised different stages of experimental ILD.

Conclusion Our preclinical proof-of-concept study suggests that specific visualisation of molecular processes in ILD by targeted nuclear imaging is feasible. If transferred into clinics, where imaging is considered an integral part of patients' management, the additional information derived from specific imaging tools could represent a first step towards precision medicine in ILD.

Key messages

What is already known about this subject?
- In interstitial lung disease, there is a lack of validated biomarkers for disease staging, prediction of disease progression and drug responses, which impairs tailored patient management.

What does this study add?
- Our preclinical data suggest that targeted nuclear imaging of pathophysiological key players allows the visualisation of specific molecular processes of interstitial lung disease.

How might this impact on clinical practice or future developments?
- If transferred into clinics, where medical imaging is already part of the routine clinical work-up, the added information derived from specific diagnostic tools could represent the first step towards precision medicine in interstitial lung disease.

INTRODUCTION

Interstitial lung disease (ILD) is an umbrella term for a group of heterogeneous chronic parenchymal lung disorders with different aetiologies. The most prevalent subtypes include idiopathic pulmonary fibrosis (IPF) and ILD in the context of connective tissue diseases (CTD), particularly in association with systemic sclerosis (SSc).[1–3] Pulmonary fibrosis is the common end stage of ILD.[4] Histologically, the most common pattern of SSc-ILD is non-specific interstitial pneumonia (NSIP), whereas in IPF it is usual interstitial pneumonia (UIP).[2 4] In contrast to IPF, in SSc-ILD, this histological classification is not considered a reliable tool for outcome prediction.[2 5] However, the differences in cellularity, cell types and degree of lung remodelling in NSIP versus UIP point to differences in the underlying pathophysiology.[2 4]

Currently, in ILD, no validated biomarkers for disease staging, prediction of disease progression and drug responses exist.[6–8] Given the highly heterogeneous nature of ILD, this largely leaves patient management at a trial-and-error stage, which stands in sharp contrast to the concept of precision medicine.[8 9]

To address this unmet clinical need, we evaluated whether nuclear imaging as a specific ('targeted') and functional imaging modality could provide molecular information on the underlying pathophysiology[10] that could be used for substratification and tailored decision-making in ILD. Nuclear imaging methodologies include single photon emission CT (SPECT) and positron emission tomography (PET), which use radiolabelled, target-specific, molecular probes, that is, radiotracers for the real-time visualisation of pathophysiological processes.[10] For a proof-of-concept study showing that targeted nuclear imaging is feasible and has the potential for clinical application in ILD, we have selected two different molecules involved in the pathophysiology

of ILD, that is, integrin alpha-v-beta-3 (αvβ3) and somatostatin receptor 2 (SSTR2), for which validated radiotracers are already available with good potential for short-term transferability into clinical application.[11 12] Compared with the current gold-standard diagnostic imaging methodologies, including high-resolution CT (HRCT) or ^{18}F-fluorodesoxyglucose (FDG)-PET/CT, integrin αvβ3-targeted and SSTR2-targeted nuclear imaging may have important advantages for the evaluation of patients with ILD. Although HRCT and ^{18}F-FDG-PET/CT are sensitive tools for the diagnosis of ILD, they are unspecific and cannot be used for the molecular subtyping of patients. Since they solely rely on the detection of changes in tissue morphology or in metabolic activity, respectively, they do not allow the discrimination of different pathophysiological stages of ILD, that is, inflammation, active fibrotic remodelling or established fibrosis,[13 14] which is paramount for informed treatment decisions and monitoring of therapeutic responses.

Alpha v integrins are key molecules in the pathogenesis of fibrosis in multiple organs due to their ability to activate matrix-bound latent transforming growth factor beta (TGF-β), the prototypical profibrotic cytokine in tissue fibrosis.[15 16] Of particular importance in this regard is integrin αvβ3, which by activating TGF-β establishes an autocrine signalling loop in fibroblasts, thus driving myofibroblast differentiation.[16 17] Targeted imaging of integrin αvβ3 can be realised with arginine-glycine-aspartic acid (RGD) tripeptide-based radiotracers, which have already been validated (pre-)clinically.[11 18 19]

SSTR2 is a G-protein-coupled receptor which is expressed on various cellular key players of lung remodelling, for example, epithelial cells, inflammatory cells[20 21] and potentially fibroblasts.[22] SSTR2 can be targeted with a series of peptides, that is, somatostatin analogues, which are already part of the routine management of neuroendocrine tumours.[12 23] Radiolabelled somatostatin analogues have recently been proposed for the visualisation of fibrotic changes in experimental[24 25] and human ILD.[26–29]

Herein, we evaluated the potential of molecular imaging of integrin αvβ3 and SSTR2 for the targeted visualisation of pathophysiological stages of ILD using the well-defined model of bleomycin (BLM)-induced lung fibrosis.

METHODS

A detailed description of the materials and methods, including information on patient characteristics, is provided in the online supplementary information.

RESULTS

Expression of integrin αvβ3 and SSTR2 is increased in different types of ILD

To assess whether the expression of our molecular imaging targets is increased in human ILD, we performed immunohistochemistry for the β3 chain of integrin αvβ3 and SSTR2 on lung sections from patients with different types of ILD, including IPF and SSc-ILD, and other types of CTD-ILD (online supplementary table S1). Tissue specimens were obtained in the context of lung transplantation. The histopathological analysis revealed severely damaged lung architecture with massive accumulation of inflammatory infiltrates and extensive interstitial collagen deposition as assessed by H&E and CD45 or Picrosirius red and alpha-smooth muscle actin (αSMA) staining, respectively, which was consistent with end-stage ILD (figure 1A, online supplementary figure S1). In these highly inflamed and fibrotic lungs, expression of integrin αvβ3 and SSTR2 was significantly increased (~3-fold to 4-fold, $p<0.05$) compared with lungs from healthy subjects (figure 1A–C). Notably, this increase in expression was independent of the underlying aetiological subtype of ILD (figure 1A–C) and of other clinical characteristics (online supplementary figures S2 and S3). However, when comparing the expression of integrin αvβ3 and SSTR2 with respect to the histological subtypes of ILD, UIP and NSIP, we found a significantly higher expression of SSTR2 in the lungs with UIP pattern compared with those with NSIP pattern ($p<0.01$). In contrast, the expression of integrin αvβ3 did not differ between both histological subtypes (figure 1D,E).

Expression of integrin αvβ3 and SSTR2 reflects different disease stages of experimental ILD

Given these promising results, we next assessed whether integrin αvβ3 and SSTR2 could also serve as surrogate markers for different pathophysiological stages in ILD. Therefore, we examined the time course of the pulmonary expression of integrin αvβ3 and SSTR2 in a representative mouse model of human ILD, the model of BLM-induced lung fibrosis.

A single intratracheal instillation of BLM (4 U/kg of body weight) in mice induced progressive lung remodelling with inflammation leading to established pulmonary fibrosis already 14 days after the BLM administration as assessed by tissue analysis (figure 2A,B) and CT scanning (online supplementary figure S4). Already on day 3, lungs from BLM-treated mice versus saline controls showed the presence of perivascular and peribronchial cellular infiltrates (figure 2A, H&E staining), which mainly consisted of CD45+ leucocytes (figure 2A, CD45 staining). In contrast, only limited fibrous thickening of the alveolar and bronchial walls (figure 2A, Picrosirius red staining) with no increase in αSMA expression (figure 2A, αSMA staining) was observed. With disease progression, the number of inflammatory infiltrates increased in BLM-treated lungs, peaked on day 7, and subsided thereafter (figure 2A, H&E and CD45 staining). In contrast, pulmonary fibrosis, characterised by extensive interstitial collagen deposition and increase of αSMA expression in the lung interstitium, reached its maximum on day 14 (figure 2A, Picrosirius red and αSMA staining). Although the progressing damage of lung architecture could also be depicted on CT (online supplementary figure S4), these morphological changes could not relay information on the underlying pathophysiology, that is, inflammation or fibrosis.

Notably, compared with saline-treated controls, the lungs of BLM-challenged mice showed significantly increased expression of integrin αvβ3 and SSTR2 at all time points. Interestingly, the expression of integrin αvβ3 was most abundant on day 7 and thus in the inflammatory stage of BLM-induced lung fibrosis, where inflammation is more dominant than fibrosis, with a median increase of $3.7_{(Q1,\ Q3=2.9,\ 4.5)}$-fold ($p<0.001$) compared with control lungs (figure 2A–C). Although integrin expression decreased on day 14, it remained significantly upregulated in the lungs of BLM-treated mice ($median_{(Q1,\ Q3)}=2.4_{(2.0,\ 4.2)}$-fold) ($p<0.01$). In contrast, the expression of SSTR2 gradually increased with the degree of lung remodelling and peaked on day 14, and thus in the fibrotic stage of this animal model, with a median of $3.8_{(Q1,\ Q3=2.2,\ 4.3)}$-fold ($p<0.01$) increase compared with the lungs from saline-treated controls (figure 2A–D).

Given the rather conflicting reports of the pulmonary expression of integrin αvβ3 and SSTR2 in the literature,[20 24] we additionally analysed the cellular expression profiles of both targets in lung sections of BLM-treated mice and respective controls. Using immunofluorescent and/or immunohistochemical double

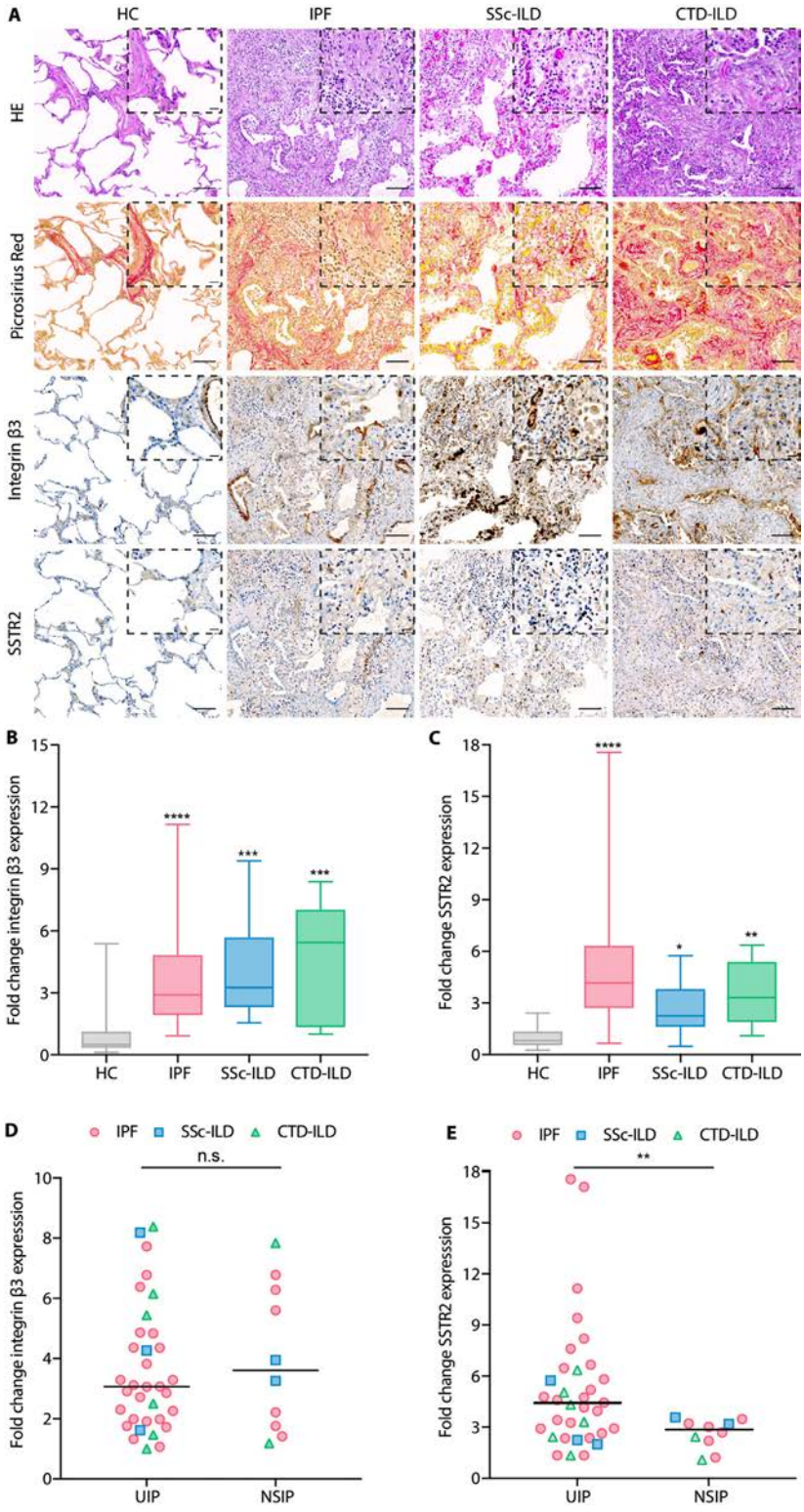

Figure 1 Expression of integrin alpha-v-beta-3 (αvβ3) and SSTR2 is increased in different types of human ILD. (A) Representative images of lung sections from healthy controls (n=26) and patients with IPF (n=39), SSc-ILD (n=11) and CTD-ILD (n=9) that were stained with H&E (first panel) and Picrosirius red (collagen=red, second panel), as well as stained for integrin αvβ3 (brown, third panel) and SSTR2 (brown, fourth panel). Representative images at 100× magnification (scale bars: 100 µm) and at higher magnification (400×, scale bars: 20 µm) are displayed. (B) Semiquantification of integrin αvβ3 expression by automatic image analysis. (C) Semiquantification of SSTR2 expression by automatic image analysis. (D) Analysis of integrin αvβ3 expression depending on the histological subtypes UIP and NSIP. (E) Analysis of SSTR2 expression depending on the histological subtypes UIP and NSIP. For B and C, data are displayed as box plots showing medians with min/max values. For D and E, individual data points for each patient and type of ILD are plotted with the black line indicating the grand median. For statistical analysis, Kruskal-Wallis test with Dunn's multiple correction or Mann-Whitney U test was applied (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). CTD, connective tissue disease; HC, healthy control; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; NSIP, non-specific interstitial pneumonia; SSc, systemic sclerosis; SSTR2, somatostatin receptor 2; UIP, usual interstitial pneumonia.

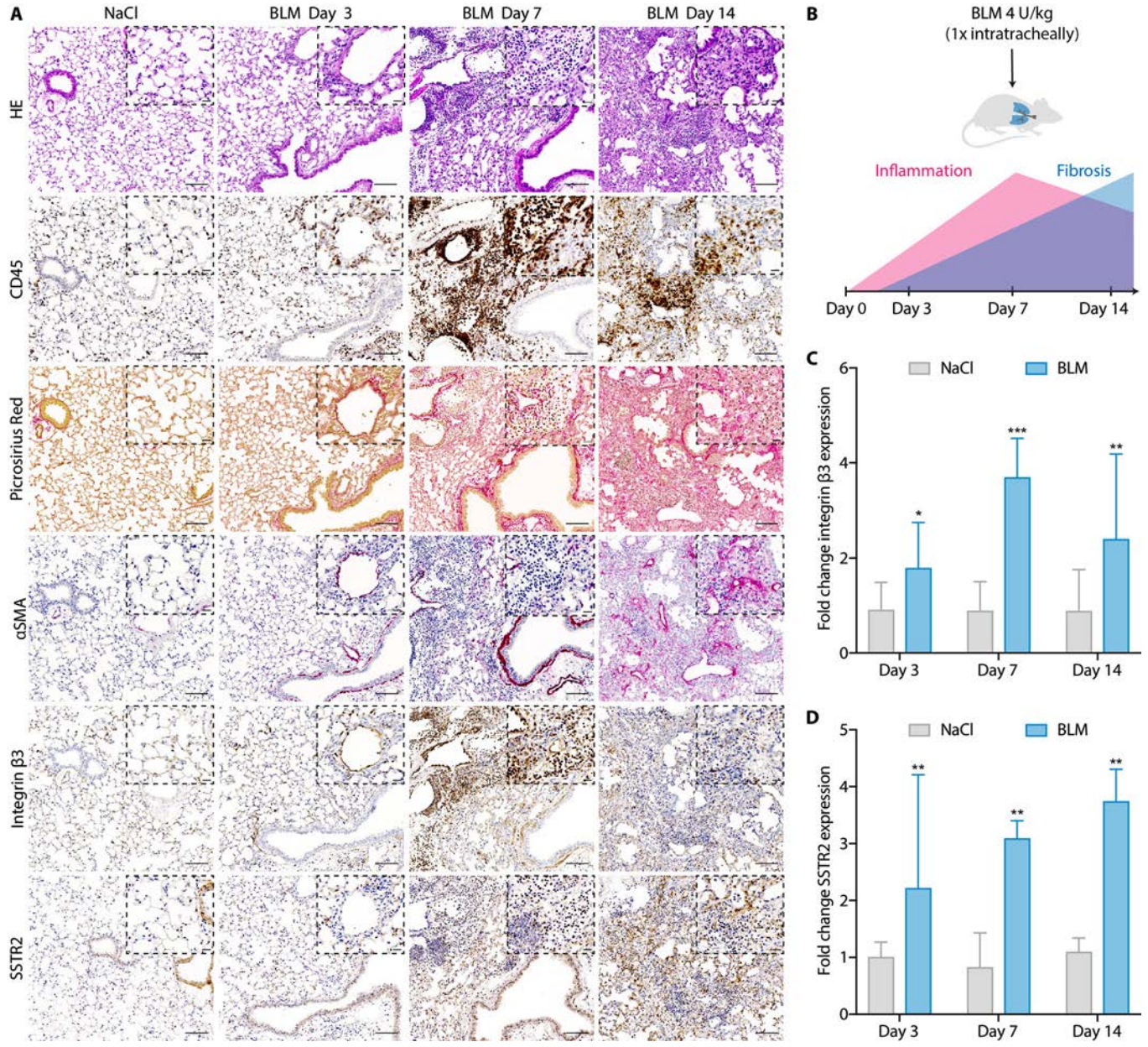

Figure 2 Integrin alpha-v-beta-3 (αvβ3) and SSTR2 are stage-specifically increased in BLM-induced lung fibrosis. (A) Representative images of lung sections from saline controls and BLM-treated mice on days 3, 7 and 14 stained with H&E (first panel), pan-leucocyte marker CD45 (brown, second panel), Picrosirius red (collagen fibres=red, third panel), myofibroblast marker (αSMA, pink, fourth panel), as well as stained for integrin β3 (brown, fifth panel) and SSTR2 (brown, sixth panel). (B) Schematic illustration of the time course of BLM-induced lung fibrosis with the inflammatory stage (days 3–7 preceding the fibrotic stage (day 14). (C) Semiquantification of integrin β3 expression by automatic image analysis. (D) Semiquantification of SSTR2 expression by automatic image analysis. For A, representative pictures at 100× magnification (scale bars: 100 µm) and at higher magnification (400×, scale bars: 20 µm) are shown. For C and D, data are presented as medians±IQR. For statistical analysis, Mann-Whitney U test was applied (*p<0.05, **p<0.01, ***p<0.001). For all experiments: n=6 for saline-treated controls and n=9–10 for BLM-treated mice. BLM, bleomycin; SSTR2, somatostatin receptor 2.

stainings with cell type-specific markers, we found both integrin αvβ3 and SSTR2 expressed on a broad range of inflammatory cell types, including leucocytes (CD45+, figure 3A,B), macrophages (F4/80+, figure 3C,D) and T cells (CD3+, figure 3E,F). Substantial expression of SSTR2 was found on pulmonary bronchial and alveolar epithelial cells (E-cadherin+), whereas expression of integrin αvβ3 was only rarely observed on epithelial cells in BLM-treated lungs and was absent on the epithelial cells in the lungs from the control mice (figure 3G,H). While integrin αvβ3 was strongly expressed on the pulmonary vasculature, including endothelial cells (von Willebrand factor (vWF)+, figure 3I), SSTR2 expression was not detected on vWF+ endothelial cells despite being expressed in vascular structures (figure 3J).

The most interesting difference in the pulmonary expression pattern between integrin αvβ3 and SSTR2 was the presence of integrin αvβ3, but absence of SSTR2 on myofibroblasts (αSMA+, figure 4A,B). The latter observation was in contrast to previous positive reports.[20 24] To confirm that the different expression of integrin αvβ3 and SSTR2 on murine lung fibroblasts also applies to human cells, we additionally analysed the mRNA and protein expression of both targets in normal human lung fibroblasts (NHLF) at basal conditions and after

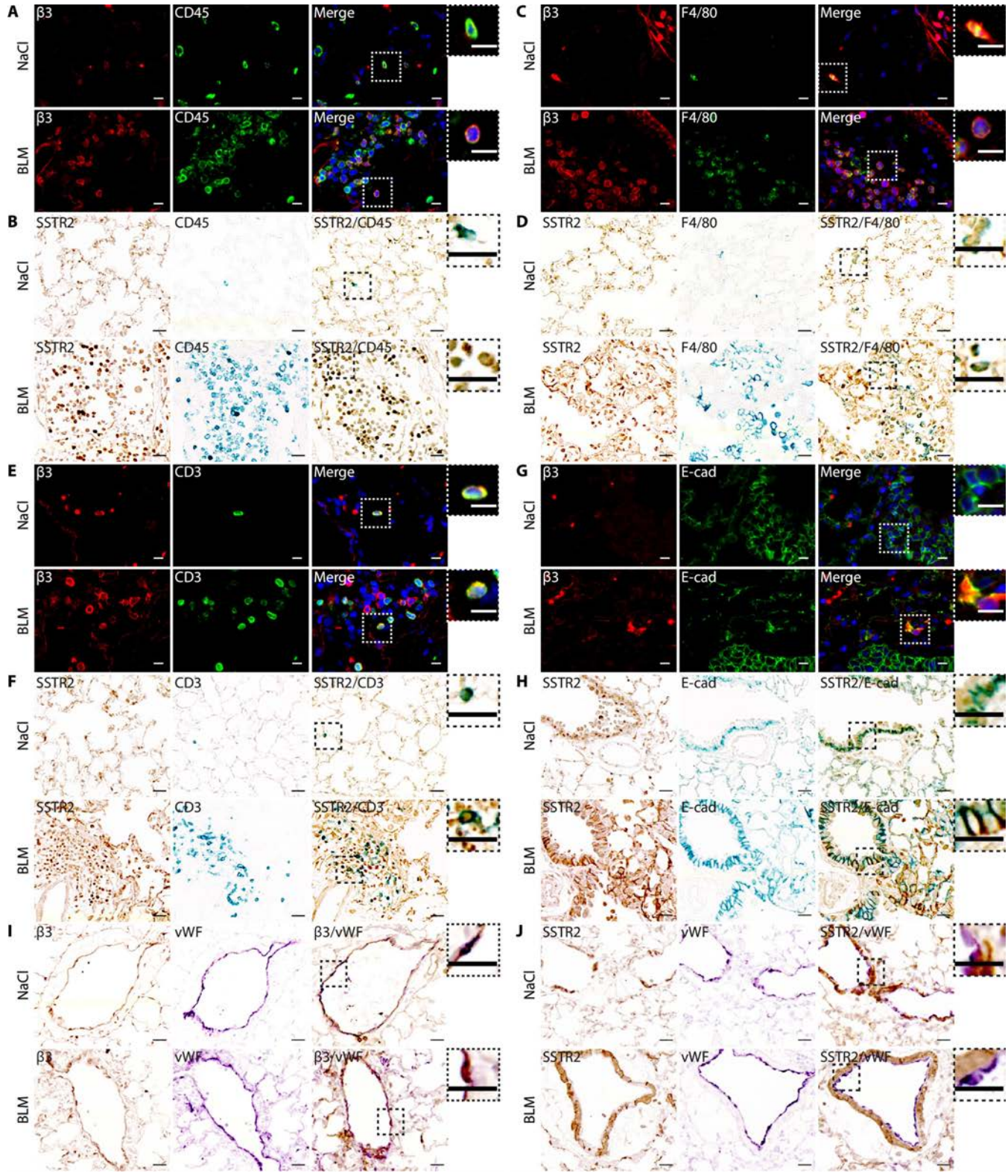

Figure 3 Cellular expression profiles of integrin alpha-v-beta-3 (αvβ3) and SSTR2 in the lungs of BLM-treated mice and saline-treated controls. (A) Immunofluorescent (IF) double staining of integrin β3 (red) and CD45 (green, leucocyte marker). (B) Immunohistochemical (IHC) double staining and sequential single stainings of SSTR2 (brown) and CD45 (green). (C) IF double staining of integrin β3 (red) and F4/80 (green, murine macrophage marker). (D) IHC double staining and sequential single stainings of SSTR2 (brown) and F4/80 (green). (E) IF double staining of integrin β3 (red) and CD3 (green, T cell marker). (F) IHC double staining and sequential single staining of SSTR2 (brown) and CD3 (green). (G) IF double staining of integrin β3 (red) and E-cadherin (E-cad, green, epithelial cell marker). (H) IHC double staining and sequential single staining of SSTR2 (brown) and E-cadherin (E-cad, green). (I) IHC double staining and sequential single staining of integrin β3 (brown) and vWF (purple, endothelial cell marker). (J) IHC double staining and sequential single staining of SSTR2 (brown) and vWF (purple). For all experiments, representative images (scale bars 10 µm for IF stainings (630× magnification) and 20 µm for IHC stainings (400× magnification) from three mice each are shown. BLM, bleomycin; SSTR2, somatostatin receptor 2; vWF, von Willebrand factor.

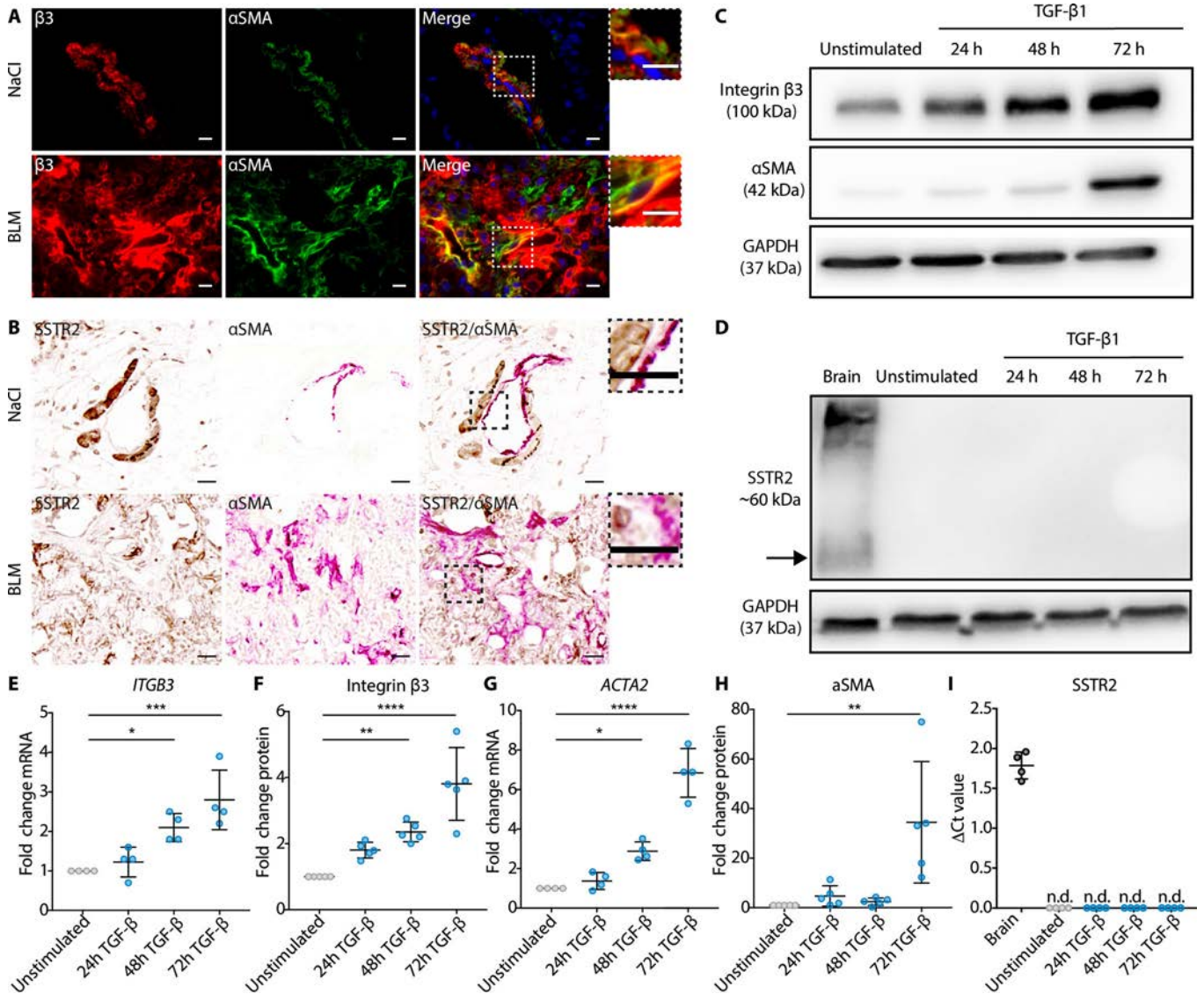

Figure 4 Integrin alpha-v-beta-3 (αvβ3) but not SSTR2 is expressed on murine and human (myo)fibroblasts. (A) Immunofluorescent (IF) double staining of integrin β3 (red) and αSMA (green, myofibroblast marker). (B) Immunohistochemical (IHC) double staining and sequential single stainings of SSTR2 (brown) and αSMA (alpha-smooth muscle actin) (pink). (C) Representative western blot for integrin β3 and αSMA expression in unstimulated NHLFs and on TGF-β stimulation (10 ng/mL) for 24 hours, 48 hours and 72 hours (10 µg of protein/lane). (D) Representative western blot for SSTR2 expression in unstimulated NHLFs and on TGF-β stimulation (10 ng/mL) for 24 hours, 48 hours and 72 hours (50 µg of protein/lane). Whole brain protein lysate served as positive control (20 µg of protein/lane). Arrow points to the expected SSTR2 protein band at ~60 kDa. (E, F) Fold change of (E) mRNA expression of integrin β3 (*ITGB3*) normalised to *RPLP0* and (F) protein expression of integrin β3 normalised to glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) at basal conditions and after stimulation with TGF-β. (G, H) Fold change of (G) mRNA expression of αSMA (*ACTA2*) normalised to *RPLP0* and (H) protein expression of αSMA normalised to GAPDH at basal conditions and after stimulation with TGF-β. (I) mRNA expression analysis of SSTR2 showing the absence of SSTR2 expression in NHLFs at basal conditions and after stimulation with TGF-β. As a positive control served total brain RNA. For A and B, representative images (scale bars 10 µm for IF stainings (630× magnification) and 20 µm for IHC stainings (400× magnification)) from three mice each are shown. For E–I, data were expressed as mean±SD. For statistical analysis, one-way analysis of variance with Turkey's post-hoc test was performed (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). BLM, bleomycin; NHLFs, normal human lung fibroblasts; SSTR2, somatostatin receptor 2; TGF-β, transforming growth factor beta.

differentiation into myofibroblasts on stimulation with TGF-β. Integrin αvβ3 was constitutively expressed in NHLF and showed a time-dependent increase after TGF-β-induced fibroblast activation at the mRNA and protein levels (figure 4C,E,F), similar to *ACTA2*/αSMA (figure 4C,G,H). In contrast, mRNA and protein levels of SSTR2 were not detectable in NHLF neither at basal conditions nor after stimulation with TGF-β (figure 4D/I and online supplementary figure S5), which so far has been a major matter of debate.[20 24] Thus, we could confirm similar expression patterns of our imaging targets on both murine and human lung fibroblasts.

Detection of integrin αvβ3 with ^{177}Lu-DOTA-RGD-SPECT/CT visualises inflammatory stages of lung fibrosis

Having confirmed integrin αvβ3 and SSTR2 as potential imaging targets in ILD, targeted nuclear imaging was performed to confirm their suitability as surrogate imaging markers for

pathophysiological processes in ILD. Since in the preclinical setting SPECT/CT often outperforms PET/CT with respect to image quality and resolution, SPECT/CT scans using the integrin αvβ3-targeting ^{177}Lu-DOTA-RGDand the SSTR2-targeting ^{177}Lu-DOTA-NOC were performed on days 3, 7 and 14 after the BLM instillation.

Consistent with the expression changes of integrin αvβ3 observed on tissue level, biodistribution studies (figure 5A and online supplementary tables S4 and S5) and ex vivo SPECT/CT scans (figure 5B) performed 2 hours postinjection (p.i.) of ^{177}Lu-DOTA-RGD revealed a significant increase of tracer uptake and signal intensity in the lungs of BLM-treated mice versus control mice at all time points. The strongest tracer accumulation in BLM-treated lungs was observed on day 7 with a mean lung uptake of 0.65%±0.13% injected activity per lung (% IA/lung) compared with 0.19%±0.04% IA/lung in the respective control lungs ($p<0.01$). Specificity of the pulmonary accumulation of ^{177}Lu-DOTA-RGD was validated by receptor blockade with an unlabelled RGD peptide, which significantly reduced the pulmonary radioactivity amounts of ^{177}Lu-DOTA-RGD in BLM-treated mice to the levels detected in control animals. This was quantified by biodistribution studies and visualised by ex vivo SPECT/CT scans. The in vivo SPECT/CT imaging closely mirrored our ex vivo imaging results with the highest signal intensity observed on day 7 (figure 5C), the inflammation-dominant stage of BLM-induced lung fibrosis.

Detection of SSTR2 with ^{177}Lu-DOTA-NOC-SPECT/CT visualises established lung fibrosis

BLM-treated mice showed a steady increase of pulmonary accumulation of ^{177}Lu-DOTA-NOC as compared with saline-treated controls in biodistribution studies and ex vivo SPECT/CT scans that were performed 2 hours p.i. of ^{177}Lu-DOTA-NOC. This was in line with the expression changes of SSTR2 at the tissue level (figure 5D,E). The highest lung accumulation in BLM-challenged mice, and hence the highest imaging signal intensity, was observed on day 14, the peak of pulmonary fibrosis, with an accumulation of 1.62%±0.32% IA/lung vs 0.66%±0.09% IA/lung in control lungs ($p<0.0001$). Due to already high basal pulmonary tracer accumulation observed in control animals, diseased lungs could only be reliably distinguished on day 14 (figure 5E and online supplementary tables S6 and S7). In vivo imaging mirrored the ex vivo findings and allowed the distinction of BLM-treated lungs from healthy lungs on day 14, the time point of established fibrosis, yet not at earlier, more inflammatory time points (figure 5F). The specificity of the pulmonary uptake of ^{177}Lu-DOTA-NOC, and thus of our imaging results, was confirmed by receptor blockade with unlabelled DOTA-NOC, which almost completely prevented the pulmonary accumulation of the radiotracer in BLM-treated mice (figure 5D,E).

DISCUSSION

This study addressed a major unmet need in ILD, the lack of pathophysiologically relevant biomarkers allowing disease staging. Our comprehensive approach integrating tissue-derived human and murine ex vivo and in vivo (imaging) data extends previously published nuclear imaging studies in ILD, where molecular analyses on tissue levels have largely not been performed.[26–29]

One of the first key findings of our study was that the expression of our imaging targets did not differ between different aetiologies of ILD comprising IPF and CTD-associated ILD. With pulmonary fibrosis as the common end stage, increasing data support the importance of dysregulated wound-healing mechanisms for both entities.[2 30] Furthermore, in contrast to previous assumptions regarding the non-inflammatory pathogenesis of IPF,[5 31] lately, the potential pathogenic role of immune cells in the development and progression of IPF has been re-evaluated.[32 33] Thus, a molecular-based rather than a clinical/histological-driven classification as the basis for substratification of patients with ILD might open novel perspectives.

Consistent with the expression of integrin αvβ3 on (activated) fibroblasts in addition to immune cells, we identified integrin αvβ3 as a valuable diagnostic tool for inflammation-dominant fibrotic stages of ILD and demonstrated that molecular-targeted SPECT/CT imaging using a radiolabelled RGD peptide can act as a surrogate marker for integrin αvβ3 expression. However, given the central roles of integrins in the pathogenesis of ILD, other integrins might also be attractive as imaging targets.[11 34] Of particular interest is, for example, integrin αvβ6, which is expressed on epithelial cells, and overexpressed in wound healing and IPF.[35–38] Preclinical imaging studies in BLM-induced lung fibrosis confirmed its potential,[34] and results from phase I clinical trials on imaging in IPF are soon to be awaited (www.clinicaltrials.gov; NCT03183570 and NCT02052297). Besides showing great promise as novel diagnostic tools, integrins represent potential therapeutic targets in ILD. Evidence from preclinical models of organ fibrosis demonstrated beneficial antifibrotic effects for the inhibition of integrin signalling.[15 16 37 38] In oncology, numerous selective integrin inhibitors are currently being tested in clinical trials,[39–41] since many tumours overexpress integrin αvβ3, αvβ5 and/or αvβ6.[42] Given the promising data from preclinical fibrosis models, these approaches could be easily applied for studies in ILD.

In contrast to integrin αvβ3, our data suggest that SSTR2 may serve as a diagnostic tool for established lung fibrosis and thus ILD severity. Targeted SPECT/CT using the radiolabelled somatostatin analogue DOTA-NOC revealed a steady increase in signal intensity over time mirroring the degree of tissue remodelling, thereby reaching its peak on day 14, the time point of established fibrosis. This is also in line with the increased expression of SSTR2 in patients with ILD with UIP pattern, where lung remodelling is more severe than in the NSIP subtype and where epithelial cells are supposed to be the central drivers of pulmonary fibrosis.[30] Further support comes from preliminary nuclear imaging studies targeting SSTR2 with different radiolabelled somatostatin analogues in patients with ILD, which showed lower signal intensity in patients with NSIP/SSc-ILD compared with patients with IPF/UIP.[27–29] Like integrin αvβ3, SSTR2 has diagnostic and therapeutic potential. Several preclinical studies using different somatostatin analogues showed beneficial effects on organ fibrosis.[24 43 44] Although increasing data suggest SSTR2 for imaging and possibly treatment of ILD, it should be pointed out that the precise pathophysiological role of SSTR2 in ILD has yet to be elucidated. Of note, SSTR-targeted (radio)pharmaceuticals are not specific for SSTR2, but also bind to other somatostatin receptors with similar affinity, including SSTR3 and SSTR5.[20 45 46] Herein, we demonstrated on several experimental levels that neither murine nor human lung fibroblasts express SSTR2. In previous studies which had reported on the SSTR2 expression on fibroblasts, fibroblasts from other sites of origin (eg, skin, retro-orbital space or liver)[47–49] and/or from embryonic stage were assessed.[50] This, and the fact that SSTR2 is expressed in two protein variants, generated by alternative splicing,[51] might well account for the observed differences. Consequently, in (experimental) ILD, it seems likely that both radiotracer

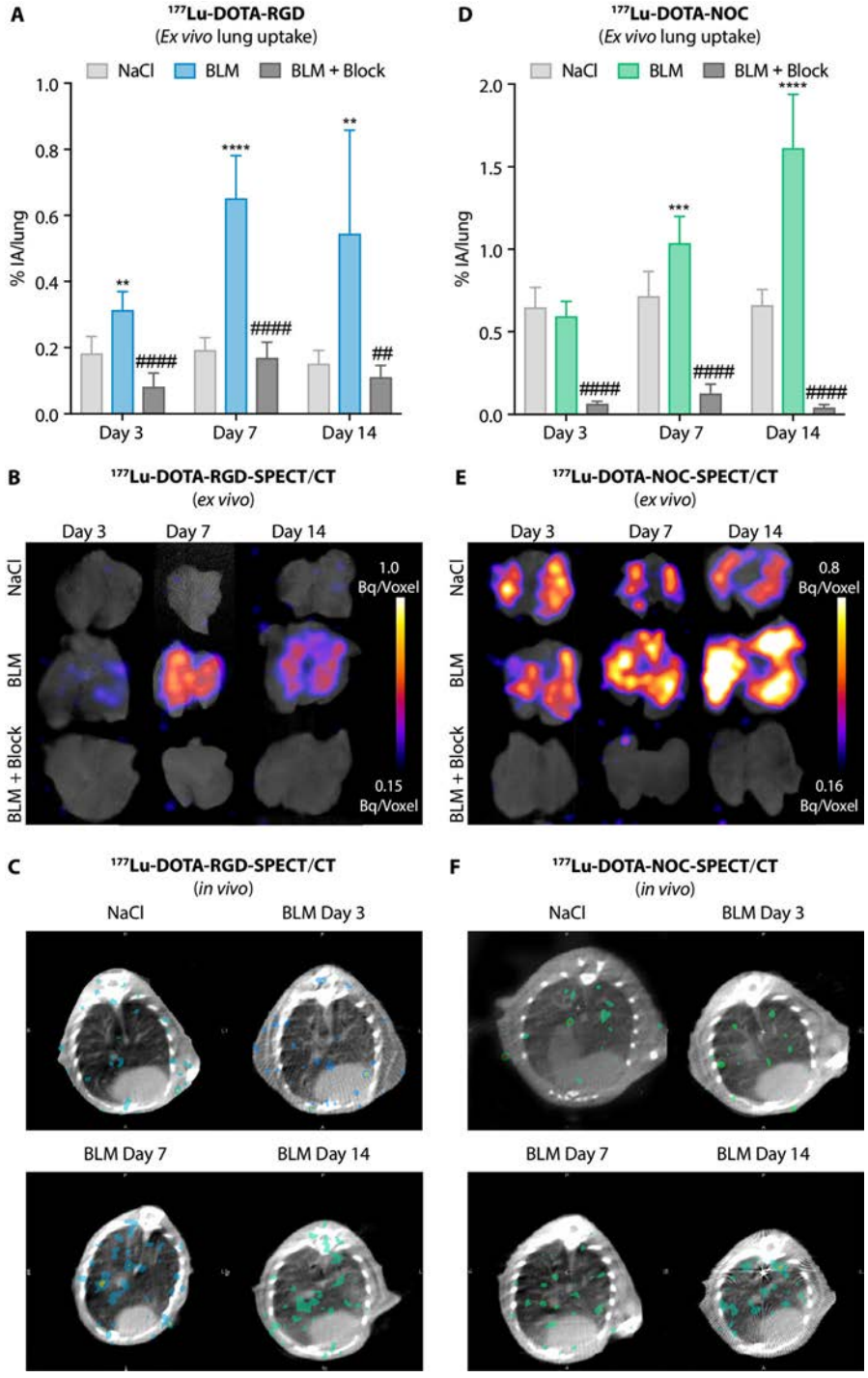

Figure 5 Integrin alpha-v-beta-3 (αvβ3) and SSTR2 can serve as surrogate imaging biomarkers for stage-specific detection of experimental ILD. (A) Ex vivo lung uptake of ^{177}Lu-DOTA-RGD (2 hours p.i.) shown as percentage of injected activity per lung (% IA/lung) in lungs from saline-treated controls and BLM-treated mice with and without receptor blockade on days 3, 7 and 14 after the BLM instillation. (B) Ex vivo SPECT/CT scans of lungs from saline-treated controls and BLM-treated mice with and without receptor blockade on days 3, 7 and 14 that were collected 2 hours after injection of ^{177}Lu-DOTA-RGD. Ex vivo scans are shown as maximum intensity projections. (C) Representative images of in vivo SPECT/CT scans of saline controls and BLM-treated mice on days 3, 7 and 14 performed 2 hours after injection of ^{177}Lu-DOTA-RGD. Transaxial projections of the lung windows are shown. (D) Ex vivo lung uptake of ^{177}Lu-DOTA-NOC (2 hours p.i.) shown as percentage of injected activity per lung (% IA/lung) in lungs from saline-treated controls and BLM-treated mice with and without receptor blockade on days 3, 7 and 14 after the BLM instillation. (E) Ex vivo SPECT/CT scans of lungs from saline-treated controls and BLM-treated mice with and without receptor blockade on days 3, 7 and 14 that were collected 2 hours after injection of ^{177}Lu-DOTA-NOC. Ex vivo scans are shown as maximum intensity projections. (F) Representative images of in vivo SPECT/CT scans of saline controls and BLM-treated mice on days 3, 7 and 14 performed 2 hours after injection of ^{177}Lu-DOTA-NOC. Transaxial projections of the lung windows are shown. For A and D, data are presented as mean±SD. For statistical analysis, one-way analysis of variance with Turkey's multiple correction was applied (**p<0.01, ***p<0.001, ****p<0.0001, vs saline; ##p<0.01, ####p<0.0001, vs BLM). For biodistribution and ex vivo SPECT/CT scans: n=3–7 for saline controls, n=5–8 for BLM-treated mice, and n=3–6 for BLM-treated mice receiving receptor blockade. BLM, bleomycin; ILD, interstitial lung disease; p.i.; postinjection; SPECT, single photon emission CT; SSTR2, somatostatin receptor 2.

uptake and antifibrotic effects of SSTR analogues can largely be attributed to inflammation and epithelial cell damage.[24 27] However, further studies are needed to assess whether pulmonary fibroblasts express SSTR3 and SSTR5 and might therefore be directly targeted by somatostatin analogues.

Additional profibrotic molecules which could be of interest for targeted nuclear imaging include extracellular matrix proteins, for example, collagens, fibronectin or fibroblast activation protein.[3 52] However, in contrast to our selected imaging targets, radiotracers for these molecules have so far only been validated in animal models[53 54] or in human non-ILD conditions.[55–57] Furthermore, imaging tools that exclusively detect fibrotic changes will probably not reflect pathophysiological changes caused by immune and/or epithelial cells and consequently may provide a less complete picture of the overall disease process. However, fibrosis markers could be valuable for monitoring therapeutic responses to fibroblast-targeting therapies in defined subcohorts of patients with ILD.

The detection of ILD in the context of multiorgan inflammation and fibrosis might represent a challenge. Thus, the performance of our radiotracers should ideally have been evaluated in a second, multisystemic animal model, which better reflects the human situation in CTD-ILD. However, the available literature on PET/CT imaging provides substantial evidence that even in patients with CTD with multiorgan involvement, ILD can reliably be diagnosed.[58–65]

In conclusion, both integrin αvβ3 and SSTR2 are intriguing candidates for the visualisation of specific molecular processes in ILD. Since SSTR2 targeting ^{68}Ga-DOTA-NOC PET/CT is already available in clinical routine for the diagnosis of neuroendocrine tumours and several RGD-targeted PET tracers such as ^{68}Ga-NOTA-RGD are in clinical trials,[11 18 19] the findings of our study could be easily and rapidly transferred into clinical application for proof-of-concept studies in patients with ILD. This could represent the first step towards molecular patients' stratification and thus precision medicine approaches in ILD.

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Competing interests JS, MBe, MBr, SH, SC, RS, CM, CAF-B and TF have no competing interests to declare. OD had consultancy relationship and/or has received research funding from Actelion, AnaMar, Bayer, Boehringer Ingelheim, ChemomAb, EspeRare Foundation, Genentech/Roche, GSK, Inventiva, Italfarmaco, Lilly, medac, MedImmune, Mitsubishi Tanabe Pharma, Novartis, Pfizer, Sanofi, Sinoxa and UCB in the area of potential treatments of scleroderma and its complications. In addition, OD has a patent mir-29 for the treatment of systemic sclerosis licensed. The real or perceived potential conflicts listed above are accurately stated. BM had grant/research support from AbbVie, Protagen and Novartis, and congress support from Pfizer, Roche and Actelion. In addition, BM has a patent mir-29 for the treatment of systemic sclerosis licensed. The real or perceived potential conflicts listed above are accurately stated.

Patient consent Obtained.

Ethics approval All animal experiments were approved by the cantonal authorities and performed according to the Swiss animal welfare guidelines. All studies with human biosamples were approved by the local ethics committees and the local institutional review boards.

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TRANSLATIONAL SCIENCE

Humanised effector-null FcγRIIA antibody inhibits immune complex-mediated proinflammatory responses

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ABSTRACT

Objective Immune complexes (ICs) play a critical role in the pathology of autoimmune diseases. The aim of this study was to generate and characterise a first-in-class anti-FcγRIIA antibody (Ab) VIB9600 (previously known as MEDI9600) that blocks IgG immune complex-mediated cellular activation for clinical development.

Methods VIB9600 was humanised and optimised from the IV.3 Ab. Binding affinity and specificity were determined by Biacore and ELISA. Confocal microscopy, Flow Cytometry-based assays and binding competition assays were used to assess the mode of action of the antibody. In vitro cell-based assays were used to demonstrate suppression of IC-mediated inflammatory responses. In vivo target suppression and efficacy was demonstrated in FcγRIIA-transgenic mice. Single-dose pharmacokinetic (PK)/pharmacodynamic study multiple dose Good Laboratory Practice (GLP) toxicity studies were conducted in non-human primates.

Results We generated a humanised effector-deficient anti-FcγRIIA antibody (VIB9600) that potently blocks autoantibody and IC-mediated proinflammatory responses. VIB9600 suppresses FcγRIIA activation by blocking ligand engagement and by internalising FcγRIIA from the cell surface. VIB9600 inhibits IC-induced type I interferons from plasmacytoid dendritic cells (involved in SLE), antineutrophil cytoplasmic antibody (ANCA)-induced production of reactive oxygen species by neutrophils (involved in ANCA-associated vasculitis) and IC-induced tumour necrosis factor α and interleukin-6 production (involved in rheumatoid arthritis). In FcγRIIA transgenic mice, VIB9600 suppressed antiplatelet antibody-induced thrombocytopaenia, acute anti-GBM Ab-induced nephritis and anticollagen Ab-induced arthritis. VIB9600 also exhibited favourable PK and safety profiles in cynomolgus monkey studies.

Conclusions VIB9600 is a specific humanised antibody antagonist of FcγRIIA with null effector function that warrants further clinical development for the treatment of IC-mediated diseases.

Key messages

What is already known about this subject?

- Immune complexes play a critical role in the pathology of a variety of autoimmune diseases. Immune complexes trigger FcγR-mediated inflammatory responses that are primarily driven through FcγRIIA. A variety of challenges have precluded the development of a therapeutic targeting this receptor.

What does this study add?

- We generated a humanised effector-deficient anti-FcγRIIA antibody, VIB9600 which specifically binds to FcγRIIA and acts by both blocking ligand and internalising the receptor.
- We demonstrated that VIB9600 suppresses immune complex-mediated activation of immune cells critical in the pathology of multiple autoimmune diseases both in vitro and in vivo.

How might this impact on clinical practice or future developments?

- VIB9600 had a favourable pharmacokinetic and safety profiles in cynomolgus monkey studies and support its clinical development.
- VIB9600 may provide a first-in-class treatment option towards immune complex-mediated autoimmune diseases.

INTRODUCTION

Autoantibodies directed against self-antigens may drive debilitating, organ specific or systemic manifestations which can be life-threatening.[1] Spikes in autoantibody titres and changes in autoantibody profiles have been associated with autoimmune disease onset and flares in disease activity.[2] Immune complexes (ICs) are found in the circulation and deposited in afflicted tissues and organs.[3 4] Engagement of ICs with immune cells bearing Fc receptors can trigger cell recruitment and activation, localised inflammation, adaptive immunity and tissue pathology.[4 5] Despite improved clinical management and the development of disease-modifying drugs, current therapies predominately target individual inflammatory pathways and many

autoimmune patients fail to achieve remission.[6] Available treatments including those targeting B cells only have modest effects on autoantibody titres, and it remains unclear if they have any impact on IC deposition.[7] Consequently, interventions that block the engagement of ICs with FcγRs, antagonise activating FcγRs, or agonise inhibitory receptors, have been pursued for their therapeutic potential.[8–10] To date, however, no therapies directly targeting FcγRs have been successfully developed.

In humans, the receptors for the Fc region of IgG (FcγRs) include the activating receptors FcγRI, FcγRIIA, FcγRIIIA and FcγRIIIB and the inhibitory receptor FcγRIIB.[8] The activating receptor FcγRIIA has low affinity (K_D ~10^{-6} M) for monomeric IgG,[11 12] but the increased avidity afforded by the higher valency of aggregated IgG ICs permit FcγRIIA binding, clustering and signalling.[11] There are two common human variants of FcγRIIA, 131 H and 131R, which exhibit differential binding toward IgG subclasses.[13] Cross-linking FcγRIIA and phosphorylation of its immunoreceptor tyrosine-based activation motif triggers a signalling cascade that leads to multiple functional responses.[5]

FcγRIIA is an attractive target for therapeutic intervention as it is expressed on multiple immune cells that trigger pathological inflammatory responses.[14 15] FcγRIIA has been specifically implicated in systemic lupus erythematosus (SLE), where its expression on plasmacytoid dendritic cells (pDC) drives IC-mediated type I interferon (IFN) production[16 17] and its presence on neutrophils promotes lupus nephritis following the passive transfer of human SLE sera.[18] In ANCA-associated vasculitis (AAV), autoantibodies targeting antigens exposed on neutrophils trigger FcγRIIA-dependent activation and tissue injury.[19–21] ICs precipitated from rheumatoid arthritis (RA) may also trigger Fc-dependent induction of proinflammatory cytokines by monocytes.[22]

There are several challenges that need to be circumvented to facilitate the development of a therapeutic targeting FcγRIIA. With respect to specificity, it is noteworthy, that the extracellular region of FcγRIIA shares 94% identity with the inhibitory receptor FcγRIIB,[23] so exposed epitopes that distinguish these receptors are limited. The two FcγRIIA 131 H and 131R variants are located in the ligand binding site,[13 24] so ligand-blocking antibodies would need to be high affinity and preferably bind both allelic variants to be effective in the presence of high serum concentrations of IgG. It is also important that the therapeutic does not agonise FcγRIIA, trigger Fc-mediated hypersensitivity or induce effector functions such as complement-dependent cytotoxicity (CDC) or antibody (Ab)-dependent cell-mediated cytotoxicity (ADCC).[11]

The aim of this study was to develop an antibody targeting FcγRIIA suitable for the treatment of patients with IC-mediated disease. To that end, we generated VIB9600, a humanised, optimised, effector-deficient anti-FcγRIIA-specific antibody. We demonstrate that VIB9600 suppresses IC-mediated activation of immune cells critical in the pathology of multiple autoimmune diseases, and the pharmacology and safety data generated in non-human primate studies support its clinical development.

RESULTS

Generation of VIB9600, a humanised effector-null FcγRIIA-specific antibody with dual mechanism of action

IV.3 is a well characterised murine IgG2b mAb specific for FcγRIIA.[25] To generate a humanised antibody suitable for clinical development, the IV.3 complementary determing region (CDR) regions of the heavy and light chains were initially grafted on to the closest human germline variable heavy chain (Vh) and variable kappa chain (Vk) genes to generate Cam IV.3. Cam IV.3 was then optimised by screening amino acid substitutions in an epitope competition assay (with IV.3) to identify variants with improved binding. The sequences of the optimised, humanised antibody, VIB9600 and its parents IV.3 and Cam IV.3 are shown in online supplementary figure 1. VIB9600 has significant improvement in binding to FcγRIIA 131 H and 131R compared with Cam IV.3, and a modest improvement compared with IV.3, as assessed by epitope competition (figure 1A) and KD affinity measurements (table 1), while retaining the specificity for FcγRIIA (figure 1B).

A triple mutation (TM) (L234F/L235E/P331S) incorporated in the heavy chain constant region of VIB9600 was included to reduce Fc-mediated effector functions.[26] To verify that the TM prevents Fc-mediated effector functions, VIB9600 and a variant incorporating an identical Fab with wild-type IgG Fc (9600 IgG1) were examined in ADCC and CDC assays. No notable ADCC or CDC was detected with VIB9600, whereas the variant with a wild-type Fc-induced cytotoxicity of FcγRIIA-expressing HEK cells in these assays (figure 1C), demonstrating that the TM renders VIB9600 effector null.

Next, we determined if VIB9600 has a competitive or non-competitive mode of action, by assessing the binding of VIB9600 to FcγRIIA-expressing neutrophils in the presence and absence of ligand. The presence of 10 mg/mL IVIG (pooled IgG) reduced the binding of VIB9600 to neutrophils from an EC_{50} of 0.03 nM to 3.35 nM (figure 1D). This competition for binding between the antibody and ligand indicates that the antibody has a ligand-blocking mechanism of action.

We next sought to determine if engagement of VIB9600 altered cell surface expression of FcγRIIA. Confocal microscopy indicated that incubation of VIB9600 with monocytes (1 hour at 37°C) resulted in the internalisation of FcγRIIA, whereas another antigen, CD14, remained expressed on the cell surface (figure 1E). VIB9600-mediated internalisation of FcγRIIA was verified using a FACS-based whole blood assay on human monocytes and neutrophils from donors with either a 131 H/H or a 131 R/R genotype (figure 1F). To justify, cynomolgus monkey as a relevant pharmacology and toxicology species, we also demonstrated that VIB9600 also reduced cell surface bound FcγRIIA on monocytes and neutrophils from cynomolgus monkey whole blood (figure 1G). Together these data demonstrate that VIB9600, has two significant modes of action: it blocks ligand and reduces the cell surface expression of FcγRIIA available for ligand engagement.

VIB9600 blocks autoantibody/IC-mediated inflammatory responses

Next, we assessed the capacity of VIB9600 to block IC-mediated inflammatory responses driven by different cell types relevant to autoimmune diseases. DNA/RNA associated ICs trigger pDC to produce the type I IFNs which are implicated in the pathogenesis of SLE.[16] Using peripheral blood mononuclear cells (PBMCs) as a source of pDC, VIB9600 potently inhibited the induction of type I IFN induced by ribonucleoprotein IC (RNP-IC) from healthy donors with either a 131 H/H or a 131 R/R genotype (figure 2A). IC can also trigger monocytes to produce TNFα and IL-6, key cytokines involved in the pathogenesis of RA.[22] Compared with control antibody, VIB9600 inhibited the IC induction of IL-6 and TNFα in whole blood by approximately 60% and 80%, respectively (figure 2B). In AAV, autoantibodies against cytoplasmic antigens such as MPO and PR3 lead to neutrophil activation, and the induction of reactive oxygen species (ROS) which induces tissue pathology.[19] Pretreatment of neutrophils with VIB9600 inhibited anti- myeloperoxidase

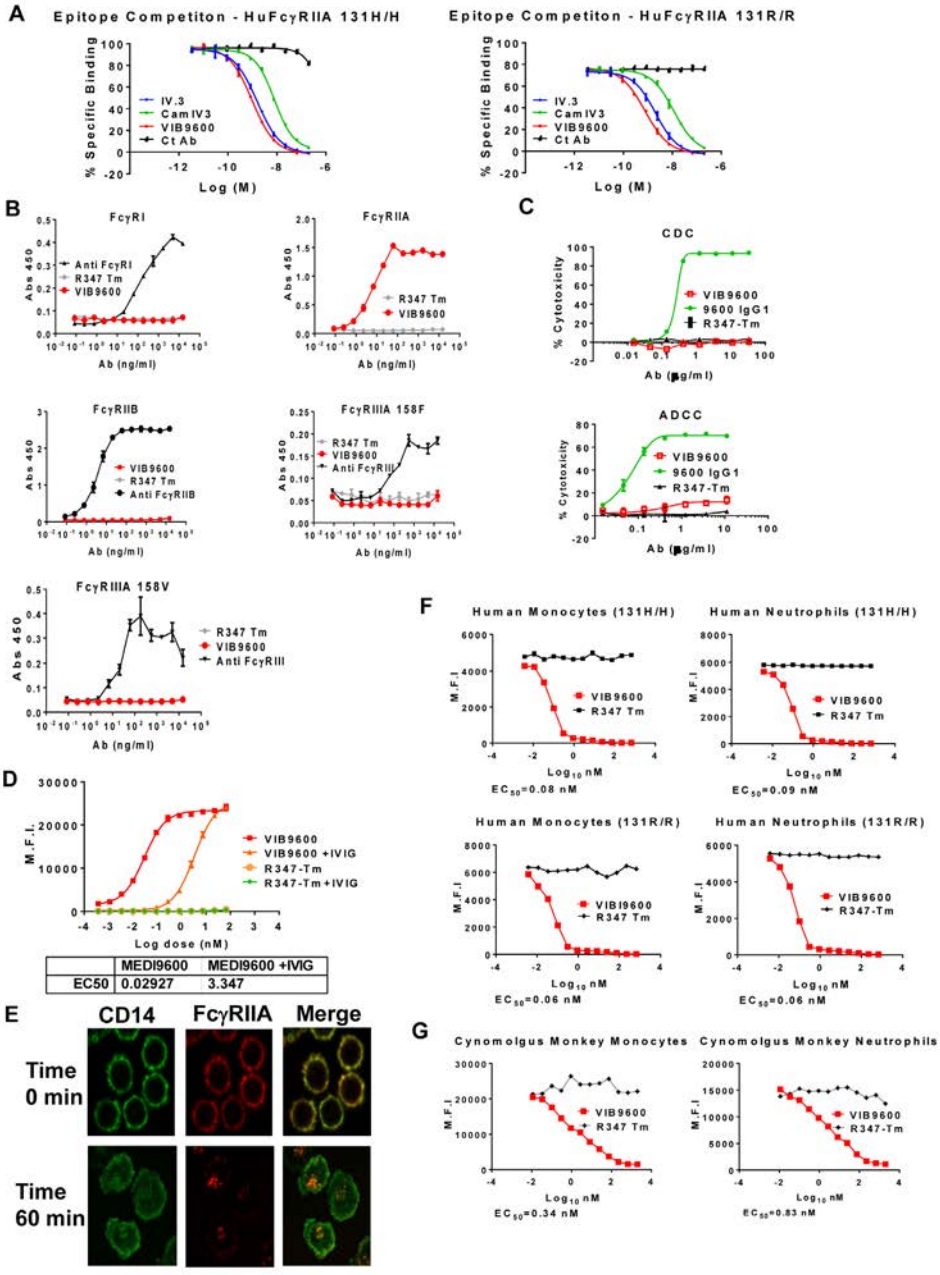

Figure 1 VIB9600 specifically binds FcγRIIA, competes with IgG for binding to FcγRIIA, causes receptor internalisation but fails to induce effector mechanisms. (A) Epitope competition data with IV.3 (mouse IgG 2b Ab), CamIV3 (humanised framework regions with IV.3 CDRs) and VIB9600 (humanised and optimised IV.3) on both human FcγRIIA 131 H (left) and FcγRIIA 131R (right). Representative data from two independent experiments are shown. (B) VIB9600 binding to human FcγRs in an ELISA-based binding assay. Plots represent the mean±SD. A representative plot of two independent experiments is shown. (C) In ADCC and CDC assays, the effects of VIB9600 were compared with wild type control 9600 IgG1 and isotype control IgG (R347-Tm) as indicated. in the ADCC assay, primary NK cells (effectors) were incubated with adherent FcγRIIA-expressing HEK-293 cells (targets) for 5 hour, and % cytotoxicity was determined. For CDC assays, baby rabbit complement was incubated with adherent FcγRIIA-expressing HEK-293 cells (targets), and % cytotoxicity was determined after 1 hour. Plots represent the mean±SD. Representative plots of three independent experiments are shown. (D) Binding of VIB9600 and control Ab (R347-Tm) to human FcγRIIA-expressing neutrophils in the presence and absence of 10 mg/mL IVIg (as indicated) was determined by flow cytometry (M.F.I). Representative data from two independent experiments. (E) Human monocytes were stained with CD14-Alexa 488 (green) and VIB9600-Alex 647 (red), and internalisation of FcγRIIA on human monocytes was visualised by confocal microscopy at time 0 and after culturing at room temperature for 1 hour. A representative image of three independent experiments is shown. (F) Available cell surface FcγRIIA on human monocytes and neutrophils in whole blood from healthy donors with either a 131 H/H or 131 R/R genotype (as indicated) was examined following a 2-hour incubation with VIB9600 or control Ab (R347-TM) by flow cytometry (M.F.I). (G) Similarly, cell surface FcγRIIA on cynomolgus monkey monocytes and neutrophils in whole blood was examined following a 12-hour incubation with VIB9600 or control Ab (R347-TM) by flow cytometry (M.F.I). Representative data from three independent humans and cynomolgus monkey experiments are shown. Ab, antibody; ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; TM, triple mutation.

Table 1 Binding affinity of IV.3 and VIB9600 Fabs to human FcγRIIA

Fab	Antigen	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)
IV.3	Human FcγRIIa 131 R	5.95×10^{6}	1.29×10^{-3}	0.22
VIB9600		4.98×10^{6}	7.35×10^{-4}	0.15
IV.3	Human FcγRIIa 131 H	3.03×10^{6}	6.81×10^{-4}	0.22
VIB9600		2.60×10^{6}	3.37×10^{-4}	0.13

(MPO) and anti-proteinase 3 (PR3) antibody-induced superoxide production as determined using either ferri-cytochrome c reduction assay (figure 2C) or oxidation of dihydrorhodamine 123 (DHR123) (figure 2D). VIB9600 also blocked ROS production from neutrophils stimulated with IgG-purified AAV patient's sera seropositive for either anti-PR3 or anti-MPO antibodies (figure 2E). Taken together these data demonstrate that VIB9600 can inhibit autoantibody and IC-mediated activation of inflammatory processes associated with autoimmune diseases.

VIB9600 does not adversely impact neutrophil function or agonise FcγRIIA in vitro

Neutrophils play a critical role in host defense by sensing infection and tissue injury and initiating an acute inflammatory response.[27 28] Therefore, it was important to determine if VIB9600 inadvertently activates neutrophils or otherwise impedes their function. Importantly, VIB9600 did not impact phorbol 12-myristate 13-acetate (PMA)-mediated ROS production (figure 3A), Pam3CysSerLys4 (TLR2)-induced CD11b upregulation (figure 3B) or IL-8 mediated neutrophil migration (figure 3C). Finally, we examined the impact of VIB9600 on antibody-dependent (anti-PsI mAb PsI0096) opsonophagic killing (OPK) of *Pseudomonas aeruginosa*, a clinically important antibiotic-resistant strain of bacteria.[29] VIB9600 had a minimal impact on *P. aeruginosa* OPK, whereas blockade of FcγRIII significantly inhibited OPK activity (figure 3D). Together these data indicate that VIB9600 does not inadvertently impact neutrophil functions.

Crosslinking FcγRs has the potential to stimulate the secretion of inflammatory cytokines[30] or induce immune hypersensitivity.[31] To assess the agonistic potential of VIB9600, whole blood was treated with 30 µg/mL of VIB9600, or ICs (positive control) for 16 hours at 37°C, and changes in secreted protein expression were examined. Cross-linking FcγRs with IgG-IC or RNP-IC-induced profound changes in the protein levels, however, there was no discernable difference between the protein profiles of untreated and VIB9600-treated samples (online supplementary table 1 and figure 3E). These data indicate that VIB9600 does not exhibit agonistic activity.

VIB9600 suppresses antibody-mediated pathology in FcγRIIA transgenic mice

Since FcγRIIA does not exist in rodents, we assessed the pharmacology of VIB9600 in FcγRIIA transgenic mice. First, we demonstrated that VIB9600 transiently reduced FcγRIIA expression on platelets and neutrophils in a dose-dependent manner over a 4-day period (figure 4A). With 10 mg/kg of VIB9600, no free surface FcγRIIA was detected on platelets and neutrophils through 48 hours (figure 4A), which is consistent with the presence of circulating VIB9600 at that time (figure 4B). In FcγRIIA transgenic models of autoimmunity, VIB9600 inhibited antiplatelet-induced thrombocytopaenia, neutrophil infiltration in acute antiglomerular basement membrane (GBM)-induced nephritis model and anticollagen Ab-induced arthritis (figure 4C–E). These data demonstrate a direct relationship between VIB9600 levels, FcγRIIA target engagement and efficacy in IgG antibody-mediated autoimmune disease models.

Pharmacokinetic and exploratory pharmacodynamic and GLP toxicity studies of VIB9600 in cynomolgus monkeys

To establish the pharmacokinetic/pharmacodynamic (PK/PD) characteristics of VIB9600, a single ascending dose study was conducted in cynomolgus monkeys (online supplementary table 2). The non-compartmental analysis indicated that the terminal half-lives for VIB9600 were 1.08–1.2 days in the 1 mg/kg group, increasing to 2.84–4.3 days in the 100 mg/kg group (figure 5A). The tendency for half-life to increase with dose is consistent with target-mediated elimination of the antibody. The PD characteristics of VIB9600 were measured by flow cytometry to assess free FcγRIIA on the surface of cells. A single-dose of VIB9600 induced a dose responsive and completely reversible reduction of FcγRIIA on monocytes ($CD45^+$, $CD14^+$) and granulocytes (side scatter/$CD45^+$) (figure 5B,C). Notably, the rapid suppression and slower recovery of FcγRIIA expression mirrored the PK profile of VIB9600. These data indicate a strong and direct relationship between PK exposure and PD response in the form of FcγRIIA expression (figure 5A–C).

To assess the safety of VIB9600, we conducted a 3-month subcutaneous and intravenous GLP toxicity study (13 weekly doses up to 100 mg/kg and an 8 week follow-up, online supplementary table 2). This GLP safety study did not result in any changes in body weight or platelet counts, and FcγRIIA levels returned to predose levels by the end of the study period (online supplementary figure 2). In addition, no adverse effects on other haematology, clinical chemistry, organ weight or histopathology were noted (data not shown).

DISCUSSION

IC and autoantibodies targeting specific cells and tissues can engage Fc-bearing immune cells and drive leucocyte recruitment and local tissue pathology. We proposed that suppressing IC-mediated cellular activation by inhibiting FcγRIIA would provide an alternate therapeutic approach for the treatment of autoimmune conditions. Herein, we generated VIB9600, a humanised, optimised, anti-FcγRIIA antibody with null effector function that potently inhibits IC-mediated responses from multiple cell types that are believed to play a critical role in autoimmune diseases. In vivo studies demonstrate a direct relationship between VIB9600 PK and target engagement, and safety assessments in vitro and in non-human primates support its clinical development.

To the best of our knowledge, no molecules directly targeting FcγRIIA have entered clinical development. Targeted therapeutic efforts that interfere with IC-mediated activation of FcγR have recently emerged. Recombinant FcγRIIB (SM101), which has the potential to sequester ICs, showed some efficacy in phase II clinical trials in immune thrombocytopenic purpura (ITP) and SLE.[32] In addition, an anti-FcγRIIB Ab (SM201) has been designed to recruit FcγRIIB to trigger and enhance inhibitory signals.[32 33] M230, a recombinant trivalent-IgG-Fc engineered for increased binding to FcγRs, was also reported to block Fc-mediated IC activation in preclinical studies.[34] Potentially, M230 could block interactions with all FcγR, so its pharmacology and impact on the clearance of IC and opsonised pathogens will be of interest. FcγRs mediate phagocytosis of large IgG-coated particles and pinocytosis of soluble IgG ICs.[11 35 36] It has been shown that neutrophil FcγRIIIB plays a dominant role in the homeostatic clearance of soluble ICs,[36] but it remains uncertain what impact the complete blockade

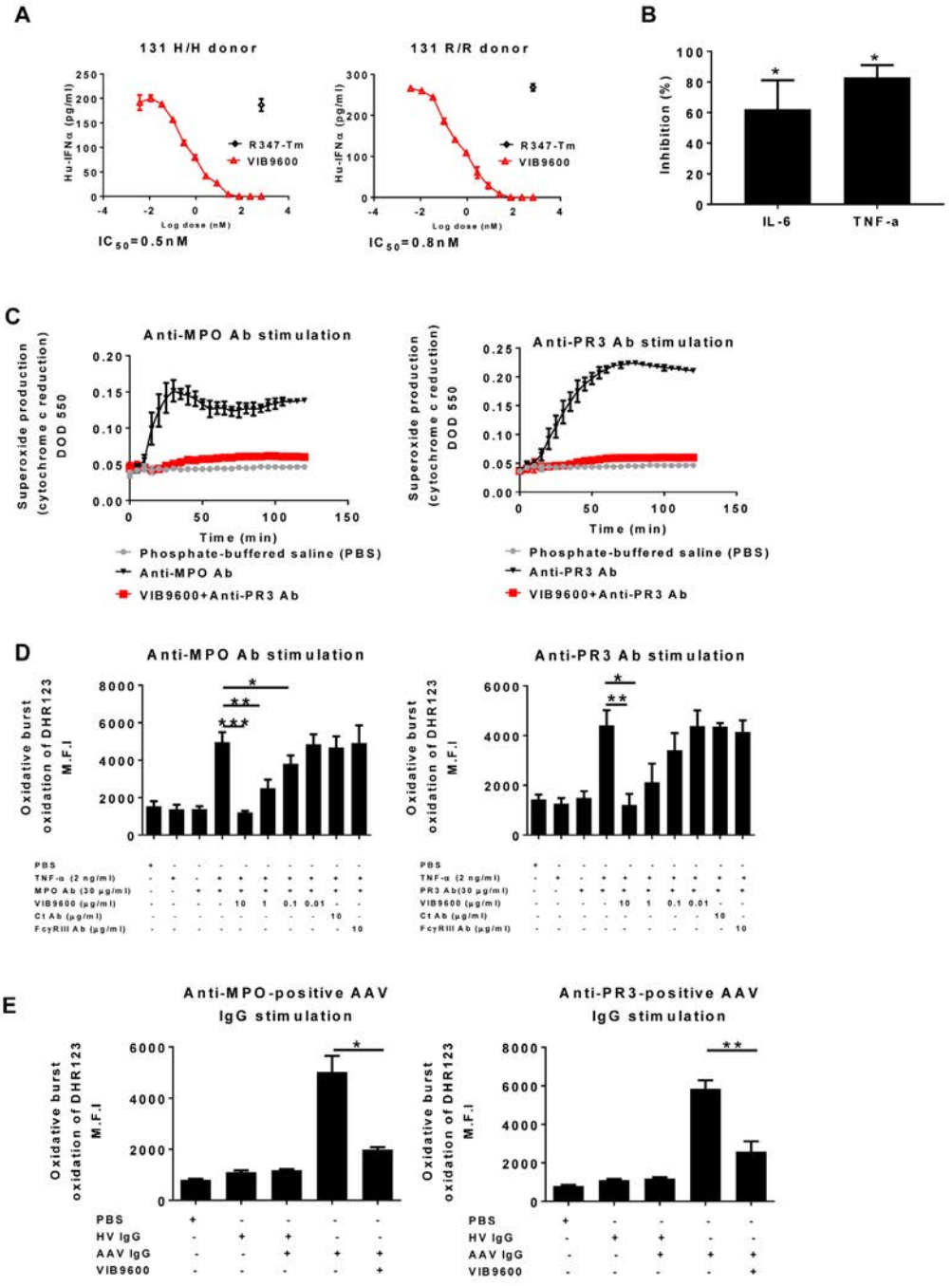

Figure 2 VIB9600 blocks autoantibody/IC-mediated inflammatory responses. (A) VIB9600 inhibited RNP-IC induced IFNα protein produced from human PBMC. Representative dose response curve from three independent experiments with human 131 H/H and 131 R/R donors are presented. (B) VIB9600 (30 µg/mL) inhibited Ig-IC-induced TNF-α and IL-6 protein in whole blood. Mean±SD percentage inhibition relative to no antibody are presented. *P<0.05, paired Student t-test. (C) VIB9600 inhibition of ANCA-induced neutrophil superoxide production measured by ferri-cytochrome C reduction assay. Human neutrophils were primed with 2 ng/mL TNF-α, with or without VIB9600 and stimulated with anti-MPO (left) or anti-PR3 Ab (right): data represent the mean±SD (n=4 replicates) of ΔOD550–490 values. Representative plots from two independent experiments are presented. (D–E) Effect of VIB9600 blockage on ANCA-induced neutrophil activation in a DHR123 assay. (D) Experiment showing the oxidative burst of neutrophils activation from TNF-α-primed human neutrophils stimulated with an anti-MPO antibody (left) or an anti-PR3 antibody (right) and treated with the indicated reagents. Oxidation of DHR123 was measured by flow cytometry, and the data show changes in M.F.I. Data were generated from three independent experiments. Error bars represent the mean±SD. (E) Left: experiment showing the oxidative burst from TNF-α-primed human neutrophils stimulated with IgG isolated from AAV anti-MPO-positive patient sera and IgG isolated from healthy volunteer (HV) sera with and without VIB9600. Right: same experiment with IgG isolated from AAV anti-PR3-positive patient sera. DHR123 oxidation was measured by flow cytometry; data show changes in M.F.I. Data were generated from three independent experiments. Error bars represent the mean±SD. *P<0.05, **p<0.01, ***p<0.001, paired Student t-test. AAV, ANCA-associated vasculitis; Ab, ANCA, antineutrophil cytoplasmic antibody; IL-6, interleukin-6; IC, immune complex; RNP-IC, ribonucleoprotein IC; Tm, triple mutation; TNF-α, tumour necrosis factor-α.

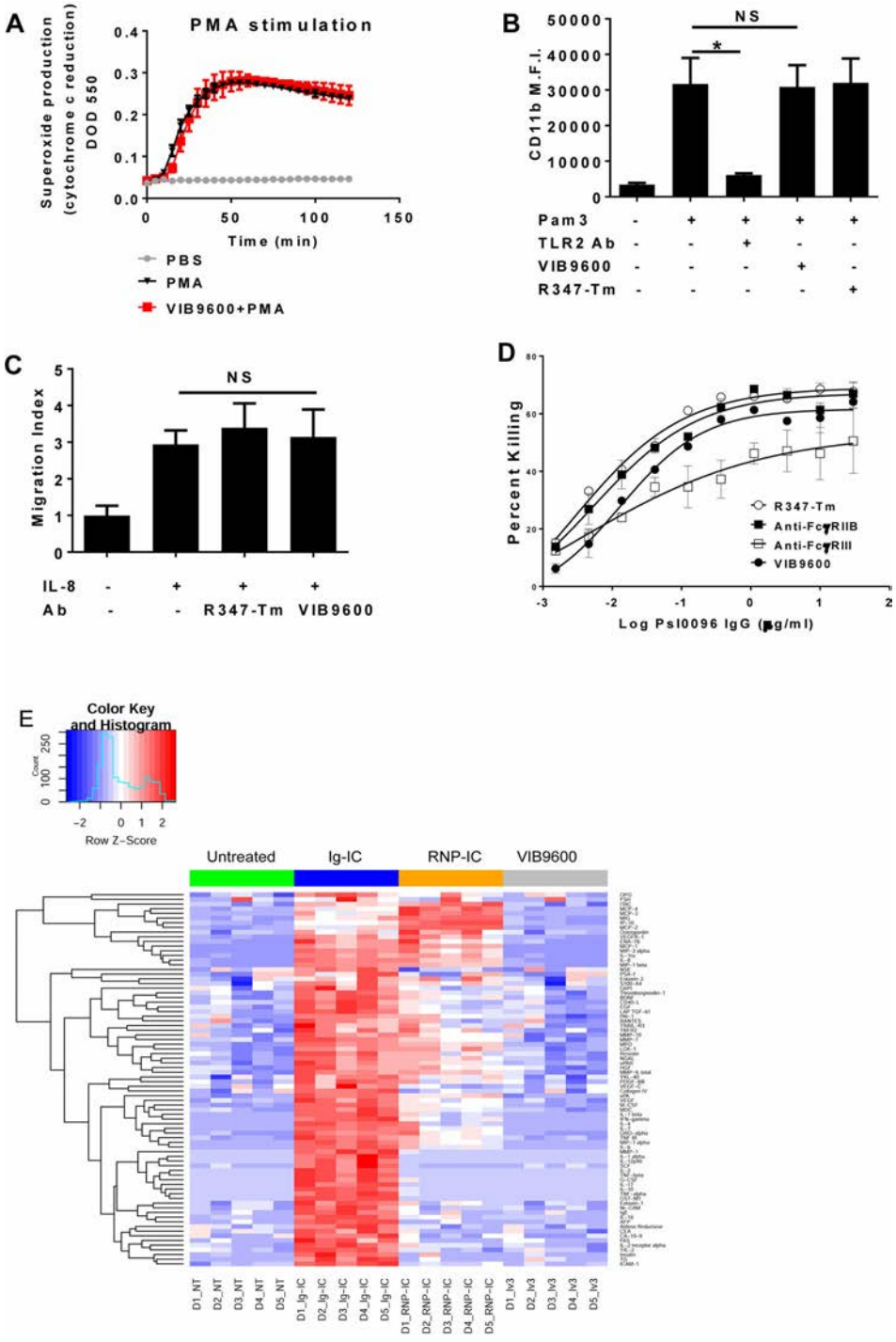

Figure 3 Blockade of FcγRIIA by VIB9600 has no adverse effects on neutrophil function and has no impact on protein expression in a whole blood proteomic assessment. (A) Effect of VIB9600 on PMA-induced reactive oxygen species production measured by ferri-cytochrome c reduction assay. $\Delta OD_{550\text{-}490}$ values obtained from TNF-α-primed human neutrophils stimulated with PBS, PMA or VIB9600+PMA. A representative plot of two independent experiments is shown. Error bars represent the mean±SD from one experiment. (n=4 replicates). (B) Effect of VIB9600 on neutrophil activation. M.F.I values for the cell surface expression of CD11b which is the indication of neutrophils activation from human neutrophils treated with the indicated reagents. Error bars represent the mean±SD from three independent experiments. (C) Effect of VIB9600 on neutrophil migration. Migration index values (the ratio of the number of cells that migrated in response to the reagent versus the number that migrated without it) obtained from human neutrophils treated with the indicated reagents. Error bars represent the mean±SD from three independent experiments. (D) Effect of VIB9600 on antibody-mediated phagocytosis. representative data of three independent opsonophagocytic killing assays. VIB9600, anti-FcγRIIB mAb or anti-FcγRIII mAb was preincubated with neutrophils. Dilutions of the anti-Psl antibody Psl0096, complement and luminescent bacteria were then added to each well and incubated for 120 min at 37°C. Relative luciferase units (RLU) were measured. The percent killing of *Pseudomonas aeruginosa* was calculated using the following formula: % Killing=100−([RLU experimental wells/RLU control wells]×100). Error bars represent the mean±SD. (E) Effect of VIB9600 in whole blood. There are five individual donors (each column) in each treatment group. Data were z-score transformed, and heatmaps were generated in R using the heatmap.2 function of the gplots package. Samples were clustered by condition, although the protein clustering structure was unsupervised. Ab, RNP-IC, ribonucleoprotien-immune complex.

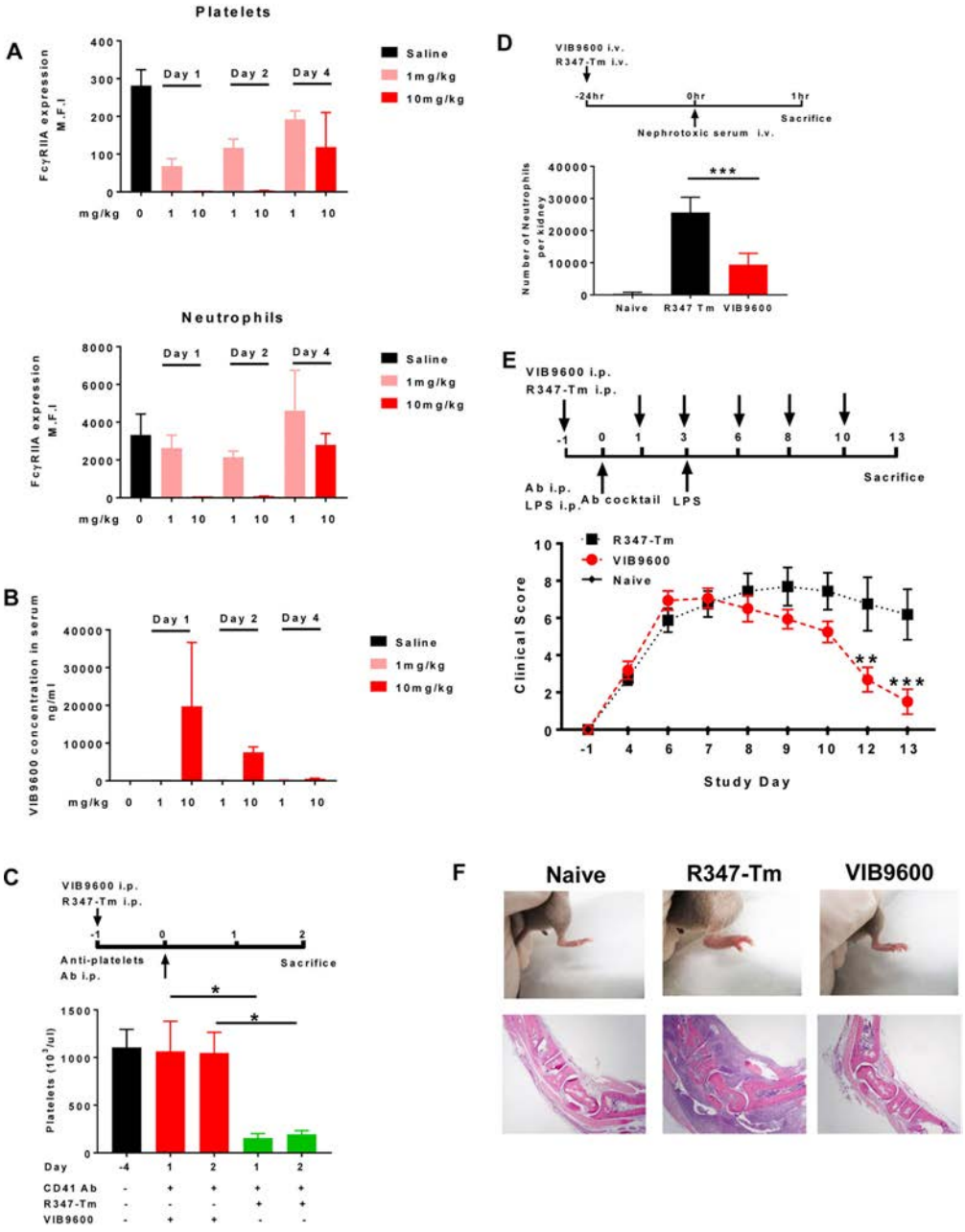

Figure 4 VIB9600 suppressed the FcγRIIA and antibody-mediated pathology in FcγRIIA transgenic mice. (A+B) The pharmacology of VIB9600 was assessed in FcγRIIA transgenic mice. Mice were treated with 1 mg/kg or 10 mg/kg VIB9600 by intraperitoneal at day 0, blood was collected at day 1, day 2 and day 4 postinjection. (A) Free FcγRIIA on platelets and neutrophils in FcγRIIA transgenic mice was measured by flow cytometry at 24, 48 and 96 hours after a single 1 mg/kg or 10 mg/kg intraperitoneal dose of VIB9600. (B) Serum concentrations of VIB9600 were measured by human IgG ELISA at 24, 48 and 96 hours after 1 mg/kg or 10 mg/kg intraperitoneal dose of VIB9600. (C) Effect of VIB9600 in FcγRIIA transgenic model of antibody-induced thrombocytopaenia. VIB9600 or control Ab (R347-Tm) was injected intraperitoneal at 10 mg/kg 24 hours (day 1) before thrombocytopaenia was induced with 2 µg rat antimouse CD41Ab delivered intraperitoneal at day 0, platelets numbers were determined at day 4 (baseline) and following induction of thrombocytopaenia (day 1 and day 2). A representative plot of two independent experiments is shown. Error bars represent the mean±SD from one experiment. (n=3 mice/group). *P<0.05 by unpaired Student's t-test. (D) Effect of VIB9600 in FcγRIIA-mediated neutrophil infiltration in an acute model of anti-glomerular basement membrane (aGBM) nephritis. Transgenic mice with FcγRIIA expression selectively in neutrophils of mice lacking endogenous murine receptors were given 20 mg/kg of VIB9600 or isotype control by intravenous injection 24 hours before intravenous injection of nephrotoxic serum (see timeline). Mice were euthanised, and kidneys and blood were collected for FACS analysis. Infiltrating renal neutrophils per kidney were quantitated by flow cytometry. naïve mice with no treatment were also euthanised and analysed. Bar graph represents mean±SD for nine animals in each treated group and four animals in the naïve, untreated group. ***P<0.001 by unpaired Student's t-test. (E–F) Effect of VIB9600 in FcγRIIA transgenic model of anticollagen Ab-induced arthritis. (E) VIB9600 or control Ab (R347-Tm) was injected intraperitoneal at 20 mg/mL at day -1,1,3,6,8 and 10, arthritis was induced with intraperitoneal. Delivery of 2 mg anticollagen Ab cocktail at day 0 and 10 µg lipopolysaccharide (LPS) at day 3. Arthritis was evaluated by clinical score at indicated timepoint. Error bars represent the mean±SD (n=8 mice/group). Two-way analysis of variance analysis, **P<0.01, ***p<0.001. (F) Top panel: representative image of hind paws at day 13 after the initial injection of anticollagen Ab cocktail. (F) Bottom panel: photomicrography analysis of H&E stained tissue sections from tarsal joint obtained from representative mice. 4X obj. Ab, antibody.

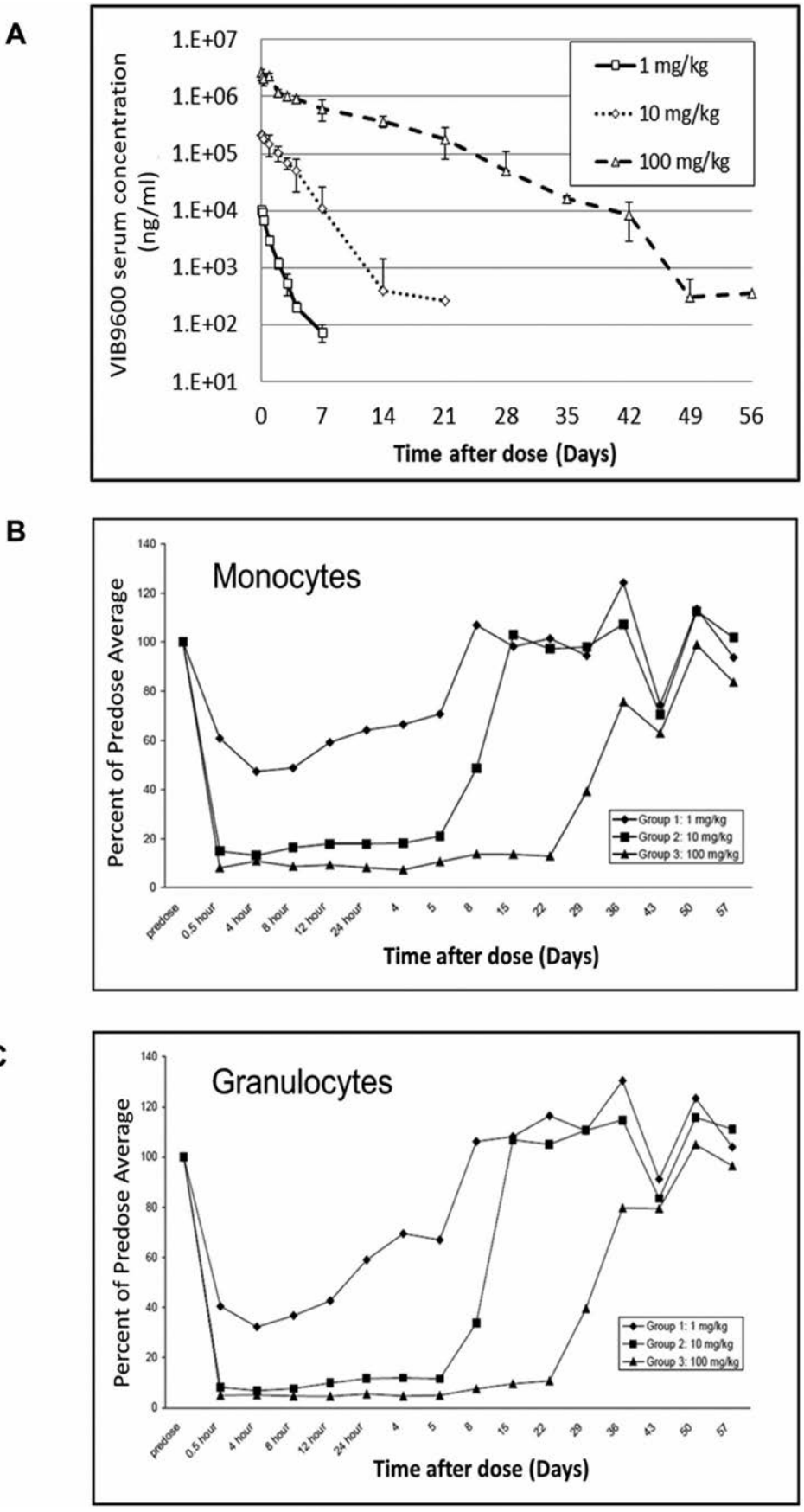

Figure 5 Single-dose pharmacokinetic and exploratory pharmacodynamic study of VIB9600 in cynomolgus monkeys. Male monkeys were given VIB9600 once at 1, 10 or 100 mg/kg in a volume of 2 mL/kg via intravenous injection. (A) Serum concentration of VIB9600 in cynomolgus monkeys at various time points after a single dose was determined by ELISA. (B) and (C) FcγRIIA levels on monocytes and neutrophils were determined at different time points by flow cytometry. The FcγRIIA levels (average percentage) relative to the mean predose levels are shown.

of FcγRs will have on the clearance of ICs. Since VIB9600 only inhibits FcγRIIA, it is predicted that other FcγRs and the complement system should adequately remove complexes from the circulation.

There remains a critical need for safe and efficacious drugs in autoimmune disease. In SLE, the only approved drug in the last 60 years, belimumab, an antibody antagonist of the B cell growth and differentiation factor BLYS, has only a modest response compared with standard of care.[37 38] We demonstrate that VIB9600 potently inhibits the induction of type I IFNs by pDCs stimulated with RNA-containing IgG complexes. A type I IFN gene signature is prominent in ~75% of patients with SLE, and a recent successful double-blinded phase II clinical trial targeting interferon-alpha/beta receptor alpha chain (IFNAR1) demonstrates the importance of this pathway.[39 40] Besides blocking IC-mediated induction of type I IFNs by pDC, targeting FcγRIIA with VIB9600 will also inhibit IC-mediated induction of other inflammatory mediates from antigen-presenting cells, granulocytes and platelets.[41] IC-mediated activation of FcγRIIA on neutrophils has also been reported to trigger formation of neutrophil extracellular traps and promote autoimmunity by providing an immunogenic source of autoantigens.[36] Therefore, it is tempting to speculate that VIB9600 could provide a greater benefit in SLE than current therapeutics.

In AAV, antibodies targeting cytoplasmic antigens MPO and PR3 exposed on the surface of neutrophils can trigger endothelial adhesion, degranulation and the release of proteolytic enzymes and ROS which drive vascular injury.[19–21] VIB9600 potently suppressed ANCA-induced neutrophil activation and the production of ROS. Importantly, despite targeting a neutrophil cell surface receptor (FcγRIIA), VIB9600, did not inappropriately activate neutrophils nor block other neutrophil functions. Current treatments for AAV include cyclophosphamide or rituximab in combination with steroids. High-dose steroids drive significant morbidity and repeated cycles of cyclophosphamide are contraindicated. Rituximab induces remission in about 60% of treated patients,[42] but it remains uncertain to what extent this effect is driven by steroids, and the relapse rate during the first year after induction remains high. Interestingly, data from rituximab in ANCA-associated vasculitis trial indicated differential response rates for the different FcγRIIA 131 H/R alleles.[43] This implies that FcγRIIA plays a critical role for in the disease pathogenesis, and importantly VIB9600 binds and inhibits both allelic variants similarly. If blockade and internalisation of FcγRIIA translate to a more rapid and sustained clinical response particularly if the use of steroids could be reduced, VIB9600 may provide clinical advantages.

The demonstration that VIB9600 can inhibit IC-mediated induction of TNFα and IL-6 among other proinflammatory molecules would suggest that VIB9600 may provide an alternative treatment option for RA. Treatments targeting the TNFα or IL-6 pathways have been approved for RA highlighting the importance of these cytokines in the pathology of the disease.[44 45] Although it is unclear to what extent the production of these cytokines is driven by FcγRIIA activation in RA, it is tempting to speculate that targeting FcγRIIA could be a more beneficial therapeutic approach than targeting downstream cytokines individually.

Besides the compelling evidence that VIB9600 can block key IC-mediated inflammatory responses in multiple human cells, we demonstrate that VIB9600 blocks antiplatelet-mediated thrombocytopaenia and anticollagen Ab-induced arthritis in FcγRIIA transgenic mice. This is consistent with previous in vivo studies that established the balance of activating and inhibitory FcγRs is important in triggering autoimmune disease,[46] experimental models of ITP, RA and haemolytic anaemia,[47–49] VIB9600 also reduced neutrophil accumulation in anti-GBM nephritis, which is consistent with the role of neutrophil FcγRIIA in the pathogenesis of glomerulonephritis[50] However, the individual expression profiles and functions of FcγR differ so significantly between humans and mice, that the role of FcγRIIA in human autoimmune diseases may only be faithfully assessed in human clinical trials.

Taken together, there remains a significant unmet need in SLE, AAV, RA and other IC-mediated and antibody-mediated autoimmune conditions for safe, fast-acting efficacious drugs that have durable effects and can significantly reduce corticosteroid usage. There is strong rationale for targeting FcγRIIA in these diseases, and VIB9600 may provide a first-in-class treatment option. VIB9600 potently inhibits FcγRIIA-mediated responses, and preclinical pharmacology and safety assessments support its clinical development to assess its efficacy in autoimmune diseases.

METHODS

Binding affinity and specificity were determined by Biacore and ELISA. Confocal microscopy, FACS-based assays and binding competition assays were used to assess the antibody mode-of-action. ADCC and CDC assays were performed using human embryo kidney (HEK)-293 stably transfected FcγRIIA cells. IC-induced ROS was measured using ferri-cytochrome C reduction and oxidation of DHR123 assays. Pam3CSK4 (TLR2)-induced neutrophil activation was assessed by CD11b upregulation. Cell migration was measured using a 96-well Chemo TX system. OPK of *P. aeruginosa* with PsI antibody PsI0096 was assessed with luminescent *P. aeruginosa* cells. FcγRIIA cross-linking was assessed in whole blood from heparin tubes, and following stimulation with VIB9600 or IC, protein analytes were measured using myriad multianalyte profiling technology platform. Target suppression was examined in FcγRIIA transgenic (B6: SJL-Tg (FcγRIIA)11Mkz/J) mice from Jackson Laboratory, and cynomolgus monkeys using a FACS-based receptor occupancy assay. In vivo efficacy was assessed using anti-CD41 Ab immune-mediated thrombocytopaenia model, acute anti-GBM nephritis model and anticollagen Ab-induced arthritis model in FcγRIIA transgenic mice. Further details of these assays are available in supplementary materials.

Statistical analysis

The statistical significance of differences between two groups was analysed using Student's *t*-test. Statistical significance was ascribed to the data when $p<0.05$.

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Competing interests MedImmune employees hold stock in AstraZeneca. Shu Wang is the emploee of the Viela Bio. Viela Bio is the sole owner of VIB9600. Bing Yao (YaoB@vielabio.com) is the CEO of Viela Bio and VIB9600 is in clinical development.

Patient consent Not required.

Ethics approval Blood from healthy volunteers was obtained with informed consent under MedImmune, LLC's blood donation program, and studies using human cells were performed in accordance with the Institutional Review Board guidelines. For animal studies, all procedures were performed in accordance with federal, state and institutional guidelines in an AAALAC-accredited facility and were approved by the MedImmune Institutional and The Brigham and Women's Hospital Animal Care and Use Committee.

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TRANSLATIONAL SCIENCE

REDD1/autophagy pathway promotes thromboinflammation and fibrosis in human systemic lupus erythematosus (SLE) through NETs decorated with tissue factor (TF) and interleukin-17A (IL-17A)

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ABSTRACT

Objectives The release of neutrophil extracellular traps (NETs) represents a novel neutrophil effector function in systemic lupus erythematosus (SLE) pathogenesis. However, the molecular mechanism underlying NET release and how NETs mediate end-organ injury in SLE remain elusive.

Methods NET formation and NET-related proteins were assessed in the peripheral blood and biopsies from discoid lupus and proliferative nephritis, using immunofluorescence, immunoblotting, quantitative PCR and ELISA. Autophagy was assessed by immunofluorescence and immunoblotting. The functional effects of NETs in vitro were assessed in a primary fibroblast culture.

Results Neutrophils from patients with active SLE exhibited increased basal autophagy levels leading to enhanced NET release, which was inhibited in vitro by hydroxychloroquine. NETosis in SLE neutrophils correlated with increased expression of the stress-response protein REDD1. Endothelin-1 (ET-1) and hypoxia-inducible factor-1α (HIF-1α) were key mediators of REDD1-driven NETs as demonstrated by their inhibition with bosentan and L-ascorbic acid, respectively. SLE NETs were decorated with tissue factor (TF) and interleukin-17A (IL-17A), which promoted thrombin generation and the fibrotic potential of cultured skin fibroblasts. Notably, TF-bearing and IL-17A-bearing NETs were abundant in discoid skin lesions and in the glomerular and tubulointerstitial compartment of proliferative nephritis biopsy specimens.

Conclusions Our data suggest the involvement of REDD1/autophagy/NET axis in end-organ injury and fibrosis in SLE, a likely candidate for repositioning of existing drugs for SLE therapy. Autophagy-mediated release of TF-bearing and IL-17A-bearing NETs provides a link between thromboinflammation and fibrosis in SLE and may account for the salutary effects of hydroxychloroquine.

Key messages

What is already known about this subject?

- In systemic lupus erythematosus (SLE), neutrophils display excessive cell death by forming extracellular chromatin traps (the so-called neutrophil extracellular traps (NETs)) but the mechanism underlying their release and the resultant tissue injury are not known.

What does this study add?

- Excessive NET production by SLE neutrophils is driven by autophagy, a process normally involved in degradation and recycling of cellular components.
- Lupus serum induces in neutrophils autophagy and NETosis by upregulating the hypoxia and stress-response protein DDIT4/REDD1.
- NETs from active SLE neutrophils show abundant expression of bioactive tissue factor and interleukin-17A, which promote thromboinflammation and fibrosis in target tissues such as kidneys and skin.
- Endothelin-1 and hypoxia inducible factor-1α are key mediators of neutrophil-driven end-organ injury in SLE, through the REDD1/autophagy axis.

How might this impact on clinical practice?

- Targeting the REDD1/autophagy axis or its mediators by existing agents through drug repositioning or other novel agents may alleviate neutrophil-mediated inflammation in SLE.

INTRODUCTION

Genome-wide association studies and gene expression analyses have implicated neutrophils and deregulated autophagy in systemic lupus erythematosus (SLE).[1–7] Specifically, neutrophils have emerged as key players in the disease pathogenesis through putative effector functions including neutrophil extracellular traps (NETs).[8] NETs are networks of extracellular fibres, comprised of extruded nuclear DNA and associated granular components, histones and cytoplasmic proteins.[9] However, the molecular mechanism underlying NET release and how NETs mediate end-organ injury in SLE are unknown.

Recent data suggest that the autophagic pathway—a homeostatic catabolic mechanism

involving degradation of cell components—may be required for NETs release.[10 11] NETs represent a common denominator across different disorders; however, depending on the inflammatory context of each pathophysiological condition, neutrophils may express and release through NETs distinct bioactive proteins involved in different biological processes.[12] To this end, the protein composition of NETs in SLE and their contribution to tissue injury have not been explored. Recently, focus has shifted on the role of NETs-expressing tissue factor (TF), the main in vivo initiator of the coagulation, as a mediator of thromboinflammation.[11] In addition, interleukin-17A (IL-17A), a proinflammatory cytokine implicated in SLE and lupus nephritis (LN),[13] promotes NET-dependent lung fibrosis.[14]

Although the presence of NETs in SLE has been associated with type I interferon production and vasculopathy,[15 16] the underlying mechanism that regulates their release and their role in SLE inflammation and fibrosis remain unknown. Here in, we demonstrate that the inflammatory microenvironment of active SLE upregulates the expression of hypoxia-response and stress-response protein DDIT4/REDD1 (herein after referred as REDD1) in neutrophils, leading to autophagy induction and formation of TF-decorated and IL-17A-decorated NETs. We also demonstrate thromboinflammatory NETs in kidney and skin sections derived from patients with active SLE, linking them with end-organ injury and fibrosis.

MATERIALS AND METHODS

Patients and sampling

Peripheral blood neutrophils and sera were isolated from six patients with SLE during active (SLEDAI-2K>8) and then inactive (SLEDAI-2K<3) disease. SLE was diagnosed according to the 1997 Update of the 1982 American College of Rheumatology classification criteria.[17] Six sex-matched/age-matched healthy individuals served as controls (online supplementary table 1). Kidney biopsies were obtained from six patients with active proliferative LN. Kidney biopsies, obtained from a patient with renal carcinoma, a patient with minimal change disease and a patient with membranous nephropathy served as controls. Skin biopsies were obtained from three patients with active discoid lupus, both from the active lesion and the normal skin of each patient. Unaffected skin tissue from three healthy individuals was used as control. Human skin specimens from healthy individuals were used to isolate primary human skin fibroblast (HSFs).[18] Written informed consent was obtained from all participants. The study protocol was in accordance with the Helsinki Declaration. Detailed information for all methods can be found in the online supplementary materials and methods. Results from each patient are provided in online supplementary figures 8–11.

RESULTS

Increased basal autophagy of peripheral neutrophils from patients with SLE is correlated with NETosis

Patients with SLE are characterised by a strong neutrophil and deregulated autophagy gene signature.[3–7 19] Since SLE neutrophils exhibit ex vivo increased NET release[15 20–22] and autophagy is a key mechanism regulating NET generation,[10 11] we assessed autophagy levels in ex vivo isolated, unstimulated neutrophils from patients with SLE. Neutrophils from patients with active SLE (active SLE neutrophils) demonstrated increased basal autophagy levels as compared with neutrophils from healthy individuals (control neutrophils) and patients with inactive SLE (inactive SLE neutrophils), as evidenced by immunofluorescence for the autophagy protein LC3B (figure 1A,B) and immunoblotting for both the lipidated LC3B-II (figure 1C) and the consumption of p62/SQSTM1 (figure 1D).

To address whether increased autophagy levels may be driven by inflammatory mediators present in SLE serum, control neutrophils were stimulated in vitro with serum derived from patients with active (active SLE serum) or inactive (inactive SLE serum) SLE. Active SLE serum induced increased autophagy levels in contrast to inactive SLE serum, as demonstrated by immunofluorescence for LC3B (online supplementary figure 1A) and diminished p62/SQSTM1 immunoblotting (online supplementary figure 1B).

Next, we observed that active SLE neutrophils exhibited increased NET release compared with control or inactive SLE neutrophils (figure 2A,B), myeloperoxidase (MPO)-DNA complex ELISA in ex vivo cell culture supernatants (figure 2C) and MPO-DNA complex ELISA measured directly in serum derived from patients with SLE (figure 2D). Inhibition of autophagy with early-stage or late-stage autophagy inhibitors, wortmannin or hydroxychloroquine (HCQ), respectively, significantly attenuated this effect (figure 2A–C). Similar findings were observed in control neutrophils cultured with active SLE serum in the presence of autophagy inhibitors (wortmannin, HCQ, AKT activator or Bafilomycin A1) (online supplementary figure 2A-C) or with low dose of rapamycin (online supplementary figure 2C). Together, these findings suggest that lupus inflammatory microenvironment induces NETs in an autophagy-dependent manner, which can be reversed by autophagy inhibitors.

NETs from patients with active SLE are decorated with TF and IL-17A

The protein composition of NETs plays a crucial role in disease pathogenesis.[12] We focused on two inflammatory mediators which have been previously implicated in NET-associated inflammatory injury, namely, TF and IL-17A. TF/thrombin axis has a key role in thromboinflammation[11 23–25] which often complicates SLE.[26] IL-17A is a proinflammatory cytokine implicated in SLE pathogenesis and severe LN.[27–30] Indeed, we have demonstrated that IL-17A-bearing NETs have a potent fibrotic role.[14] Accordingly, we investigated whether TF and IL-17A are externalised via SLE NETs and could represent a link between increased thrombogenicity and fibrosis observed in the disease.

We observed that NETs from patients with active SLE (active SLE NETs) expressed TF (figure 3A,B) and IL-17A (figure 3C,D), as assessed by immunofluorescence and immunoblotting on NET structures. Importantly, TF on active SLE NETs was bioactive, since NET structures increased thrombin levels in healthy platelet-poor plasma, as assessed by thrombin-antithrombin assay (figure 3E). This effect was TF-dependent as shown by inhibition of TF with an anti-TF neutralising antibody (figure 3E). NETs from patients with inactive SLE (inactive SLE NETs) demonstrated reduced thrombin generation when compared with active SLE NETs (figure 3E). These findings were further supported in vitro, where active SLE serum upregulated intracellular *TF* and *IL17A* mRNA expression in control neutrophils (online supplementary figure 3A-B) and induced NETs bearing functional TF (online supplementary figure 3C-D) and IL-17A (online supplementary figure 3E). Thus, the inflammatory microenvironment of SLE induces NETs decorated with TF and IL-17A.

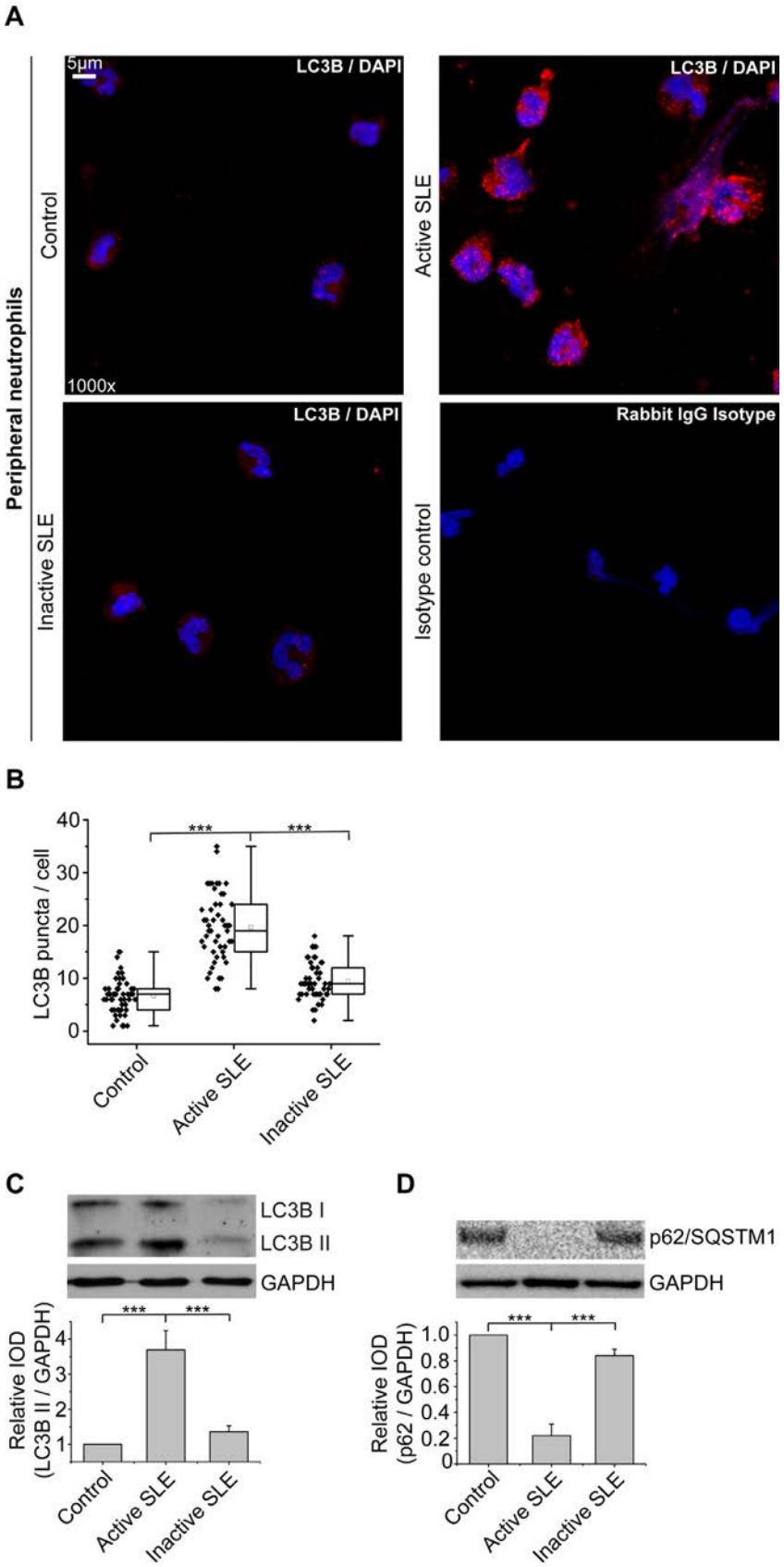

Figure 1 Peripheral blood neutrophils isolated from patients with active systemic lupus erythematosus (SLE) are characterised by increased basal autophagy levels. (A) Autophagy induction assessed with LC3B staining (confocal microscopy; red: LC3B, blue: 4′,6-diamidino-2-phenylindole (DAPI)/DNA, 45 min incubation) in active SLE neutrophils compared with inactive SLE or control neutrophils. (B) LC3B puncta/cell are depicted. (C) LC3B-I/II (45 min incubation) and (D) p62/SQSTM1 (90 min incubation) immunoblotting. For (C) and (D) integrated optical density (IOD) ratio of LC3BII/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and p62/GAPDH relative to control. For (B)–(D), data presented as mean±SD, ***p<0.001. For (A)–(D), one representative experiment of 6 is shown, n=6 patients.

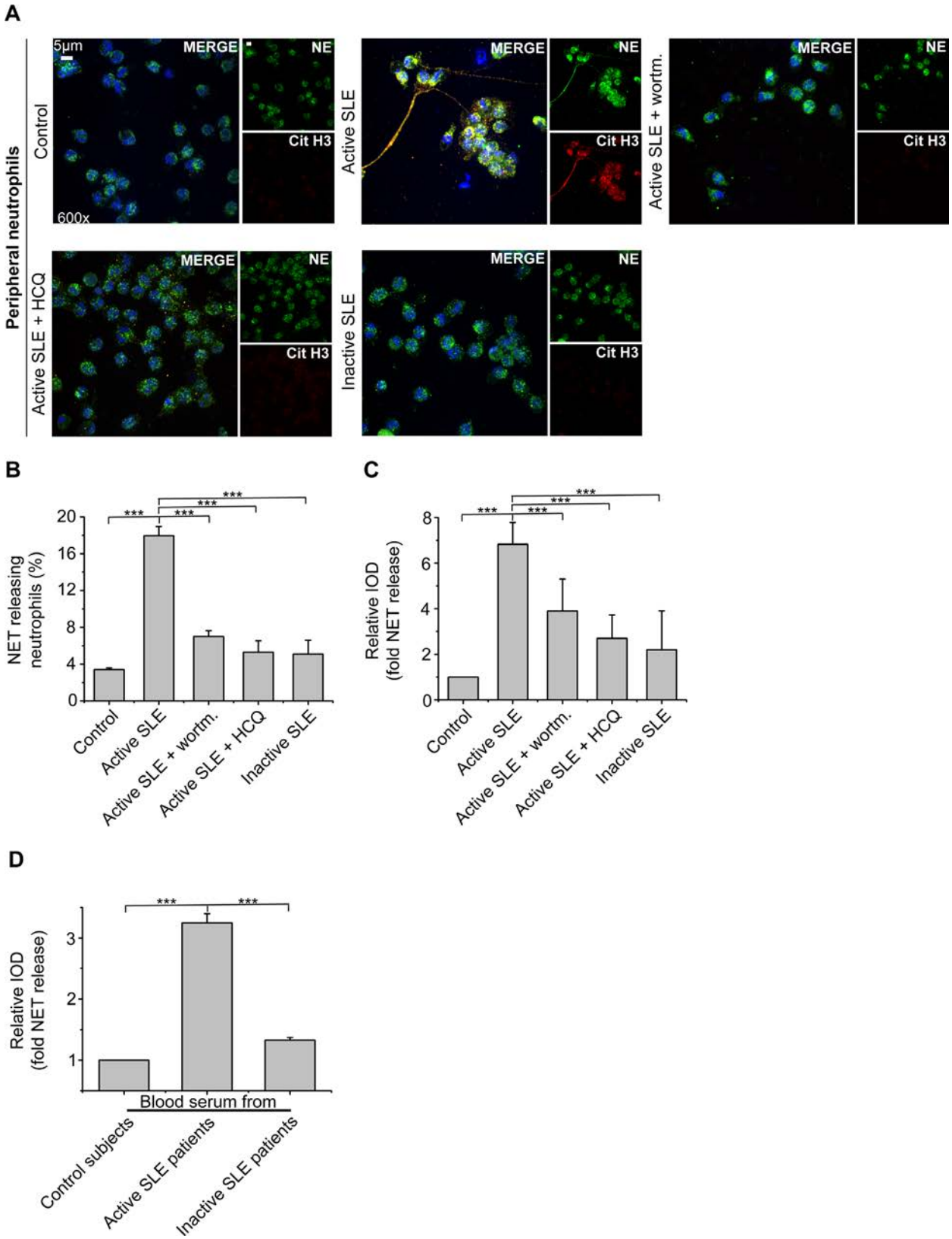

Figure 2 Neutrophil extracellular trap (NET) formation is increased in peripheral blood neutrophils of patients with active systemic lupus erythematosus (SLE) and is mediated by autophagy. (A) NET formation (3 hours incubation) in isolated peripheral neutrophils from patients with active SLE compared with inactive SLE or control subjects. Wortmannin (30 min pretreatment) was used as an early stage autophagy inhibitor, while hydroxychloroquine (HCQ, 30 min pr-treatment) as a late stage autophagy inhibitor (confocal microscopy; green: neutrophil elastase (NE), red: citrullinated histone H3 (CitH3), blue: 4′,6-diamidino-2-phenylindole/DNA). (B) Percentage of NET-releasing neutrophils (3 hours incubation). Myeloperoxidase-DNA complex measured (C) in NET structures released from patients' neutrophils or (D) directly in blood serum derived from patients with active SLE compared with patients with inactive SLE or control subjects. For (B)–(D), data presented as mean±SD, ***p<0.001. For (A)–(D), one representative experiment of 6 is shown, n=6 patients.

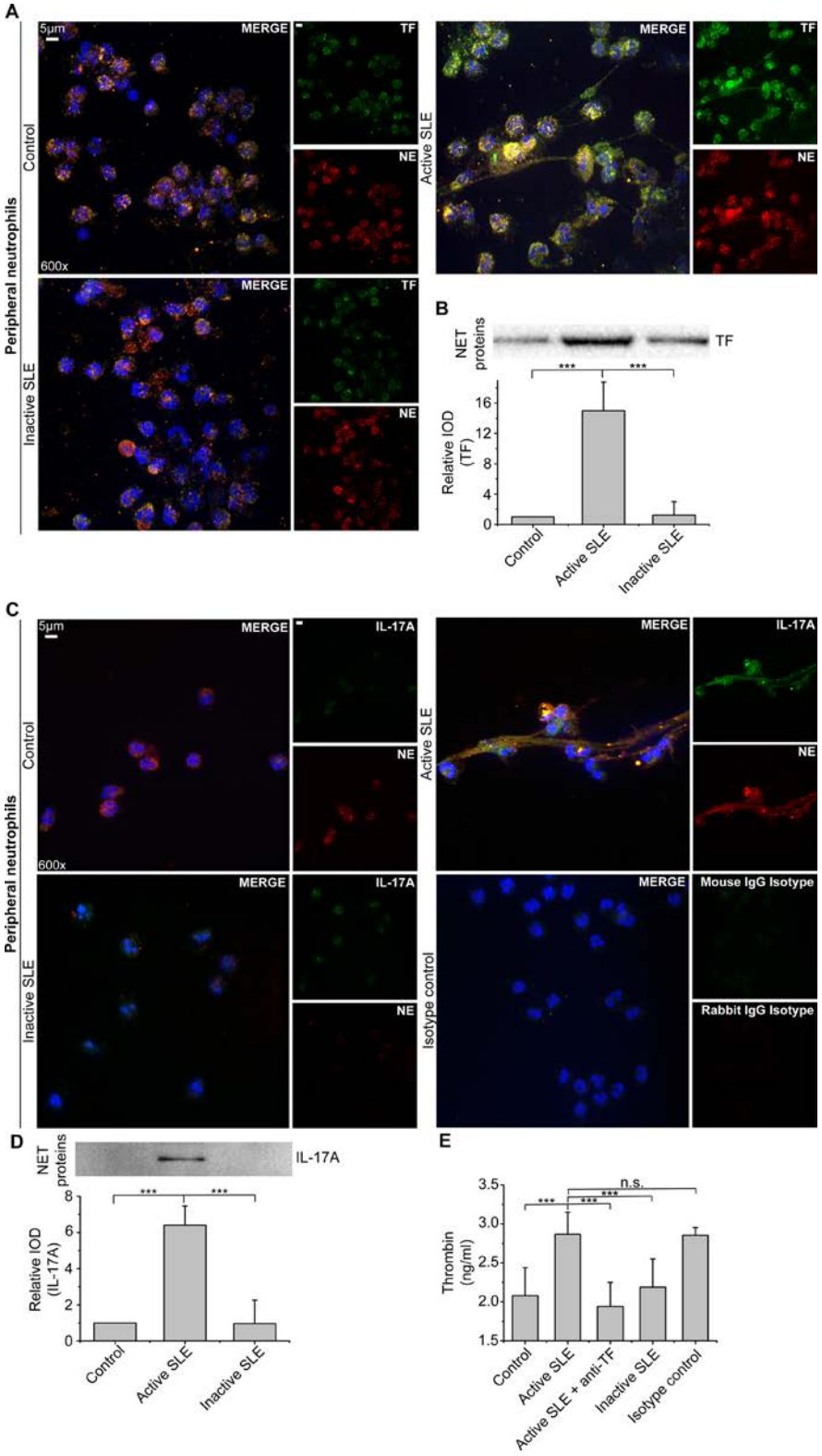

Figure 3 Patients with active systemic lupus erythematosus (SLE) release tissue factor (TF) and interleukin (IL)-17A-bearing neutrophil extracellular traps (NETs). (A) Localisation of TF on NETs released by isolated peripheral neutrophils from patients with active SLE compared with inactive SLE (confocal microscopy; green: TF, red: neutrophil elastase (NE), blue: 4′,6-diamidino-2-phenylindole (DAPI)/DNA). (B) TF expression in purified NET proteins from peripheral neutrophils from patients with active SLE. (C) Localisation of IL-17A on NETs released by isolated peripheral neutrophils from patients with active SLE compared with inactive SLE (confocal microscopy; green: IL-17A, red: NE, blue: DAPI/DNA) and (D) IL-17A expression in purified NET proteins. (E) Thrombin levels in control plasma incubated with NET structures (thrombin-antithrombin assay). Neutralising anti-TF antibody was used to inhibit TF-mediated thrombin generation. A mouse immunoglobulin (Ig)G antibody was used as isotype control. For (B) and (D), integrated optical density (IOD) relative to control, one representative experiment of 4 is shown, n=4 patients. For (B), (D), and (E), data presented as mean±SD, ***p<0.001. For (A), (C) and (E), one representative experiment of 6 is shown, n=6 patients. For (A)–(E), neutrophils were harvested after 3 hours of incubation.

Hypoxia-inducible factor-1α (HIF-1α) and endothelin-1 (ET-1) induce REDD1 expression in SLE neutrophils that activates the REDD1/autophagy pathway mediating NET release

Next, we sought to identify pathways that drive the autophagy-mediated NET release in SLE. Since we have recently shown that the stress-induced protein REDD1 regulates NET release through autophagy induction,[31] we examined the involvement of REDD1/autophagy pathway in the formation of SLE NETs. Active SLE neutrophils demonstrated increased REDD1 mRNA and protein expression as compared with control or inactive neutrophils (figure 4A,B). Active SLE serum was also able to induce REDD1 in control neutrophils (figure 4C,D).

To define the mechanism of increased REDD1 in SLE neutrophils, we focused on the upstream mediators HIF-1α and ET-1. HIF-1α, an oxygen sensitive transcription factor affecting numerous immune cells,[32] involved in the mechanistic target of rapamycin (mTOR) system[33] and an upstream regulator of REDD1 signalling[34] is expressed in the kidneys and is found increased in urine of patients with LN.[35–37] To this end, control neutrophils were pretreated with L-ascorbic acid, a HIF-1α inhibitor or a specific HIF-1α inhibitor ($C_{26}H_{29}NO_5$) resulting in significant reduction in REDD1 levels (figure 4C,D, online supplementary figure 4A), autophagy induction (figure 4E, online supplementary figure 4B) and subsequent NET release (figure 4F, online supplementary figure 4C). ET-1, a potent vasoconstrictor involved in the mTOR pathway,[38] is increased in SLE sera[39 40] and correlates with disease activity and LN.[41–43] Thus, control neutrophils were treated with the ET-1 receptor antagonist bosentan or a neutralising antibody against ET-1 prior to stimulation with active SLE serum. Both agents abolished REDD1 upregulation (figure 4C,D, online supplementary figure 4A), resulting in a significant reduction of autophagic levels (figure 4E, online supplementary figure 4B) and subsequent NET formation (figure 4F, online supplementary figure 4C). Importantly, recombinant ET-1 alone in concentration similar to that present in sera from patients with active SLE[39 40] neither upregulated REDD1 expression nor induced autophagy and NETs in control neutrophils (online supplementary figure 5A-C). However, combination of recombinant ET-1 with inactive SLE serum upregulated REDD1 expression, enhanced autophagy and led to NET release (online supplementary figure 5A-C) in control neutrophils, similar to stimulation with active SLE serum. These findings suggest that ET-1 may synergise with HIF-1α or/and other mediators within the lupus microenvironment to mediate the phenomenon.

Taken together, these data demonstrate the regulatory role of REDD1/autophagy pathway in the formation of NETs in SLE and point to ET-1 and HIF-1α as emerging key mediators of neutrophil-driven thromboinflammation in SLE, through REDD1 axis.

REDD1-mediated NETs bearing TF and IL-17A activate and differentiate human fibroblasts in vitro

To investigate the effect of TF-bearing and IL-17A-bearing NETs on tissue resident cells, human skin fibroblasts (HSF) were incubated with NET structures induced by active SLE serum (active SLE NETs). This stimulation resulted in the activation of fibroblasts, as evidenced by α-smooth muscle actin (α-SMA) mRNA (*ACTA2*) and protein overexpression (figure 5A,B), compared with untreated HSF. Treatment of HSF with active SLE NETs also enhanced CCN2 expression (figure 5A), a matricellular protein implicated in fibrosis, collagen production (figure 5C) and proliferation/migration rates (figure 5D,E).

Contrary to NETs, direct stimulation of HSF with active SLE serum did not induce their activation/differentiation towards a fibrotic phenotype (figure 5A), suggesting a specific effect of NETs. In the same context, dismantling of NETs with DNaseI or inhibition of autophagy with HCQ further abolished the fibrotic potential of HSF (figure 5A–E).

To confirm that HIF-1α and ET-1 are involved in REDD1-mediated active SLE NET release and subsequent HSF activation/differentiation, neutrophils were treated with either L-ascorbic acid or bosentan prior to incubation with active SLE serum. A significant attenuation of HSF activation/differentiation, collagen production and proliferation/migration rate were observed (figure 5A–E). A similar finding was also detected on treatment of active SLE serum with a specific HIF-1α inhibitor or with an ET-1 neutralising antibody (online supplementary figure 4D). Moreover, NETs mediated by the combination of recombinant ET-1 and inactive SLE serum induced enhanced collagen production by HSF (online supplementary figure 5D).

Next, we tried to elucidate the NET components responsible for the activation/differentiation of HSF and particularly the role of TF and IL-17A that were found to decorate active SLE NETs. As assessed by *ACTA2* expression, TF or IL-17A neutralisation on NETs did not mediate HSF activation/differentiation (figure 5A). Conversely, their neutralisation affected the fibrotic potential of HSF, as evidenced by the marked decrease in CCN2, collagen production and proliferation/migration rate (figure 5A–E). To further investigate TF-thrombin axis involvement and considering that thrombin signals through protease-activated receptors (PAR)-1, HSF were pretreated with FLLRN, a PAR-1-specific peptide. A significant reduction in the fibrotic potential of differentiated HSF was noted (figure 5C).

Collectively, these results demonstrate that active SLE NETs contribute to the activation/differentiation of HSF, while TF and IL-17A present on SLE NETs enhance the fibrotic activity of differentiated HSF. These findings suggest multiple potential targets for therapeutic interventions in end-organ injury in SLE.

TF-bearing and IL-17A-bearing NETs are present within the kidneys and skin biopsies of patients with SLE

To gain further insights into the role of NETs in end-organ injury in SLE, we studied NET deposition in kidney biopsies of patients with proliferative LN and skin biopsies from active lesions and non-affected skin areas of patients with discoid lupus. Even in the absence of intact neutrophils in the kidneys, remnants of neutrophil activation (such as NET structures) were prominent in the glomerular (online supplementary figure 6A) and tubulointerstitial compartment close to the Bowman's capsule and adjacent to renal tubular cells (figure 6A, online supplementary figure 6A and C). These NET structures were co-localised with TF and IL-17A (figure 6A). Conversely, neutrophils/NETs were absent in kidney biopsies from patients with renal carcinoma, minimal change disease or membranous nephropathy (online supplementary figure 6B-C).

NETs were also detected in skin biopsies obtained from lesions of patients with active discoid lupus, as observed by the extracellular localisation of elastase and citrullinated histone 3 (figure 6B). Skin biopsies obtained either from non-inflamed skin of the same patients with SLE or from healthy subjects (controls) did not demonstrate the presence of NETs (figure 6B). Similar to kidney specimens, NETs present in skin lesions were decorated with TF or IL-17A (figure 6C).

Together, these findings indicate the presence of NET-derived components, such as TF and IL-17A, in skin or renal biopsies,

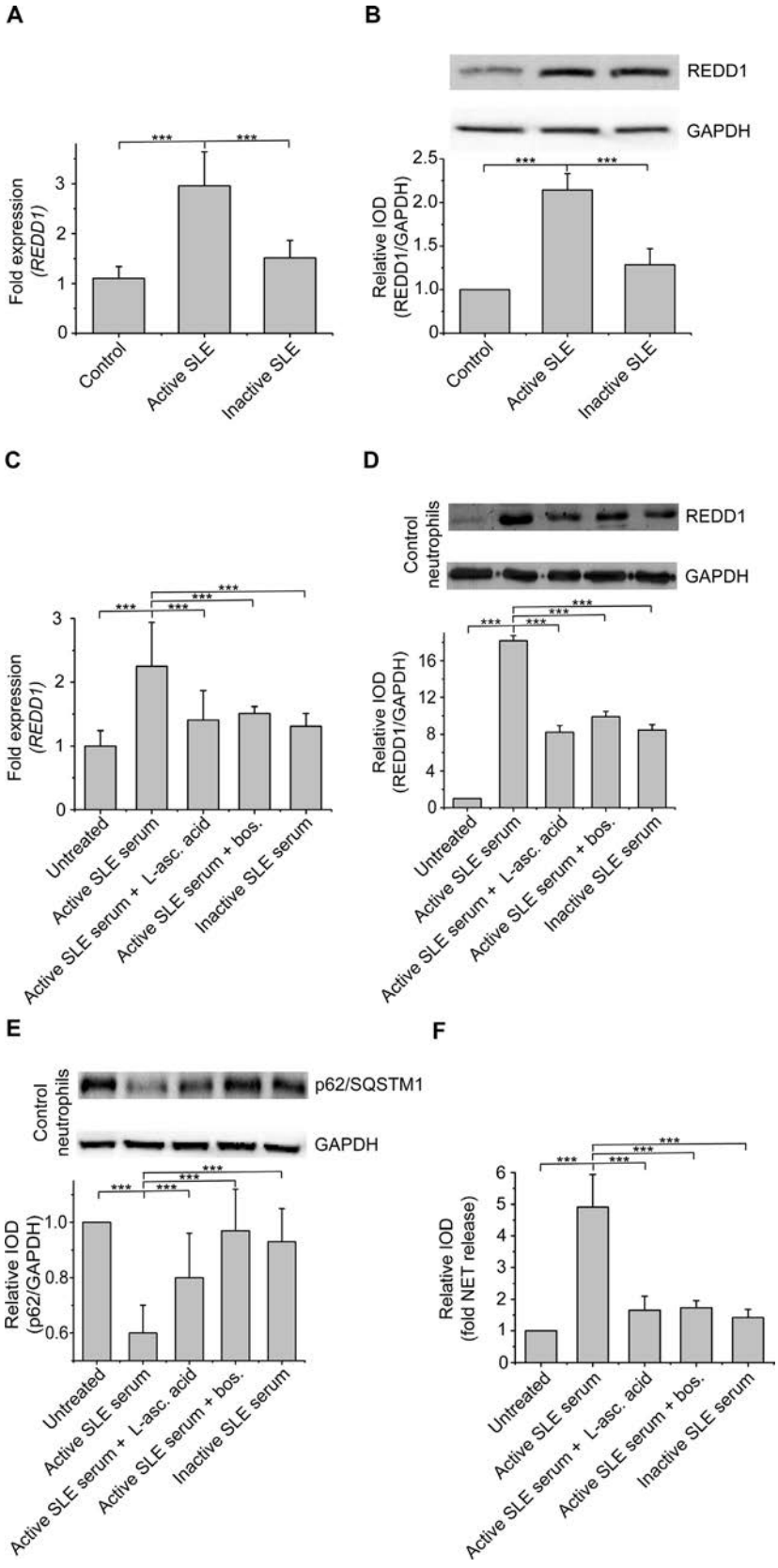

Figure 4 Hypoxia factor-1α (HIF-1α) and endothelin-1 (ET-1) are involved in REDD1-driven neutrophil extracellular trap (NET)osis in active systemic lupus erythematosus (SLE). (A) *REDD1* mRNA (45 min incubation) and (B) REDD1 protein (45 min incubation) levels in active SLE neutrophils, compared with inactive SLE or control neutrophils. (C) *REDD1* mRNA levels and (D) REDD1 (45 min incubation) or (E) p62/SQSTM1 (90 min incubation) immunoblotting in control neutrophils stimulated with active SLE serum in the presence of specific inhibitors (30 min pretreatment); L-ascorbic acid (10 mM), a HIF-1α inhibitor or bosentan (10 µM), an ET-1 receptor antagonist. (F) MPO-DNA complex in isolated NET structures. For (B), (D) and (E), integrated optical density (IOD) relative to control. For (A)–(F), data presented as mean±SD, ***p<0.001, one representative experiment of 6 is shown, n=6 patients.

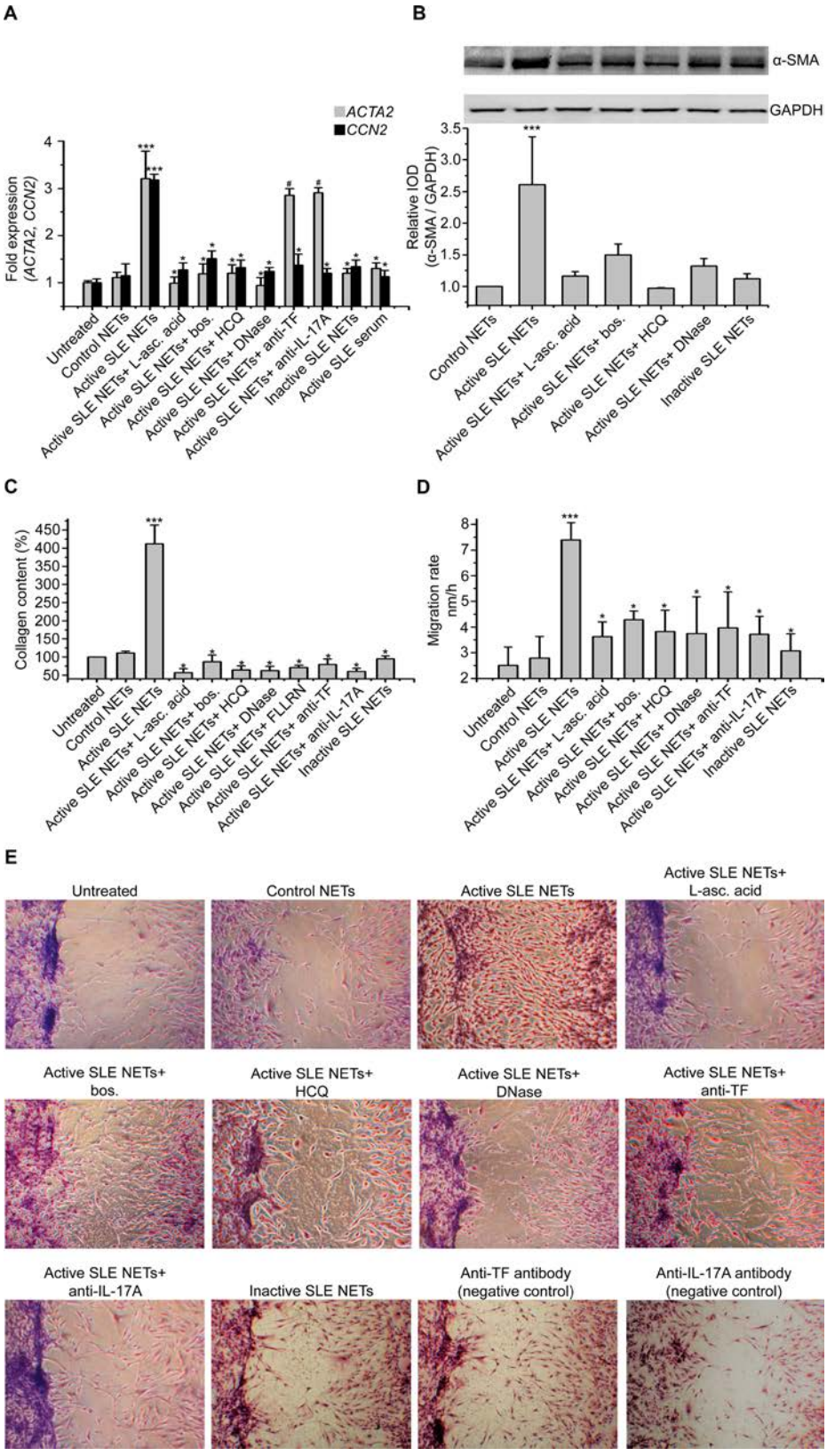

Figure 5 Active systemic lupus erythematosus (SLE) neuttrophil extracellular traps (NETs) bearing tissue factor (TF) and interleukin (IL)-17A promote the fibrotic activity in human skin fibroblasts (HSF) in vitro. (A) *ACTA2* (24 hours incubation) and *CCN2* (48 hours incubation) mRNA expression; (B) α-SMA protein expression (26 hours incubation); (C) collagen production (48 hours incubation) and (D–E) migration rate (18 hours incubation) in HSF treated with active SLE NET structures in the presence or absence of specific inhibitors (30 min pre-treatment). For (A), treatment of HSF with active SLE serum instead of NETs was used as negative control. For (B), integrated optical density (IOD) relative to control. For (E), anti-TF or anti-IL17 antibody directly on HSF was used as negative control. For (A)–(D), data presented as mean±SD, ***p<0.001 compared with respective control; *p<0.001 compared with respective 'active SLE NETs' and ♯non-significant compared with respective 'active SLE nets'. For (A)–(E), one representative experiment of 6 is shown, n=6 patients.

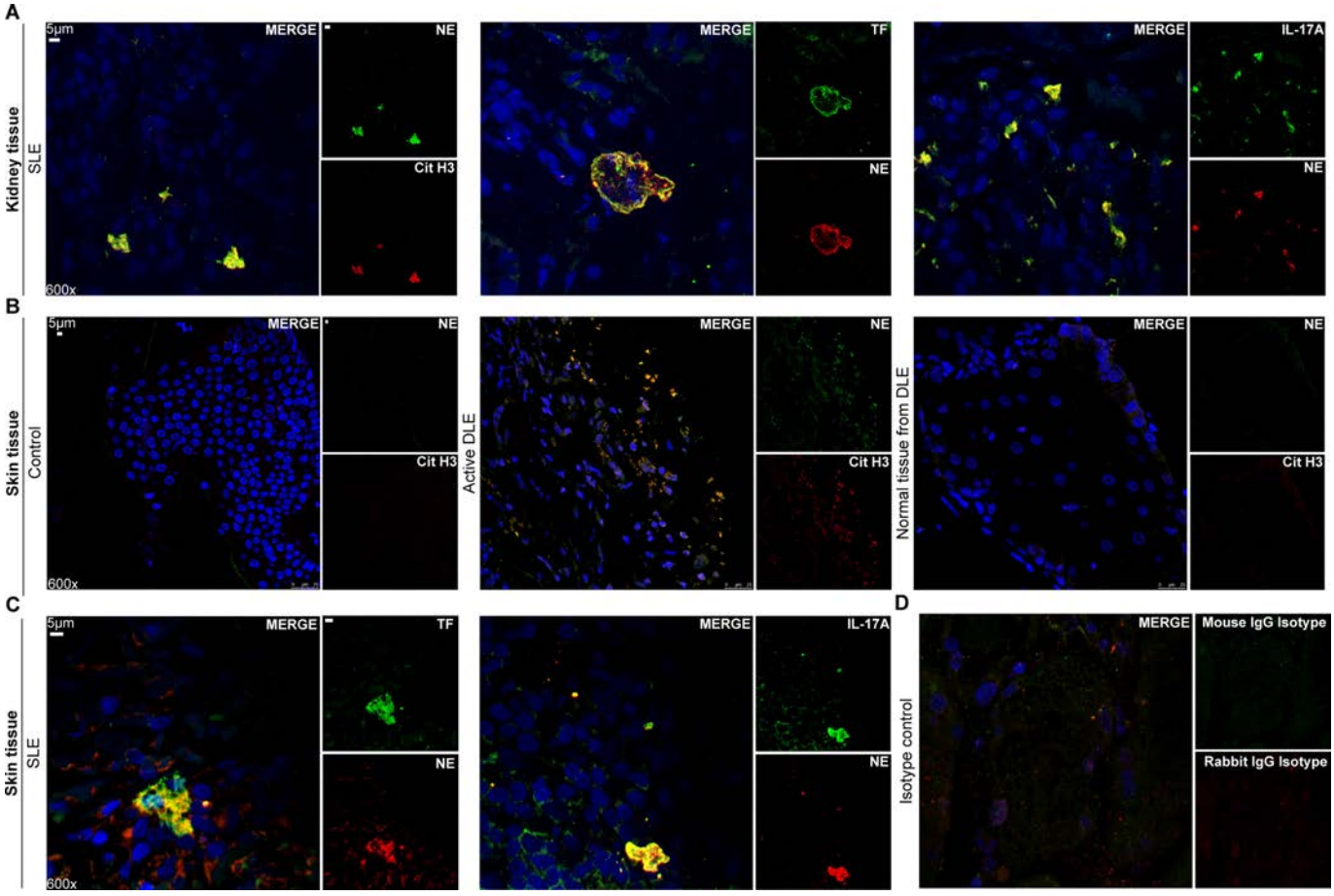

Figure 6 Neutrophil extracellular traps (NETs) expressing tissue factor (TF) and interleukin (IL)-17A are identified in kidney and skin biopsy specimens from patients with active systemic lupus erythematosus (SLE). (A) NETs visualised in kidney specimens from a patient with proliferative lupus nephritis (LN), as extracellular structures by staining with neutrophil elastase (NE) and citrullinated histone H3 (CitH3) (confocal microscopy; green: NE, red: CitH3, blue: 4′,6-diamidino-2-phenylindole (DAPI)/DNA), expressing both TF (confocal microscopy; green: TF, red: NE, blue: DAPI/DNA) and IL-17A (green: IL-17A, red: NE, blue: DAPI/DNA). Representative data from six patients. (B) NETs were identified in skin biopsy specimens from patients with active discoid lupus (DLE) by staining with NE and CitH3, compared with normal tissue obtained either from the same patient with SLE or from healthy subject (control) (confocal microscopy; green: NE, red: CitH3, blue: DAPI/DNA). (C) Presence of TF (confocal microscopy; green: TF, red: NE, blue: DAPI/DNA) and IL-17A (confocal microscopy; green: IL-17A, red: NE, blue: DAPI/DNA) on NET structures observed in skin specimens from patient with active DLE. For (B) and (C), representative data from four patients. (D) Tissue specimen stained with isotype control antibodies.

suggesting their involvement in the thromboinflammatory and fibrotic aspects of SLE.

DISCUSSION

Herein, we implicate for the first time the REDD1/autophagy pathway in neutrophil-mediated end-organ injury in SLE. Serum from patients with active SLE—a surrogate of the inflammatory microenvironment in SLE—through ET-1 and HIF-1α, upregulates neutrophil REDD1 expression, resulting in autophagy induction and subsequent NET release. Bioactive IL-17A- and TF-decorated NETs, detected in active lupus kidney and skin, activate tissue resident cells mediating inflammation and fibrosis.

Autophagy is enhanced in lupus T cells[44] and B cells.[45] Herein, to elucidate the mechanism underlying NET release in SLE, we investigated autophagy in SLE neutrophils and its association to NET release. We demonstrate that active SLE neutrophils display increased basal autophagy levels mediated by inflammatory mediators within active SLE sera. We link NETs with autophagy in SLE and provide evidence that HCQ, a late-stage autophagy inhibitor, has a key role in NETs reduction through autophagy inhibition. Our data extend previous observations suggesting that chloroquine abrogated NET formation in lupus low-density granulocytes[46] and may account, at least in part, for the beneficial effects of HCQ in various organ manifestations in SLE, including skin and kidneys.

Upstream regulatory molecules, governing autophagy and NET release, remain elusive. To date, SLE pathogenesis was partially attributed to impaired clearance of NETs, due to decreased serum DNaseI activity.[20 21] The stress-induced protein REDD1 represents a ‘gate’ to inflammation by linking environmental triggers to cell response through autophagy.[47 48] We show that increased autophagy in SLE neutrophils and subsequent NET release are mediated by REDD1 upregulation, induced by lupus inflammatory mediators. We also provide novel insights into the disease pathogenesis by demonstrating that the REDD1/autophagy pathway is critically involved in SLE NETosis and show that this pathway represents a shared mechanism between autoinflammatory[31] and autoimmune disorders.

ET-1 and HIF-1α are potent mediators of the REDD1/autophagy pathway in SLE. HIF-1α inhibition by L-ascorbic acid or ET-1 inhibition with bosentan, used for treatment of pulmonary hypertension and scleroderma, reduces REDD1 overexpression and abrogates autophagy and subsequent NET release in vitro. Of note, pharmacological inhibition of HIF-1α activity also blocked

NET formation by LPS-stimulated neutrophils.[49] Importantly, HIF-1α or ET-1 inhibition, prior to stimulation of neutrophils with active serum, ameliorated the activation/differentiation of HSF to myofibroblasts, indicating the importance of NETs in the activation/differentiation of fibroblasts. These findings are in accordance with our previous in vitro data demonstrating the involvement of autophagy-dependent NET release in pulmonary fibrosis through lung fibroblasts activation and differentiation.[14] Although further studies are needed to identify additional triggers, our findings demonstrate that ET-1 and HIF-1α could be therapeutically targeted as mediators of the REDD1/autophagy pathway that regulates NET release in SLE.

Disease-specific bioactive proteins on NETs could contribute to different biological processes and histological phenotypes in various diseases.[50–53] We therefore searched for SLE-specific proteins on NETs and asked whether increased autophagy is associated with their expression on NETs. We demonstrate that active SLE serum upregulates the expression of the thromboinflammatory TF and profibrotic IL-17A in neutrophils and mediates their expression on NETs in an autophagy-dependent manner. We demonstrate that these proteins on NETs are bioactive, inducing thrombin generation and activation/differentiation of HSF to collagen-producing myofibroblasts. We also demonstrate that the NET scaffold is essential for these proteins to exert their function and provide evidence that TF-expressing and IL-17A-expressing NETs represent a link between increased thromboinflammation and fibrosis in patients with active SLE.

Since NETs are associated with lupus nephritis,[16 20 54] we reasoned that NETs may be involved in tissue inflammation and fibrosis via the TF/thrombin axis and IL-17A, respectively. We demonstrate the presence of TF-decorated and IL-17A-decorated NETs within end-organ tissues of SLE, in the absence of intact neutrophils. In the kidneys of patients with proliferative LN, TF- and IL-17A-decorated NETs are found within glomeruli whereas TF-decorated NETs are observed within the tubulointerstitial compartment close to the Bowman's capsule, suggesting their possible involvement in capsule rapture and crescent formation (features of rapidly progressive glomerulonephritis).

Pathogenic events at more easily accessible organs in SLE may mirror pathogenic processes in the kidney.[55] NETs have been identified in the skin of patients with SLE[56 57]; however, the disease-associated proteins externalised on NETs were remained unknown. Thus, we analysed skin biopsies from patients with active SLE and found TF-decorated and IL-17A-decorated NETs within affected skin areas. We further demonstrated that the blockade of TF and IL-17A on NETs attenuated the activation/differentiation, collagen production and proliferation/migration in HSF. Accordingly, we provide evidence supporting the important role of TF-bearing and IL-17A-bearing NETs in end-organ injury in SLE, suggesting NETs as a connecting link between the thromboinflammatory and fibrotic aspects of the disease. To this end, agents targeting the IL-17A pathway—currently used for treatment of psoriasis, psoriatic arthritis and ankylosing spondylitis—and/or thrombin inhibitors or PAR blockers could potentially attenuate tissue injury in SLE.

In summary, our findings identify upstream regulators and downstream molecules that mediate NET release in human SLE linking immunometabolism, thromboinflammation and fibrosis towards end-organ injury. ET-1 and HIF-1α in active SLE serum activate the REDD1/autophagy pathway to induce NETs. Active SLE NETs represent scaffolds with high concentration of bioactive IL-17A and TF that remain in end-organ tissues even in the absence of intact neutrophils, activating resident cells and promoting thromboinflammation and fibrosis. To this end, we propose a multistep model for end-organ injury in SLE that can be targeted at multiple levels by repositioning of available drugs to ameliorate tissue injury (online supplementary figure 7). Accordingly, ET-1 receptor antagonists (eg, bosentan) and HIF-1α inhibitors (eg, L-ascorbic acid) could disrupt the 'pre-NETotic' step; autophagy inhibitors (eg, hydroxychoroquine) could prevent the 'NETotic' step, and finally, agents targeting the IL-17A pathway (eg, secukinumab) and/or TF/thrombin axis (eg, thrombin inhibitors or PAR blockers) could offset the 'post-NETotic' deleterious effects in SLE.

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Contributors EF and AC designed and conducted experiments, acquired and analysed the data and wrote the manuscript. AM, KK, SA, IA, AA and HG performed experiments and contributed to data analysis. PV and GB reviewed and analysed the data. DB and KR conceived, designed, wrote and critically reviewed the data and they equally cosupervised this manuscript. All authors have approved the submitted manuscript.

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Competing interests None declared.

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TRANSLATIONAL SCIENCE

Phosphatidylinositol 3-kinase delta pathway: a novel therapeutic target for Sjögren's syndrome

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ABSTRACT

Background The phosphatidylinositol 3-kinase delta isoform (PI3Kδ) belongs to an intracellular lipid kinase family that regulate lymphocyte metabolism, survival, proliferation, apoptosis and migration and has been successfully targeted in B-cell malignancies. Primary Sjögren's syndrome (pSS) is a chronic immune-mediated inflammatory disease characterised by exocrine gland lymphocytic infiltration and B-cell hyperactivation which results in systemic manifestations, autoantibody production and loss of glandular function. Given the central role of B cells in pSS pathogenesis, we investigated PI3Kδ pathway activation in pSS and the functional consequences of blocking PI3Kδ in a murine model of focal sialoadenitis that mimics some features of pSS.

Methods and results Target validation assays showed significant expression of phosphorylated ribosomal protein S6 (pS6), a downstream mediator of the phosphatidylinositol 3-kinase delta (PI3Kδ) pathway, within pSS salivary glands. pS6 distribution was found to co-localise with T/B cell markers within pSS aggregates and the CD138+ plasma cells infiltrating the glands. In vivo blockade of PI3Kδ activity with seletalisib, a PI3Kδ-selective inhibitor, in a murine model of focal sialoadenitis decreased accumulation of lymphocytes and plasma cells within the glands of treated mice in the prophylactic and therapeutic regimes. Additionally, production of lymphoid chemokines and cytokines associated with ectopic lymphoneogenesis and, remarkably, saliva flow and autoantibody production, were significantly affected by treatment with seletalisib.

Conclusion These data demonstrate activation of PI3Kδ pathway within the glands of patients with pSS and its contribution to disease pathogenesis in a model of disease, supporting the exploration of the therapeutic potential of PI3Kδ pathway inhibition in this condition.

Key messages

What is already known about this subject?
► The phosphatidylinositol 3–kinase pathway is involved in the pathogenesis of proliferative disorders and autoimmunity.

What does this study add?
► Our study demonstrate that this pathway is active in SS and that pharmacological targetting of this pathway drives disease amelioration in an animal model of sialoadenitis that recapitulates some feayures of SS.

How might this impact on clinical practice or future developments?
► This proof of concept study support future development of therapeutics against PI3KD in SS.

INTRODUCTION

The phosphatidylinositol 3-kinase delta isoform (PI3Kδ) belongs to the class 1 phosphoinositide-3-kinase family of intracellular lipid kinases that regulate metabolism, survival, proliferation, apoptosis, growth and cell migration.[1] Extensive data demonstrate a central role for PI3K signalling in several aspects of adaptive immune responses. Expression of the catalytic subunit of PI3Kδ is greatly enriched in lymphocytes. In B cells, PI3Kδ represents the predominant PI3K isoform to transduce signals derived from the B cell receptor and receptors binding B cell survival factors, cytokines, chemokines and costimulatory molecules.[2–4] Downstream signalling on PI3Kδ activation results in the activation of AKT and mTOR; the latter exists in two major protein complexes, the rapamycin-sensitive mTORC1 (in complex with raptor) and the rapamycin-insensitive mTORC2 (in complex with rictor). A key substrate of mTORC1, ribosomal protein S6 kinase (S6K), phosphorylates ribosomal protein S6 (pS6), which can thereby act as a marker of active PI3K-mTOR signalling. The sensitivity of pS6 expression to PI3Kδ signalling has been demonstrated in both T and B cells.[5 6]

The significant role of PI3Kδ in regulating B cell biology has led to the development of PI3Kδ inhibitors as therapeutics for B cell malignancies.[7–9] Idelalisib, a PI3Kδ selective inhibitor, has recently received Food and Drug Administration approval for the treatment of chronic lymphocytic leukaemia and non-Hodgkin's lymphoma (NHL). Clinical trials have demonstrated the ability of idelalisib to inhibit B cell survival and interfere with microenvironment-derived signals responsible for maintenance of malignant cells within the lymph node.[7] The established role of PI3Kδ in B cell hyperactivity suggest that this pathway is an attractive target for

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autoimmune conditions characterised by B cell hyperactivation, such as primary Sjögren's syndrome (pSS).

pSS is characterised by systemic autoantibody production and local, predominantly B cell infiltration of the exocrine glands that often results in functional loss. Cellular infiltrates are characterised by ectopic production of lymphoid chemokines, T/B cell segregation and formation of follicular dendritic cell networks within ectopic germinal centres (GC).[10 11] Moreover, local expression of *AICDA*, the gene encoding for the activation-induced cytidine deaminase (AID), the enzyme instrumental for B cell affinity maturation, is expressed in pSS GC where it is believed to support local autoantibody production.[12] Progressive enlargement of pSS inflammatory foci is characterised by increased accumulation of activated B cells, and in some cases, local emergence of post-GC malignant clones responsible for the development of NHL.[13–18] Dysregulated B cell activation, locally manifested by salivary gland swelling and production of anti-SSA and anti-SSB autoantibodies, is also accompanied by systemic increases in immunoglobulins and autoantibodies, including rheumatoid factor and cryoglobulins.[19–24] Additional systemic features associated with B cell hyperactivity, such as lymphadenopathy, night sweats and loss of weight are often observed during lymphoma development.[21 24 25] The dysfunctional humoral response present in these patients supports the investigation of PI3Kδ in pSS pathogenesis and its blockade as a therapeutic option for this condition.

MATERIALS AND METHODS

Mice and salivary gland cannulation

C57BL/6 mice were purchased from Charles River and were maintained under specific pathogen-free conditions in the Biomedical Service Unit at the University of Birmingham according to Home Office and local ethics committee regulations. Under ketamine/domitor anaesthesia, the submandibular glands of female C57BL/6 (8–12 weeks) were intraductally cannulated with 10^8–10^9 plaque-forming unit (pfu) of luciferase-encoding replication-defective adenovirus (AdV5), as previously described.[26] Mice were sacrificed at day 15 post-cannulation (pc) (peak of organisation of the lymphoid aggregates). To collect samples, mice were given general anaesthesia as mentioned above and were then secured in the supine position. Salivation was induced by subcutaneous administration of 10 mg/kg pilocarpine (Sigma-Aldrich) in phosphate buffered saline (PBS). Saliva was collected with a pipet over a 10 min period and transferred into weighed eppendorf tubes, the tubes were then reweighed and the volume of saliva calculated (1 mg=1 µL saliva). Results were expressed as mg saliva/10 min/g body weight.

Seletalisib inhibitor

The in vitro and in vivo properties of seletalisib have been described previously.[27] Mice were gavaged at a dose of 10 mg/kg with seletalisib every day starting from day 0, day 3, day 5 and day 8 pc.

Human salivary gland biopsies from patients with pSS

Minor salivary gland (mSGs) samples were obtained from the Human Biomaterials Resource Centre at the University of Birmingham under ethics number 10-018 and from the Sjögren's cohort at the University of Rome, Sapienza under ethics Harmonics H2020. Specimens were identified among samples obtained by patients diagnosed with pSS according to the 2002 American European Consensus Group Criteria criteria[28] and fulfilling the histological criteria for the diagnosis of pSS (presence of aggregates>1 focus score). All patients included were untreated with immunosuppressive drugs including steroids.

Non-specific sialoadenitis samples were selected among patients undergone investigation for pSS, because of clinical symptoms of dryness (eyes and/or mouth) but either did not fulfil the classification criteria for pSS and/or were not clinically diagnosed as primary or secondary SS by the leading physician table 1.

On patients collected between 2012 and 2018, EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) data were available and reported in table 1.

Table 1 Baseline characteristics of subjects included in the study

Cohorts 1 and 2 Birmingham		
Baseline characteristics	**pSS (cohort 1)**	**Sicca (cohort 1)**
Age (years)*	63.0 (55, 67)	47 (46, 52)
Female†	9/9 (100)	3/3 (100)
Anti-Ro antibody positive†	7/9 (78)	0/0 (0)
Anti-La antibody positive†	6/9 (67)	0/0 (0)
IgG (g/L)*	17.6 (12.9, 42.5)	–
Focus score	>1	N/A
Germinal centre†	–	N/A
ESSDAI	–	–
N/A, non applicable.		
Baseline characteristic	**pSS (cohort 2)**	**Sicca (cohort 2)**
Age (years)*	56.0 (24, 57)	41 (32, 76)
Female†	4/5 (80)	3/5 (60)
Anti-Ro antibody positive†	4/5 (80)	0/0 (0)
Anti-La antibody positive†	2/5 (40)	0/0 (0)
IgG (g/L)*	13.05 (9.95, 19.94)	9.26 (7.51, 13.98)
Focus score*	1.29 (0.8, 2.14)	N/A
Germinal centre†	2/4 (50)	N/A
ESSDAI	9 (8, 17)	N/A
Cohort 3. Rome		
Baseline characteristic	**pSS**	**Sicca**
Age (years)*	48 (26,72)	55 (37,70)
Female†	15/17 (88.2)	15/15 (100)
Anti-Ro antibody positive†	11/17 (64.7)	0/0 (100)
Anti-La antibody positive†	8/17 (47.1)	0/0 (100)
Anti-nuclear antibody positive†	12/17 (70.6)	0/0 (100)
Hyperglobulinemia†	7/17 (41.2)	0/0 (100)
Focus score*	2.8 (1,10)	N/A
Germinal centre†	11/17 (64.7)	N/A
ESSDAI*	1 (0, 7)	0 (0, 2)

*Median (range).
†Number positive (%).
BAFF, B cell activating factor; ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; Ig, immunoglobulin; pSS, primary Sjögren's syndrome.

Histology and immunofluorescence

Immunofluorescence (IF) staining was performed as previously described on formalin-fixed, paraffin-embedded (FFPE) labial salivary gland biopsies from patients with SS[10 29 30] and on murine SGs obtained from virus cannulated and control mice.[30]

The following antibodies were used: for mouse CD45 clone 30-F11, CD19 clone eBio1D3 and CD3e clone ebio500A2 (from eBiosciences) and for humans CD3 polyclonal rabbit or monoclonal mouse (Dako), CD20 clone L26 (Dako), CD138

and CD68 (Abd Serotech) and pS6 polyclonal rabbit (Cell signalling).

RNAScope

IF staining was performed as previously described on FFPE labial salivary gland biopsies from patients with SS.[10 29 30] Samples were probed for PI3KCD ref.520988 (ACDBio) following manufacturer's instructions (ACDBio). Samples were double stained with antihuman CD45 NCL-L-LCA (Leica).

Enzymatic digestion and isolation of cells

ADV5 infected SGs from seletalisib-treated and vehicle-treated mice were isolated from culled animals at different time points. Glands were dissected and placed in 1 mL of RPMI-1640 (with 2% fetal calf serum (FCS)) on ice. Once all SGs were collected, RPMI-1640 was removed, replaced with 2 mL enzyme mix (RPMI with 2% FCS, 0.8 mg/mL dispase, 0.2 mg/mL collagenase P and 0.1 mg/mL DNase I) and digested as previously described.[31]

Flow cytometry analysis and sorting

Single cell suspensions were incubated with 100 µL diluted antibodies for 30 min at 4°C in ice-cold fluorescence-activated cell sorting (FACS) buffer (0.5% bovine serum albumin, 2 mM EDTA in PBS) with 'cocktails' of the following antibodies: CD45 clone 30-F11, CD3e clone 145–2 C11, CD4 clone RM4-5, CD62L clone MEL-14, CD44 clone IM7, CD8a clone 53–6.7, B220 clone RA3-6B2, CD23 clone B3B4, CD19 clone 1D3 and CD5 clone 53–7.3 (all from eBiosciences), CD21 clone 7G6 (BD biosciences) and CD11c clone N418, F4/80 clone BM8, CD64 clone X54-5/7.1 and NK1.1 clone PK136. Intracellular staining for Ki67 clone B56 (BD Biosciences) and pS6 PE and pAKT Alexa Fluor 488 (Cell signalling) was performed by using the Cytofix/Perm kit (BD biosciences) and Fixation/Permeabilization Buffer set (ebiosciences) according to the manufacturer's protocol. Cells were resuspended in FACS buffer and then analysed using a Cyan-ADP (Dako) or Fortessa (BD) with forward/side scatter gates set to exclude non-viable cells. Cells of interest were sorted by using BD FACSAria. Data were analysed with FlowJo software (Tree Star).

Microdissection, mRNA isolation, qRT-PCR

Microdissection and laser catapulting were performed on Cresyl-violet (0.1% in ethanol)-stained frozen tissue sections from salivary gland samples and tonsil GCs as previously described.[32]

Total RNA was isolated either from murine and human SGs with an RNeasy mini kit (Qiagen), from microdissected tissue or from sorted cells. RNA was then reverse transcribed using the high capacity reverse transcription cDNA synthesis kit (Applied Biosystems) according to the manufacturer's specifications. Reverse transcription was carried out on a Techne 312 Thermal Cycler PCR machine. Quantitative real-time (qRT)-PCR (Applied Biosystems) was performed on cDNA samples for *ccl19*, *cxcl13*, *lta*, *ltb* and *baff* mRNA expression. β-actin and pdgfrβ were used as an endogenous control. The primers and probes used were from Applied Biosystems (table 2). qRT-PCR was run in duplicates on a 384-well PCR plate (Applied Biosystems) and detected using an ABI PRISM 7900HT instrument. Results were analysed with the Applied Biosystems SDS software (SDS V.2.3) as previously described.[30]

Table 2 Primers and probes used for quantitative PCR

Gene	Assay ID
Mouse β-actin	Mm01205647_g1
Mouse Pdgfrβ	Mm00435546_m1
Mouse AICDA	Mm00507774_m1
Mouse BAFF	Mm00840578_g1
Mouse CXCL13	Mm00444533_m1
Mouse CXCR5	Mm00432086_m1
Mouse CCL19	Mm00839967_g1
Mouse CCR7	Mm01301785_m1
Mouse CXCL12	Mm00445553_m1
Mouse CXCR4	Mm01292123_m1
Mouse LTβ	Mm00484254_m1
Mouse LTα	Mm00484254_m1
Mouse IL-23	Mm00484254_m1
Mouse IL-6	Mm00434256_m1
Mouse IFNγ	Mm00434774_g1
Mouse TNFα	Mm00443258_m1
Mouse IL-1β	Mm00434228_m1

Lipid analysis

Salivary gland tissue was pulverised in liquid nitrogen using a mortar and pestle and determination of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) levels, including lipid extraction, derivatisation and mass spectrometric analysis, was carried out as described previously.[33]

RESULTS

Target validation of PI3Kδ pathway engagement in SGs of patient with pSS

We confirmed the expression of PI3KCD transcript mRNA name for PI3Kδ in sorted peripheral blood mononuclear cell from patients with pSS (figure 1A) and in total mRNA isolated from minor SGs from pSS and sicca controls (figure 1B). Transcript levels of PI3KCD significantly correlated with the focus score (FSC) calculated in the same SGs (figure 1C) and associate with immune activation markers such as the presence of autoantibodies, hyperglobulinaemia and the presence of GCs (online supplementary figure 1). qRT-PCR on microdissected tissue and RNAScope confirmed localisation of the transcript for PI3Kδ within the foci and in particular within GC+foci (figure 1D,E and control tonsil in the online supplementary figure 1).

In order to assess activation of the PI3Kδ pathway in minor SG biopsies and confirm its local engagement, we used IF to detect the presence of the phosphorylated ribosomal protein S6 (pS6),[27 34] in pSS and non-specific sialoadenitis control (NSCS) tissue. Significant expression of pS6 was observed in salivary gland biopsies of patients with pSS as compared with non-specific sialoadenitis. In NSCS, pS6 staining was only detected within the epithelium and not present in all samples analysed (figure 1F). On the contrary, in pSS, intense pS6 staining was detected within the lymphoid aggregates and on the periphery of the foci, in co-localisation with T (CD3+) and B (CD20+) cells and myeloid cells (figure 1G,H and online supplementary figure 1 for pSS and tonsil GC, used as control). This correlated with the extent of infiltration of the glands (online supplementary figure 1).

Interestingly, intense pS6 staining was detected in co-localization with CD138+ plasma cells in pSS SGs as demonstrated by IF and flow cytometry (figure 1H,I). pS6 positive

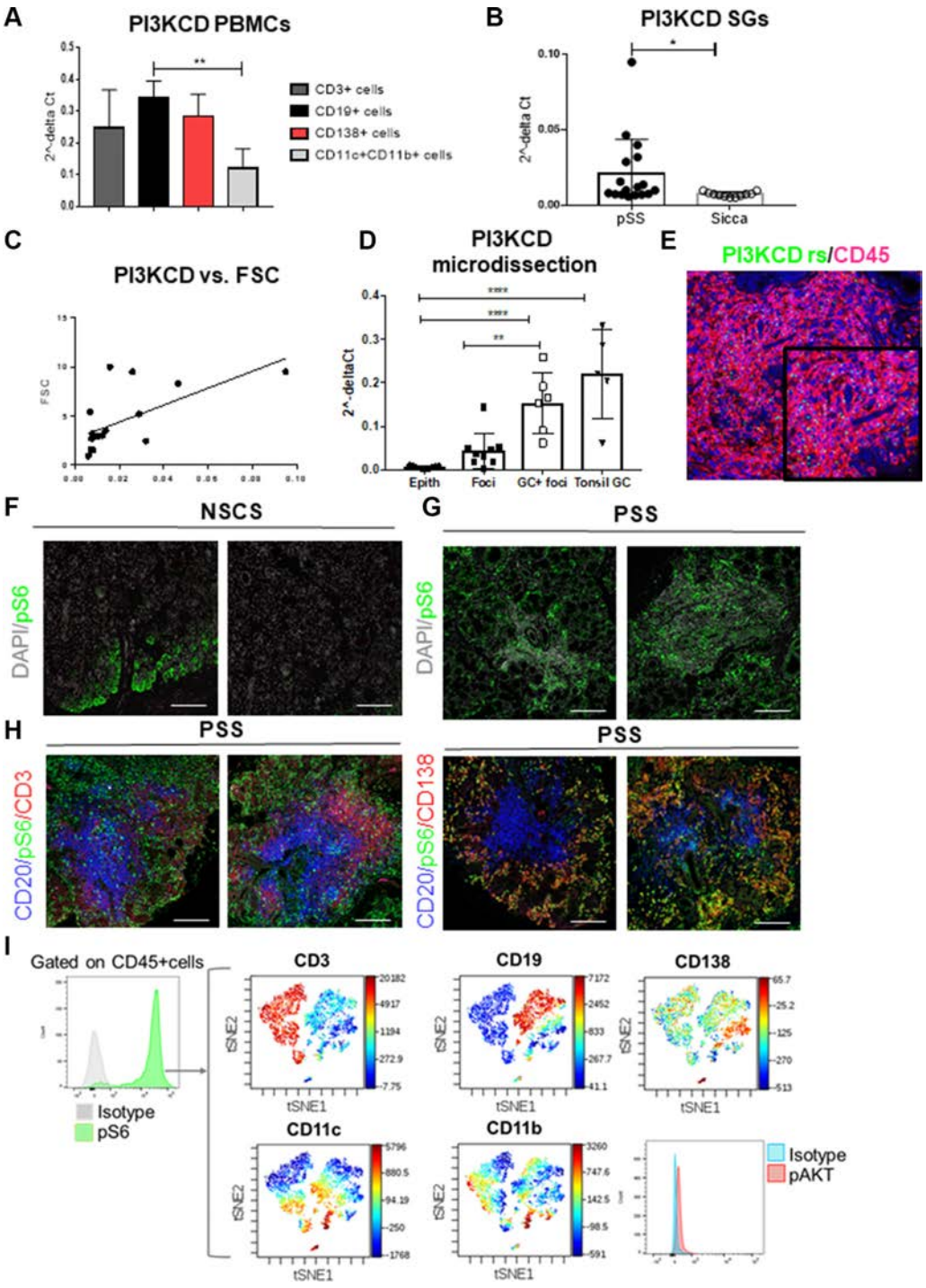

Figure 1 (A) Quantitative real-time (qRT)-PCR analysis of PI3KCD transcripts in peripheral blood mononuclear cell (PBMC) isolated from patients with primary Sjögren's syndrome (pSS). CD3+ cells (dark grey bar), CD19+ cells (black bar), CD138+ cells (red bar), CD11c+CD11b+ cells (light grey bar). Results represented as mean±SD of five patients; **p<0.01, one-way analysis of variance (ANOVA). (B) qRT-PCR analysis of PI3KCD transcripts in total mRNA isolated from salivary glands of patients with pSS (black circles) and sicca controls (open circles). Results represented as mean±SD of 15–17 patients in each group; *p<0.05, unpaired t-test. (C) Correlation between focus scores (FSC) and levels of PI3KDC expressed as 2^-DCT detected in frozen salivary galnds from patients with pSS. R^2 0.3941, p=0.0092. (D) qRT-PCR analysis of PI3KCD transcripts in microdissected epithelium, foci, germinal centre positive (GC+) foci from salivary glands of patients with pSS and GCs isolated from tonsils. Results represented as mean±SD of 5–10 biological replicates in each category; **p<0.01, ****p<0.0001, one-way ANOVA. (E) Microphotograph of minor salivary glands from patients with pSS, showing in red CD45 staining and in green PI3KCD RNA (visualised with RNAScope). (F) Representative microphotograph of salivary glands from non-specific sialoadenitis control (NSCS) patients stained for the PI3Kδ pathway activation marker phosphorylated ribosomal protein S6 (pS6; green) and 4′,6-diamidino-2-phenylindole (DAPI; grey); scale bars=100 µm. (G) Representative microphotograph of salivary glands from patients with pSS with pS6 (green) and DAPI (grey). (H) Representative microphotographs showing pS6 (green) expression within CD20 (blue) and CD3 or CD138 (red) cells in salivary glands from patients with pSS; scale bars=100 µm. (I) Representative histogram showing flow cytometry staining for pS6 (green) and isotype control (grey) in CD45+ cells present in salivary glands of patients with pSS. viSNE plots of flow cytometry of pSS salivary gland CD45+pS6+ cells. Colours indicate cell expression level of labelled marker. Histogram showing pAkt expression in CD45+pS6+ cells.

cells encompassed also T, B and dendritic cells (DCs) and AKT activation (figure 1I).

These data suggest that PI3Kδ is engaged in several cell types within pSS inflammatory infiltrates and might be involved in the perpetuation of the local autoimmune response.

Blockade of PI3Kδ pathway reverses lymphocytic infiltration in a mouse model of focal sialoadenitis

The in vivo functional role and downstream effect of PI3Kδ inhibition in pSS was tested taking advantage of a mouse model of focal sialoadenitis induced by direct delivery of a replication-deficient ADV5 within murine wild-type SGs.[26] Localised viral infection in this model mimics features of pSS, including the formation of focal lymphocytic aggregates, expression of lymphoid chemokines and cytokines as well as antinuclear antibodies.[26] First, expression of PI3KCD was confirmed in the CD45+ compartment of cannulated SGs from mice sacrificed at day 15 pc (figure 2A). Engagement of the pathway was confirmed by upregulation of pS6 and pAKT on isolated CD45+ cells, with a predominant expression in DCs, T cells, B cells and plasma cells (figure 2B–D). The large predominance of pS6+ DC in our model is probably related to the viral nature of the stimulus and is not reflecting entirely human pSS where the percentage of pS6+ cells only accounted for a minority of the CD11c+ and CD11b+ cells. Treatment of mice with seletalisib resulted in a significant decrease in the ratio between PIP3 and phosphatidylinositol (4,5)-biphosphate (PIP2), which demonstrated blockade of the PI3Kδ pathway (figure 2E). Moreover, seletalisib treatment induced downregulation of S6 phosphorylation in CD45+ cells isolated from infected SGs in treated mice but not in vehicle controls (figure 2F). Together, these data confirmed the activation of the PI3Kδ pathway in our model and the ability to modulate it by using seletalisib. ADV5 infected mice treated with seletalisib, either prophylactically (day 0 pc) or therapeutically (at day 3, 5 or 8 pc) showed a reduction in the absolute number of CD45+ cells in active treatment groups as compared with the vehicle-treated mice. This significant decrease was maintained in a full therapeutic regime when mice were treated from either day 3 or 5 pc (figure 3A). Although this significant reduction in CD45+ cell counts was not maintained when treated day 8 pc, a significant reduction was observed in specific immune cell populations, notably T and B cells (online supplementary figure 2). Together, these data confirm the therapeutic potential of this drug in established disease. Flow cytometry analysis revealed a marked reduction in absolute numbers of CD3+ T cells (both CD4 and CD8 cells) (figure 3B–D) as well as CD19+ B cells in all active treatment groups relative to controls (figure 3E). Within the overall T cell population, memory and effector CD4 and CD8+ cells were both affected (online supplementary figure 3). Moreover, all subsets of B cells (B1A, B1b, B1c, B2, marginal zone and follicular B cells) displayed marked decreases in absolute cell numbers (figure 3F–G and online supplementary figure 4). In addition, the proliferative ability of both T and B lymphocytes was impaired as demonstrated by a significant decrease in Ki67 staining in both the T and B compartment (figure 3H–I).

Following our observation of PI3Kδ activation in CD138+ plasma cells, we also explored the effect of seletalisib on this cell type in cannulated mice treated either with the compound or its vehicle. Inhibition of PI3Kδ resulted in a significant decline in the number of CD138+ plasma cells in all treatment groups, suggesting that the PI3Kδ pathway also regulates plasma cell homeostasis (figure 3J).

Interestingly, the effects observed on specific subpopulations can be different depending on the treatment regime used. While we did not observe a selective effect in samples treated prophylactically or from day 3 pc, we have observed a significant effect on all B cells as percentages (as well as absolute numbers) and in particular on B1a and MZ B cells in animals treated from day 5 pc (online supplementary figures 4 and 5).

Aggregate formation during salivary gland inflammation is abrogated in mice treated with seletalisib

Having observed a reduction in lymphocyte accumulation within SGs following seletalisib by flow cytometry, we wanted to confirm these observations by IF staining for CD3+ and CD19+ cells as well as to visualise any impact on the organisation of infiltrating lymphocytes. These data revealed impaired lymphoid aggregate formation in seletalisib-treated mice compared with those treated with vehicle. It was particularly marked in mice treated prophylactically with seletalisib, in which no visible lymphoid aggregate formation was evident. This was confirmed by quantification of the FSC, foci size and aggregate organisation, with all parameters demonstrating a significant reduction in the treated animals at day 15 pc as compared with the controls (figure 4A–C). Importantly, the abrogation of lymphocytic foci formation and organisation coincided with a decrease in antinuclear autoantibody production in mice treated with the PI3kδ inhibitor compound as compared with vehicle controls (figure 4 and online supplementary figure 4). Analysis of stimulated salivary flow also showed a significant improvement in saliva production in seletalisib-treated mice (figure 4E).

Inhibition of PI3Kδ pathway impairs the expression of ectopic lymphoneogenesis associated cytokines and chemokines

The reduced lymphocyte aggregation following seletalisib treatment led us to investigate its impact on the expression of factors that drive ectopic lymphoneogenesis. In accordance with the histological findings, qRT-PCR performed on whole SG tissue demonstrated significantly reduced transcript levels for the lymphoid cytokines (LTβ and LTα) in mice treated with seletalisib as compared with controls. Moreover, a significant reduction in CXCL13 and CXCL12 transcript levels was observed in seletalisib-treated mice, while a modest effect was observed for CCL19, one of the chemokines responsible for T cell migration within the affected glands. To further support the lymphoid chemokine expression and aggregate histological data, qRT-PCR analysis for CXCR5, CCR7 and CXCR4 mRNA also showed significantly lower transcript levels in seletalisib-treated mice when compared with vehicle controls. A significant reduction in B cell activating factor (BAFF) expression across all treatment groups tested as compared with vehicle-treated mice was also detected. Furthermore, marked suppression in AICDA mRNA transcripts (the gene encoding for AID) was observed in mice treated with PI3Kδ inhibitor (figure 5A).

IF analysis demonstrated decreased protein expression for CXCL13 and CCL21 in the mice analysed (figure 5B).

Overall, these results suggest that inhibition of the PI3Kδ pathway disrupts the positive feedback loop of lymphocytic infiltration and lymphoid chemokine production which is required for the establishment of ectopic GC and plasma cell survival niches in the affected SGs.

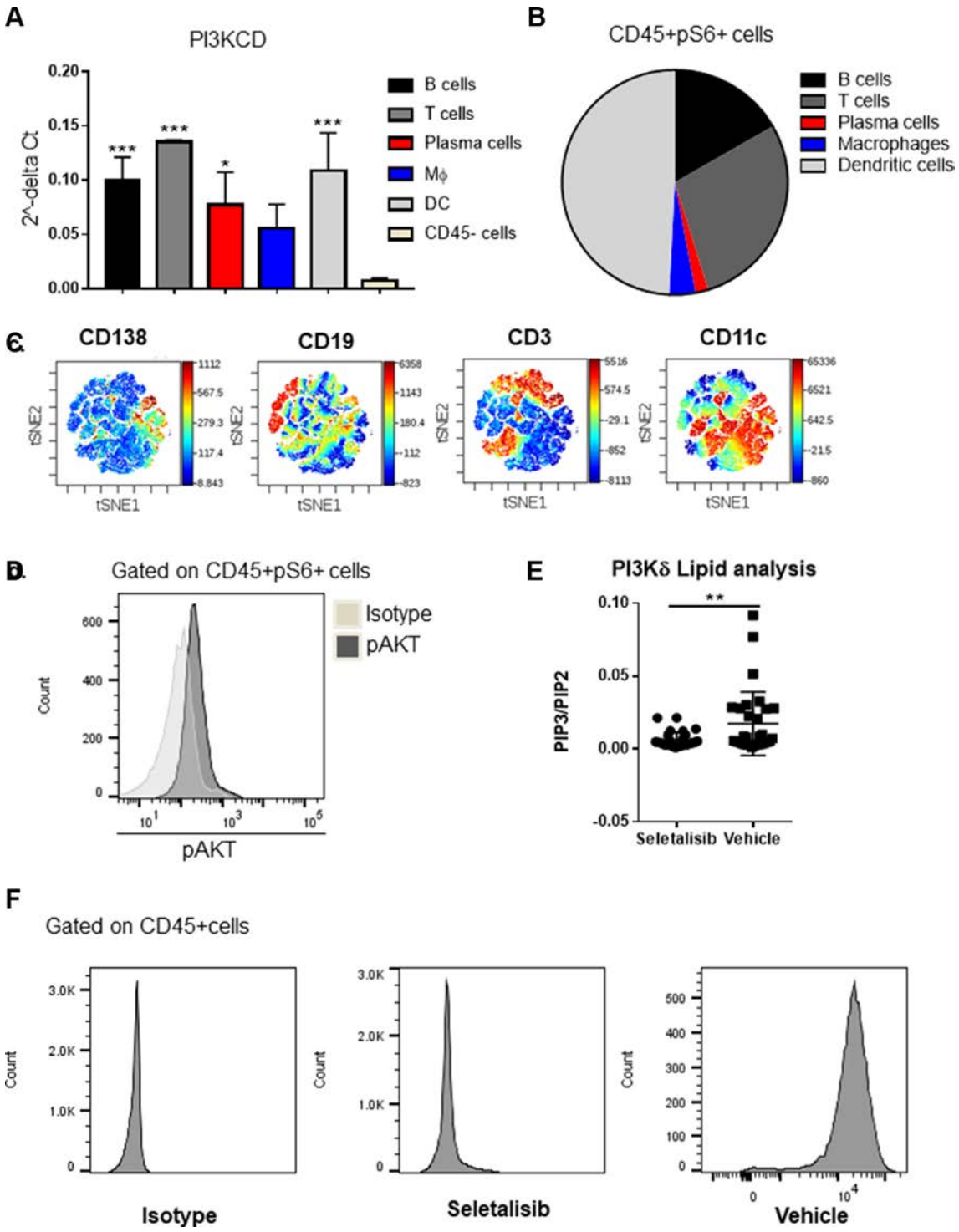

Figure 2 (A) Quantitative real-time PCR analysis of PI3KCD transcripts isolated cells from salivary glands of cannulated mice at day 15 postcannulation (pc). B cells (black bar), T cells (dark grey bar), plasma cells (red bar), macrophages (blue) and dendritic cells (light grey bar), CD45– cells (light yellow bars). Results represented as mean±SD from five mice; *p<0.5, ***p<0.001, one-way analysis of variance. (B) Pie chart showing distribution of different leucocyte populations within CD45+ phosphorylated ribosomal protein S6 (pS6+) cells present in salivary glands of wild-type (WT) mice at day 15 pc (C) viSNE plots of flow cytometry of day 15 pc salivary gland CD45+pS6+ cells. Colour indicates cell expression level of labelled marker. Data is representative of two independent experiments with five mice. (D) Histogram showing phosphorylation of Akt in CD45+pS6+ cells in salivary glands of WT mice at day 15 pc. (E) Graphs showing phosphatidylinositol (3,4,5)-trisphosphate (PIP3)/phosphatidylinositol (4,5)-biphosphate (PIP2) ratio in salivary glands of mice treated with seletalisib versus vehicle control to demonstrate effect of the compound directly in the salivary glands. Results represented as mean±SD of three independent experiments with five mice per group; **p<0.01, unpaired t-test. (F) Histogram showing pS6 expression levels within the CD45+ cells in day 15 pc salivary glands of mice treated with seletalisib as compared with the vehicle-treated mice. Isotype control also shown. The mice were treated with seletalisib or vehicle from day 12 pc onwards. Data is representative of experiments with three mice in each group.

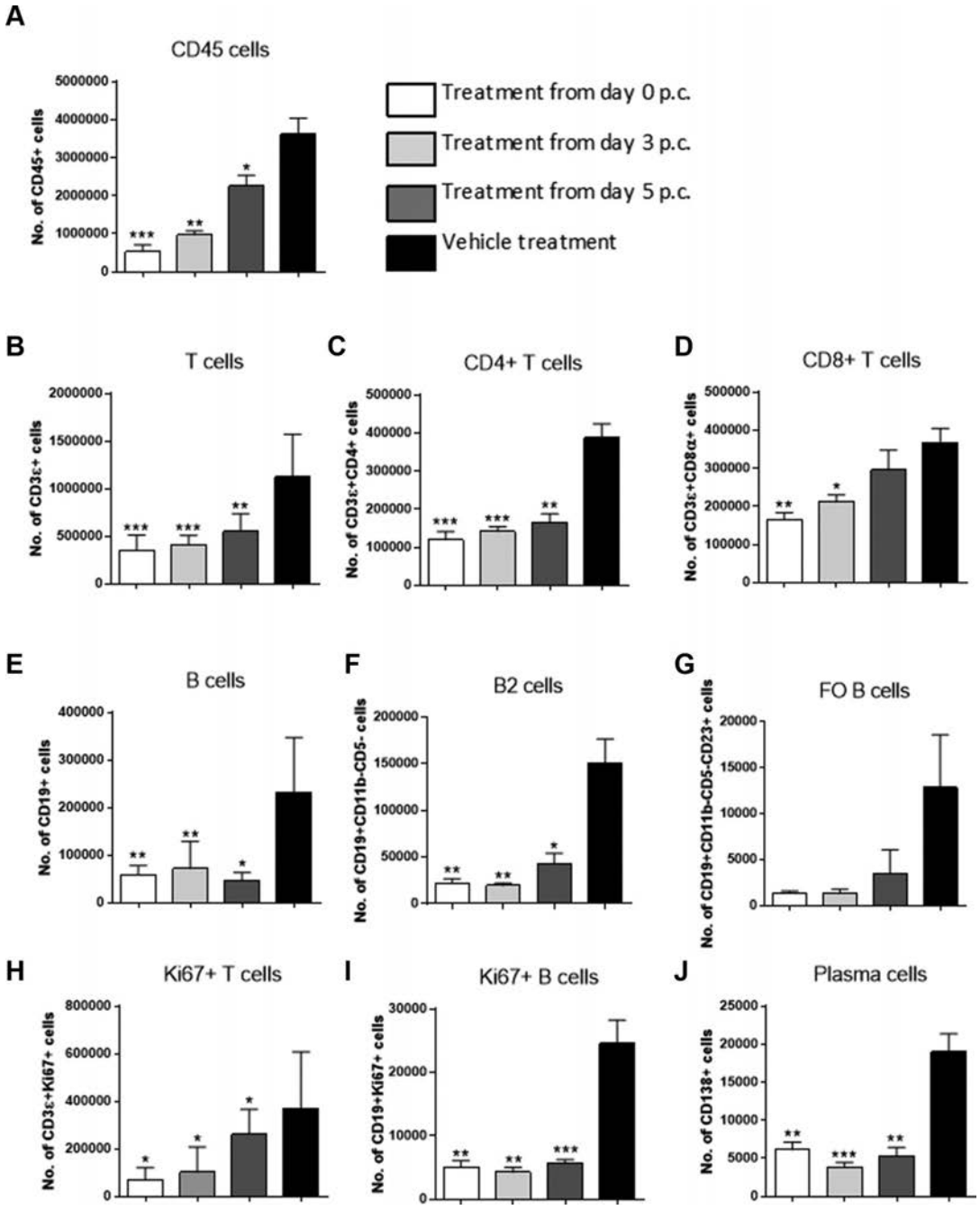

Figure 3 (A) Graphs summarising flow cytometry data for absolute numbers of CD45 cells in salivary glands of wild-type (WT) mice at day 15 postcannulation (pc) treated with seletalisib at day 0 (white bars), day 3 (light grey) and day 5 (dark grey) pc as compared with vehicle controls (black bars). Results represented as mean±SD of two independent experiments with five mice per group; *p<0.05, **p<0.01, ***p<0.001, one-way analysis of variance (ANOVA). (B) Graphs summarising flow cytometry data for absolute numbers of CD3+ T cells in salivary glands of WT mice at day 15 pc treated with seletalisib at day 0 (white bars), day 3 (light grey) and day 5 (dark grey) pc as compared with vehicle controls (black bars). Results represented as mean±SD of two independent experiments with five mice; *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA. (C and D) Graphs summarising flow cytometry data for absolute numbers of CD4+ T cells, CD8+ T cells in salivary glands of WT mice at day 15 pc treated with seletalisib at day 0 (white bars), day 3 (light grey) and day 5 (dark grey) pc as compared with vehicle controls (black bars). Results represented as mean±SD of two independent experiments with three mice per group; *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA. (E) Graphs summarising flow cytometry data for absolute numbers of CD19+ B cells in salivary glands of WT mice at day 15 pc treated with seletalisib at day 0 (white bars), day 3 (light grey) and day 5 (dark grey) pc as compared with vehicle controls (black bars). Results represented as mean±SD of two independent experiments with five mice glands per group; *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA. (F–I) Graphs summarising flow cytometry data for absolute numbers of CD19+CD11b−CD5−B2 B cells, follicular (CD23+) B cells and Ki67+ (proliferating) T and B cells in salivary glands of WT mice at day 15 pc treated with seletalisib at day 0 (white bars), day 3 (light grey) and day 5 (dark grey) pc as compared with vehicle controls (black bars). Results represented as mean±SD of two independent experiments with three mice per group; *p<0.05, **p<0.01, ***p<0.001, one-way ANOVA. (J) Graphs summarising flow cytometry data for absolute numbers of B220+ CD138+ plasma cells in salivary glands of WT mice at day 15 pc treated with seletalisib at day 0 (white bars), day 3 (light grey) and day 5 (dark grey) pc as compared with vehicle controls (black bars). Results represented as mean±SD of two independent experiments with five mice per group; **p<0.01, ***p<0.001, one-way ANOVA.

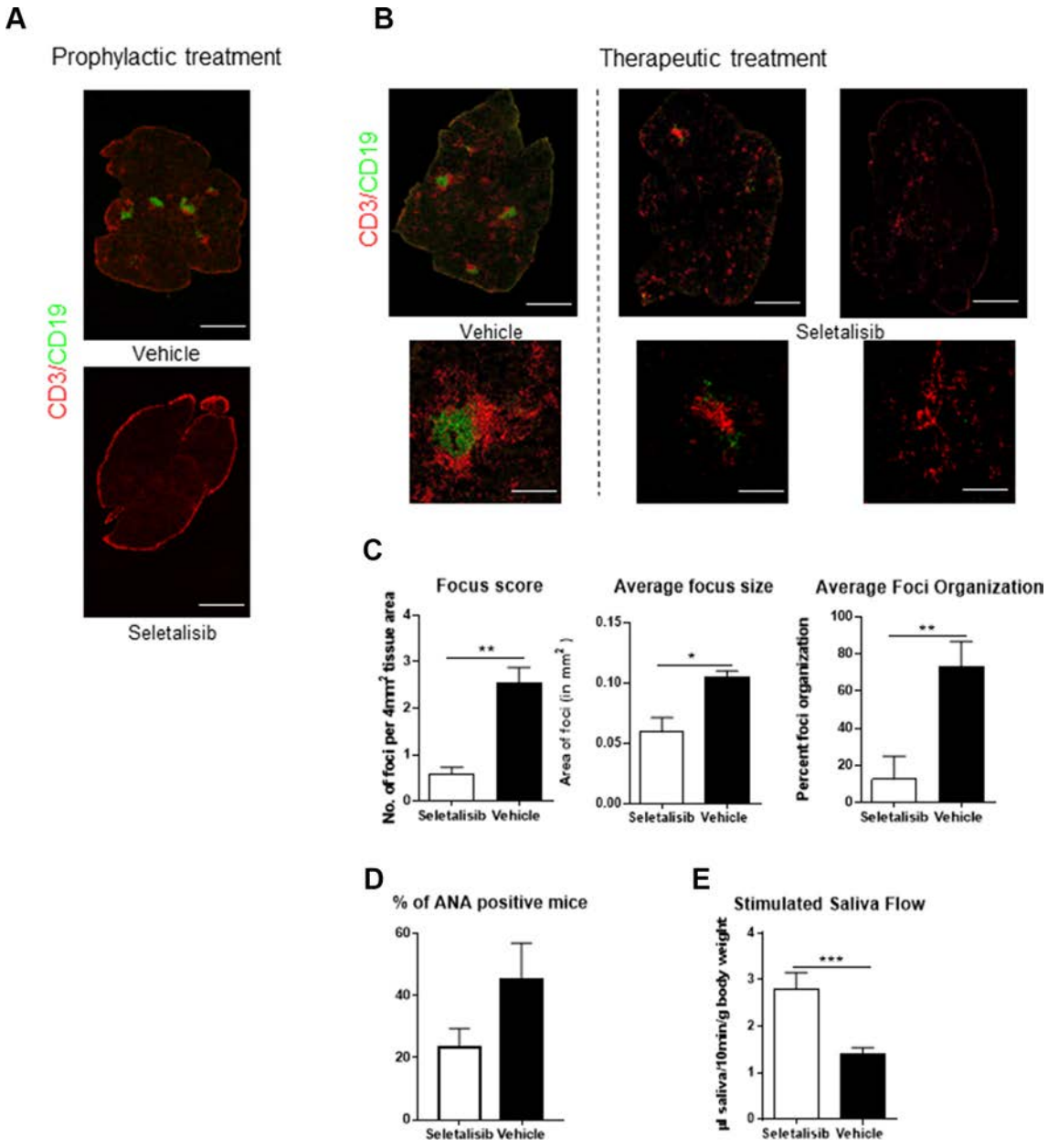

Figure 4 (A and B) Microphotograph of lymphoid aggregates in salivary glands of wild-type (WT) mice at day 15 postcannulation (pc) treated with seletalisib prophylactically or therapeutically as compared with vehicle controls (black bars) stained for CD3 (red) and CD19 (green). Scale bars=500 µm (tile scans) and 100 µm (foci snapshots). (C) Graphs represent the focus score (number of lymphocytic foci (>50 lymphocytes) per 4 mm^2), average size of foci and percentage of segregated aggregates in cannulated salivary glands from therapeutically treated mice as compared with controls. Results represented as mean±SE of two independent experiments with five mice per group; *p<0.05, **p<0.01, ***p<0.001, unpaired t-test. (D) Graphs represent percentage of antinuclear antibodies (ANA) positive mice from seletalisib-treated mice as compared with controls. Results represented as mean±SD of two independent experiments with 10 mice per group, unpaired t-test. (E) Graph comparing salivary flow in seletalisib-treated mice and vehicle controls measured at day 15 pc. Salivary flow is measured as milligrams of saliva produced in 10 min/body weight following pilocarpine stimulation (see the Methods section). Results represented as mean±SD of three independent experiments with 10 mice per group, unpaired t-test.

Interestingly, control lymphoid tissue obtained from mice treated with seletalisib (lymph node and blood) showed minimal impact of the drug on circulating B cells and in the lymph node on the CD4/CD8 ratio (online supplementary figure 6). The anatomical structure of the secondary lymphoid organs was fully conserved in these animals (data not shown).

DISCUSSION

Here, we provide evidence that the PI3Kδ pathway is active and functional in pSS and its blockade in vivo interferes with local and systemic disease progression in an animal model of focal sialoadenitis.

Aberrant B cell activation is the hallmark of pSS. B cell number rises in the SGs during disease progression, correlating with a higher FSC, higher autoantibody titres and the presence of systemic manifestations.[10 19 22 35 36] The increased risk of lymphoma development also correlates with the progressive aggregation of B cells within the SGs and, while a positive association between lymphoma development and GC formation has not been established, the negative

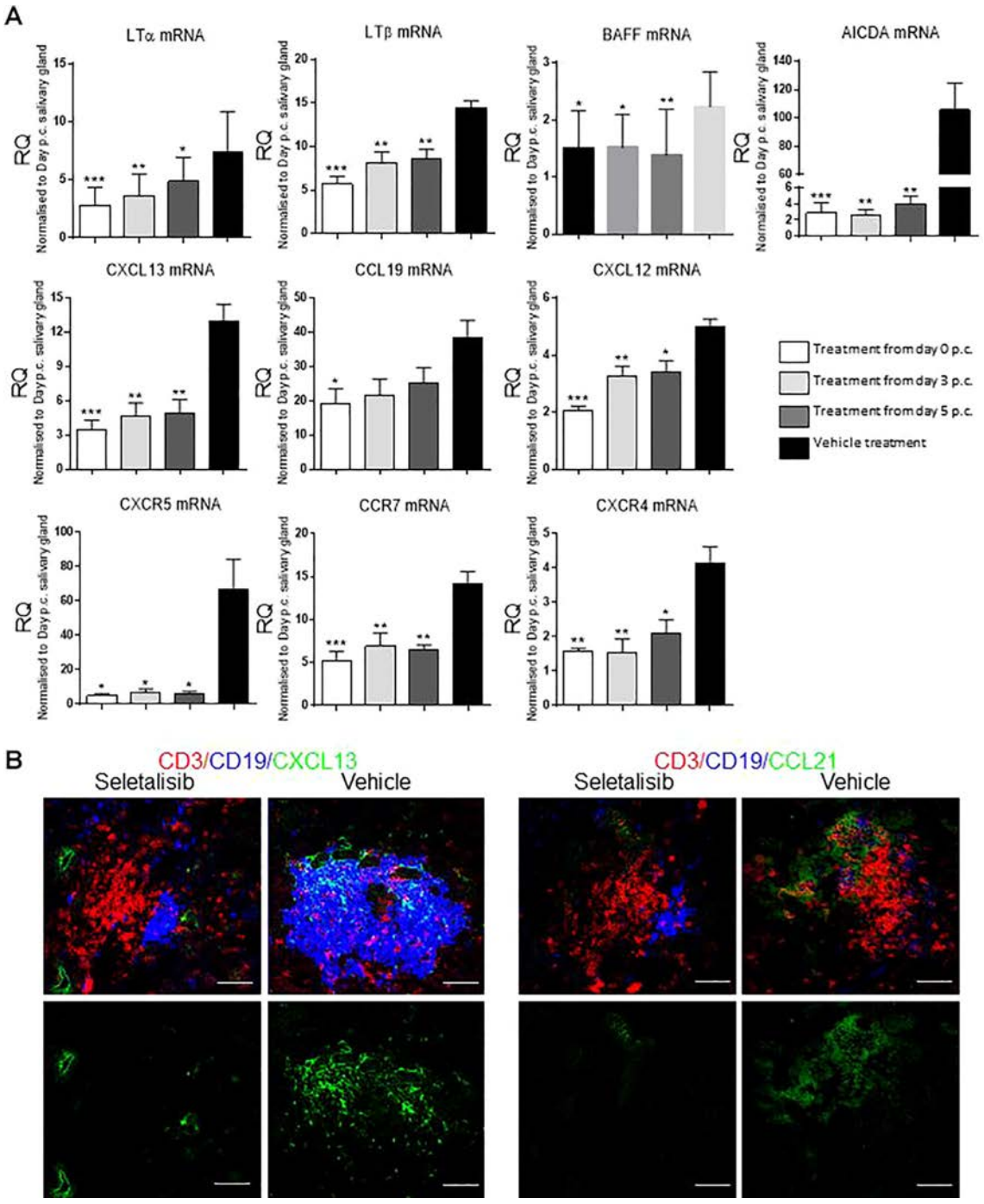

Figure 5 (A) Quantitative real-time-PCR analysis of ltα, ltβ, cxcl13, ccl19, cxcl12, cxcr5, ccr7,cxcr4, baff and aicda mRNA transcripts in salivary glands of wild-type mice at day 15 postcannulation (pc) treated with seletalisib at day 0 (white bars), day 3 (light grey) and day 5 (dark grey) pc as compared with vehicle controls (black bars). Results represented as mean±SD of two independent experiments with five mice per group; *p<0.05, **p<0.01, ***p<0.001, one-way analysis of variance (ANOVA). (B) Microphotograph showing CXCL13 and CCL21 protein expression (green) in day 15 ADV5-infected salivary glands from seletalisib-treated mice as compared with vehicle. T cells (CD3 red) and B cells (CD19 green) are also shown. Scale bars=20 µm.

predictive value of the absence of GC in lymphomagenesis seems clear.[13 17 22 37] More recently, an increased frequency of transitional B cells and mature naive B cells expressing polyreactive antibodies has been demonstrated in the peripheral blood of patients with pSS, confirming that impaired peripheral B cell tolerance plays a critical role in pSS pathogenesis.[38] Accordingly, we previously demonstrated that altering B cell recruitment by blocking the interleukin (IL)-22 mediated production of CXCL13 reduces the formation of SG aggregates and abrogates production of autoantibodies in a mouse model of pSS.[30]

PI3Kδ regulates key aspects of B cell homeostasis. B cells derived from mice deficient in PI3Kδ activity or wild type B cells treated with the PI3Kδ inhibitor all display reduced proliferative ability and increased susceptibility to apoptosis in response to anti-CD40, IL-4 or anti-IgM stimulation.[39 40] Moreover, both B cell response to the BAFF[41] and to the chemoattractant CXCL13 and shingosine-1-phosphate

(S1P) largely relies on PI3Kδ via activation of Rap1, a key GTPase in B lymphocyte migration.[42 43] Memory T cell generation and function is also impaired in the absence of PI3Kδ[6]; thus, unsurprisingly, T cell-dependent antibody responses are also affected in the absence of PI3Kδ isoform.[44] Finally, PI3Kδ-deficient lymphocytes are unable to form polarised synapses efficiently.[44 45]

While the rationale for PI3Kδ targeting in B cell driven autoimmune condition is clear, target validation has not been reported for pSS. Here we demonstrate that the PI3Kδ pathway is activated in pSS and clearly differentiates pSS samples from control sialoadenitis. Expression of the PI3Kδ transcript correlates with manifestations of B cell hyperactivity, including autoantibody production, formation of GCs and hyperglobulinaemia. The expression of pS6, a downstream adaptor of the PI3Kδ pathway, was anticipated in T and B lymphocytes but this marker was also detected in myeloid cells and plasma cells. This finding suggests that patients with pSS and in particular those manifesting B cell symptoms and those characterised by a 'plasma cell signature'[46] have an increased engagement of the PI3Kδ pathway and would benefit from a treatment targeting its activation.

We used a small molecule seletalisib (UCB Celltech), previously demonstrated to be safe and efficacious in patients with psoriasis,[27 47] to target this pathway in vivo, in a model of inducible sialoadenitis.[26] Treatment of cannulated mice with seletalisib resulted in downregulation of S6 phosphorylation and decreased conversion of PIP2 in PIP3, demonstrating the ability of seletalisib to inhibit PI3Kδ activation in treated samples. PI3Kδ blockade in the SGs of our murine model resulted in significantly decreased lymphocyte infiltration, both in terms of T and B cells, disrupted lymphocyte organisation, reduction in autoantibody production, abrogated transcription of lymphoid chemokines and cytokines and improvement in saliva production. In peripheral organs, we observed a non-significant decreased on total cellularity and some changes in the T/B cell ratio and CD4/CD8 ratio. In the blood, we observed a more profound effect on cellularity, probably due to bioavailability and a decrease in total B cell number. Importantly and in agreement with previous publications in human,[27 47] including a recent study in pSS,[48] the mice did not show any sign of infection or unexplained weight loss.

It has been previously demonstrated by us and others that lymphocytes, and in particular T cells, imprint the local microenvironment by releasing LTα, β and proinflammatory cytokines, such as IL-22 or IL-17 that, in turn, regulate the expression of lymphoid chemokines and survival factors necessary for ectopic lymphocyte homing and maintenance in the tissue.[30 49–58] Here we establish that inhibition of PI3Kδ, in seletalisib-treated mice, affects both T and B cells, directly interfering with the establishment of the pathogenic SG microenvironment, preventing the formation of the GC and the perpetuation of local disease.[10 29 30 59–62] These data are in line with previous reports highlighting the role of PI3Kδ in the differentiation of T cells into T helper cells, required for effective GC responses and antibody production.[45 63 64] Accordingly, in our model, abrogation of tissue pathology was accompanied by decreased autoantibody production.

While the effect on antigen presentation and B cell function have been largely described[4 45] and hereby confirmed by the decrease in IL-23 and DC number in the SGs, blood and lymph node, our data highlight a clear requirement for this pathway on plasma cells in our model. In patients with pSS, the aberrant levels of autoantibodies and immunoglobulin are used as biomarkers for disease activity and prognosis.[20 21] GC in the SGs are able to support B cell affinity maturation; moreover, Ro+ and La+ plasma cells have been demonstrated at the periphery of large intraglandular foci. The detection of long-lived CD138+ Bcl-2+ plasma cells in pSS SG has also been associated with higher FSCs,[60 65] more severe systemic manifestation and increased lymphoma risk,[23 66–69] thus establishing that in pSS, local and systemic activation of the plasma cell compartment is involved in disease progression. Here, we demonstrate intense pS6 staining within SG infiltrating plasma cells, suggesting that even on activation, plasma cells are reliant on the PI3Kδ pathway for homeostatic maintenance. Accordingly, in vivo treatment with seletalisib significantly affects plasma cell numbers and abrogates autoantibody production in murine sialoadenitis. Similar data on the efficacy of a PI3Kδ blocking agent have been reported in a phase 2 study, showing a decrease in immunoglobulins in pSS-treated patients as compared with placebo. While primary endpoints were not met in this first study, effects on plasma cells and safety profile from this study support the continued investigation of PI3Kδ inhibitors such as seletalisib in pSS.[48]

All together, these data and the significant correlation between PI3Kδ expression in the glands and clinical manifestations associated with B cell hyperactivation strongly support the evaluation of seletalisib in patients characterised by systemic manifestations, including high levels of immunoglobulins, presence of GCs and high FSC in the biopsies, often identifiable with high levels of ESSDAI.[70–75]

In pSS, B cell-depleting agents, such as rituximab, failed to demonstrate significant clinical success in phase 3 randomised clinical trials, and disease relapse has been observed in patients with pSS (and lymphoma) treated with rituximab.[76–78] While these disappointing findings with rituxumab might in part be due to trial design and choice of outcome measure, biologically, there is evidence of expansion of pathogenic B cell clones following depletion, allegedly supported by the persistent production of survival and chemotactic factors in the SG microenvironment.[77 79–84] Of note, rituximab is unable to target long-lived plasma cells (CD20 negative) directly, thus leaving the autoantibody producing reservoir intact.[85] Consequently, a strategy that aims to target plasma cells directly, alongside T and B lymphocytes, using an agent such as seletalisib, would be desirable in patients with pSS presenting a clear plasma cell signature.[46] Our findings, confirm that in pSS, PI3Kδ has a pleotropic effect on the homeostasis of T, B lymphocytes (including GC B cells) and plasma cells. Selective targeting of PI3Kδ using seletalisib significantly impacts pathogenic microenvironment in the inflamed murine glands, while affecting, systemically, the production of autoantibodies. Overall, these results appear to confirm a mechanistic role for PI3Kδ activity in the immunopathogenesis of pSS supporting the presence and engagement of this pathway in patients characterised by local and systemic B cell hyperactivity. Overall, these results appear to confirm a mechanistic role for PI3Kδ activity in the immunopathogenesis of pSS supporting the presence and engagement of this pathway in human pSS salivary gland and warranting the further evaluation of seletalisib in clinical trials in patients with pSS.

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EPIDEMIOLOGICAL SCIENCE

Cardiovascular, thromboembolic and renal outcomes in IgA vasculitis (Henoch-Schönlein purpura): a retrospective cohort study using routinely collected primary care data

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ABSTRACT

Background IgA vasculitis (IgAV, Henoch-Schönlein purpura) is a small-vessel vasculitis most common in children but also occurring in adults. Case series have suggested that IgAV may be associated with cardiovascular disease and venous thromboembolism, but this has not been evaluated in population-based studies. Renal disease and hypertension are possible complications of the disease with unknown incidence.

Methods Using a large UK primary care database, we conducted an open retrospective matched cohort study of cardiovascular, venous thrombotic and renal outcomes in adult-onset and childhood-onset IgAV. Control participants were selected at a 2:1 ratio, matched for age and sex. Adjusted HRs (aHRs) were calculated using Cox proportional hazards models.

Results 2828 patients with adult-onset IgAV and 10 405 patients with childhood-onset IgAV were compared with age-matched and sex-matched controls. There was significantly increased risk of hypertension (adult-onset aHR 1.42, 95% CI 1.19 to 1.70, $p < 0.001$; childhood-onset aHR 1.52, 95% CI 1.22 to 1.89, $p < 0.001$) and stage G3–G5 chronic kidney disease (adult-onset aHR 1.54, 95% CI 1.23 to 1.93, $p < 0.001$; childhood-onset aHR 1.89, 95% CI 1.16 to 3.07, p=0.010). There was no evidence of association with ischaemic heart disease, cerebrovascular disease or venous thromboembolism. All-cause mortality was increased in the adult-onset IgAV cohort compared with controls (aHR 1.27, 95% CI 1.07 to 1.50, p=0.006).

Conclusions Patients with IgAV are at increased risk of hypertension and chronic kidney disease (CKD) compared with individuals without IgAV; analysis restricted to adult-onset IgAV patients showed increased mortality. Appropriate surveillance and risk factor modification could improve long-term outcomes in these patients.

Key messages

What is already known about this subject?
- IgA vasculitis (IgAV) is recognised to occur in both children and adults; however, the incidence and prevalence, especially in adults, is unknown in large populations based in primary care.
- IgAV is associated with long-term complications including chronic kidney disease.
- Small case series have previously suggested a predisposition to ischaemic heart disease and venous thromboembolism in some patients.

What does this study add?
- The risk of hypertension and CKD is significantly increased in adult-onset and childhood-onset IgAV compared with the general population.
- IgAV is not significantly associated with ischaemic heart disease or venous thromboembolism in this study.
- The young age of most patients and short follow-up in this study mean longer follow-up is required to address the risk of ischaemic heart disease.

How might this impact on clinical practice or future developments?
- Clinicians looking after patients who have had IgAV should routinely monitor for hypertension and CKD

INTRODUCTION

IgA vasculitis (IgAV), also termed Henoch-Schönlein purpura, is a small-vessel vasculitis most frequently affecting children.[1] IgAV is the most common childhood vasculitis in the UK, with estimated annual incidence of 20/100 000 children under the age of 17 years according to the largest regional study.[2] However, incidence rates vary widely between study populations, and, furthermore, these may represent underestimates of true incidence.[3] The epidemiology of adult-onset IgAV is less well-studied, but hospital-based studies indicate an estimated annual incidence of 0.8–1.8/100 000 population.[3]

IgAV may be complicated by glomerulonephritis[4] and it is thought that adult-onset IgAV is associated with increased risk and severity of renal involvement compared with childhood disease.[5 6] However, long-term health outcomes of adult-onset IgAV are not well characterised. Most evidence regarding complications of IgAV in adults derives from case reports and case series[7] ; there is need for controlled epidemiological studies to address this question.

Other outcomes associated with IgAV are unknown. Multiple case reports have raised the possibility of associations between IgAV and venous thromboembolism (VTE), hypertension and

ischaemic heart disease (IHD) in both children and adults.[8–19] However, many cases involved patients with additional risk factors, making the role of IgAV unclear. To date, the incidence of these outcomes has not been examined in a large cohort study. Furthermore, there is emerging evidence that patients with other vasculitides have increased incidence of cardiovascular disease[20 21] and receive inadequate management of cardiovascular risk.[22]

This study aims to calculate incidence of IgAV in adults and children, and to quantify risk of important complications in adult and childhood-onset disease, in particular, risk of cardiovascular, thromboembolic and renal outcomes. These data will facilitate prognostication in such patients, thus informing strategies for surveillance and risk factor modification in routine care.

METHODS

Study design

Incidence and prevalence of IgAV

To calculate IgAV incidence, annual cohort studies were performed between 1 January 2005 and 31 December 2016. To estimate prevalence, sequential cross-sectional studies were carried out on 1 January each calendar year from 2005 to 2016.

Chronic outcomes

An open retrospective matched cohort study was performed to compare long-term cardiovascular, venous thromboembolic and renal outcomes in adults and children diagnosed with IgAV and randomly selected age-matched and sex-matched controls without a diagnosis. The study period was 1 January 1995 to 15 May 2017.

Data source

Data were extracted from The Health Improvement Network (THIN) database, which comprises anonymised medical records for 3.6 million active patients from >675 general practices, as previously reported.[23 24] Patient data are derived from practices using Vision electronic medical record software, which stores information in a hierarchical system of clinical (Read) codes.[25] THIN includes information on patient demographics, diagnoses, prescriptions and investigations. THIN has previously been validated for studies of cardiovascular and renal outcomes,[26 27] and for studies of VTE risk.[28] It is broadly representative of the UK population in terms of demographics, disease prevalence and mortality rates.[23] To maximise data quality, general practices were only included in this study from the latest of 1 year after they began using Vision software and 1 year after their acceptable mortality recording date.[24] Diagnosis was based on Read codes which registered a clinical diagnosis of Henoch-Schönlein purpura, and not IgA deposition within tissues.

Study population

Incidence and prevalence of IgAV

Adults and children with no record of a IgAV diagnosis at the beginning of each 1-year study period were included in the annual incidence cohorts. The eligible populations were followed from 1 January every year until the earliest of the following dates: IgAV diagnosis, patient left the practice, death or 31 December of that year. Annual prevalence is reported per 100 000 population.

Chronic outcomes

In the adult-onset IgAV cohort, patients were eligible for inclusion if they had a clinical code for IgAV recorded at age ≥16 years. Inclusion in the childhood-onset IgAV cohort was restricted to patients with recorded diagnosis of IgAV before the age of 16 years. For each patient with IgAV, two age-matched and sex-matched control patients were randomly selected from a pool of eligible controls. All patients were required to be registered with their general practice for at least 1 year before study entry.

Index date in the exposed group was the date of first documentation of IgAV after study entry for incident cases (newly diagnosed patients) or date of study entry for prevalent cases (patients with an existing diagnosis). To avoid immortal time bias,[29] controls were assigned the same index date as their corresponding exposed patient. Participants were followed up until the earliest of the following dates: outcome event, death, patient left practice, practice stopped contributing to the database and study end.

Outcomes

In patients with adult-onset IgAV, primary outcomes were IHD, VTE, stroke/transient ischaemic attack (TIA), hypertension, stage G3–G5 CKD and all-cause mortality. IHD, VTE, stroke/TIA and hypertension were defined by clinical (Read) codes; stage G3–G5 CKD was defined by new-onset estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2 on two consecutive measurements separated by at least 90 days.[30] Clinical codes were selected based on quality and outcomes framework (QOF) business rules and previously published studies.[31 32]

For hypertension, a sensitivity analysis was performed using a lag period of 1 year to test whether this represented a chronic outcome or was solely related to the acute illness.

In the childhood-onset IgAV cohort, primary outcomes were hypertension, VTE and CKD. IHD, stroke/TIA and mortality were not studied in this cohort due to low incidence in this age group and short follow-up period.

Analysis

Annual incidence rates (IRs) of IgAV were calculated by dividing the number of newly diagnosed IgAV patients by person-years at risk for adults and children separately.

Cox proportional hazards models were used to calculate crude HRs and adjusted HRs (aHR) for each outcome in IgAV compared with controls. Breslow's method was used to handle tied survival times where required. All models were adjusted for the following covariates: age, sex, body mass index (BMI) category, Townsend deprivation quintile and smoking status. Additionally, the models for IHD and stroke/TIA were adjusted for baseline diabetes, hypertension and lipid-lowering drug prescription; the model for hypertension was adjusted for baseline diabetes and lipid-lowering drug prescription; the model for CKD was adjusted for baseline diabetes and hypertension; and the model for all-cause mortality was adjusted for lipid-lowering drug prescription and Charlson comorbidity index (CCI). For childhood-onset IgAV, BMI category, lipid-lowering drug prescription and smoking status were not included in the models. BMI recorded closest to index date was categorised as '<25 kg/m^2', '25–30 kg/m^2' (overweight) and '>30 kg/m^2' (obesity); smoking status was categorised as 'smoker', 'ex-smoker' and 'non-smoker'. Social deprivation was categorised according to Townsend deprivation quintile.[33]

CCI was categorised as '0', '1', '2' or '>2'.[34] Separate categories were created for missing data, which were included in the regression analyses.

Baseline renal function was not adjusted for in the primary analysis due to limited availability of creatinine measurements before the index date. A sensitivity analysis was performed in which all models were adjusted for baseline eGFR.

For the adult-onset and childhood-onset studies, all patients without a record of the outcome under study at baseline were included in the primary analysis. For the CKD study, primary analysis included only patients with an eGFR >60 mL/min/1.73 m^2 at baseline. Sensitivity analyses were performed in which (1) patients with missing baseline eGFR values were included and categorised as having normal renal function and (2) all patients were included regardless of baseline eGFR, assuming that baseline eGFR might reflect transient residual renal impairment.

To ensure that results are applicable to disease of adult onset, a sensitivity analysis was performed using incident (newly diagnosed, definite adult-onset) adult IgAV cases only.

Analyses were performed using STATA V.14.0. Statistical significance was set at $p<0.05$.

RESULTS

Incidence of IgAV in adults and children

Between 2005 and 2016, incidence of childhood-onset IgAV was 27.22 per 100 000 person-years; incidence of adult-onset IgAV was 2.20 per 100 000 person-years (figure 1; baseline characteristics are summarised in online supplementary table S2. Mean (SD) age at diagnosis was 6.68 years (3.41) years for children and 38.1 (18.8) years for adults. While IgAV incidence remained stable, prevalence of both adult-onset and childhood-onset IgAV increased over the study period.

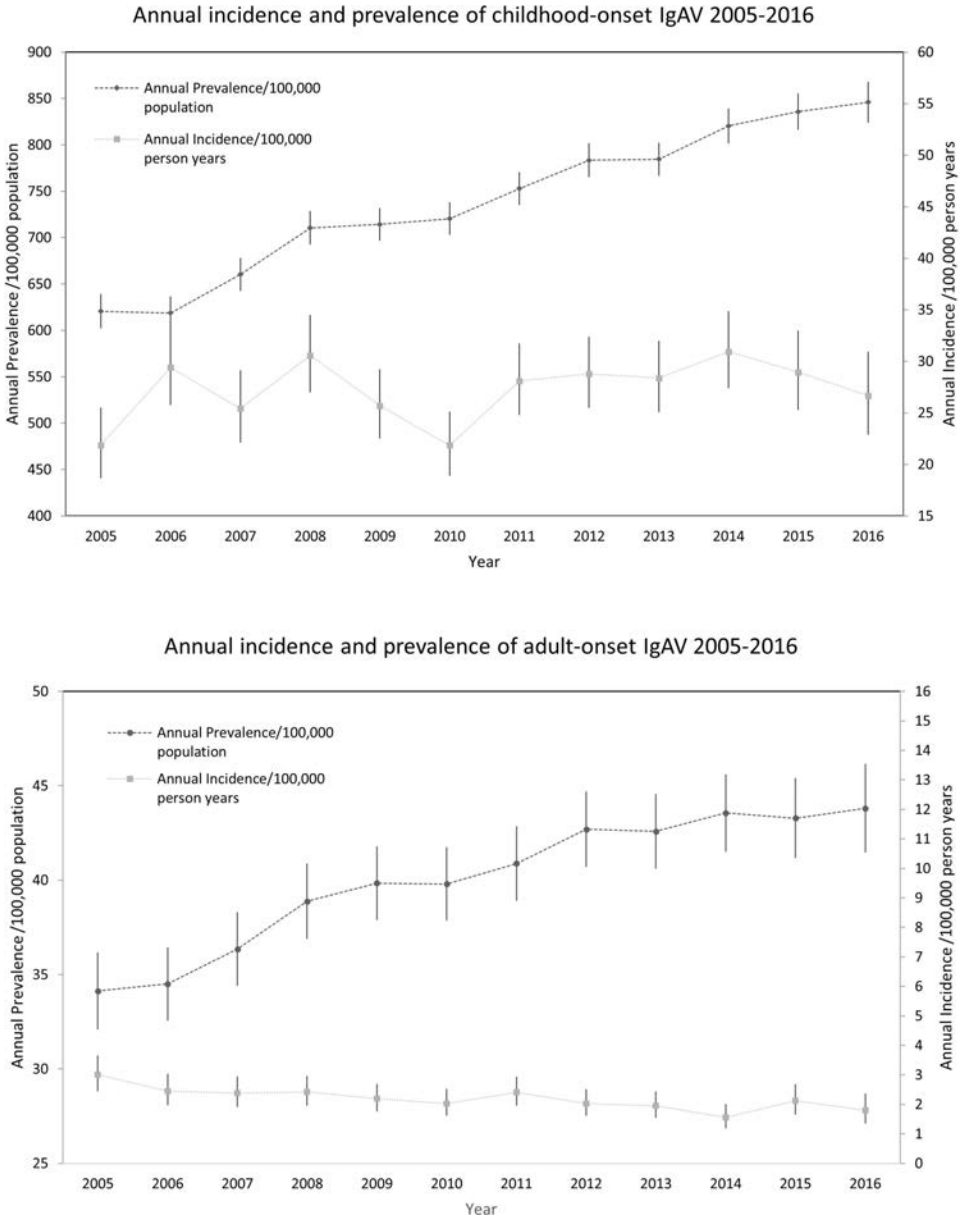

Figure 1 Annual incidence and prevalence for childhood-onset and adult-onset IgA vasculitis (IgAV) from 2005 to 2016. (A) Annual incidence (squares) and prevalence (circles) for childhood-onset IgAV (95% CIs shown). (B) Annual incidence (squares) and prevalence (circles) for adult-onset IgAV (95% CIs shown).

Between 2005 and 2016, prevalence of adult-onset IgAV increased from 34 to 44 per 100 000 population; prevalence of childhood-onset IgAV increased from 621 to 846 per 100 000 population.

Outcomes in adult-onset IgAV

Baseline characteristics

There were 2828 patients with adult-onset IgAV and 5655 controls. Median (IQR) follow-up was similar in both cohorts: 4.91 2.07–9.08 years in the IgAV cohort and 4.99 2.18–9.05 years in the control cohort.

Mean age at study entry was 43 years in both exposed and unexposed cohorts. Both cohorts had 48.4% males. At baseline renal impairment (eGFR <60 mL/min/1.73 m^2) was more common in patients with IgAV (6.86% vs 4.05% in unexposed). Similar differences between those with and without IgAV were observed for hypertension (18.5% vs 13.0%), diabetes mellitus (5.3% vs 3.7%), VTE (1.8% vs 1.1%), lipid-lowering drug prescription (11.1% vs 5.0%) and CCI (15.28% vs 7.35% with ≥2 comorbidities). Patients with IgAV were less likely to be current smokers 18.5% versus 22.8%. The cohorts were similar with respect to BMI and Townsend deprivation quintile (table 1).

Table 1 Baseline characteristics of the adult-onset and childhood-onset IgA vasculitis (IgAV) cohorts with corresponding controls. adult-onset patients include all incident and prevalent IgAV cases with a date of diagnosis after the age of 16 years

Baseline characteristics (SD or percentage)				
	Adult-onset IgAV		Childhood-onset IgAV	
	Exposed	Unexposed	Exposed	Unexposed
Patients, n	2828	5655	10 405	20 810
Median follow-up period (years)	4.91 (IQR 2.07–9.08)	4.99 (IQR 2.18–9.05)	4.86 (IQR 2.06–9.08)	4.98 (IQR 2.17–9.86)
Mean age at study entry (years)*	43.33 (18.8)	43.33 (18.7)	17.57 (13.12)	17.59 (13.13)
Mean age at IgAV diagnosis	38.09 (18.8)	N/A	6.68 (3.41)	N/A
Gender (male)	1370 (48.4%)	2739 (48.4%)	5545 (53.29%)	11 090 (53.29%)
Gender (female)	1458 (51.6%)	2916 (51.6%)	4860 (46.71%)	9720 (46.71%)
Mean body mass index	26.9 (6.1)	26.2 (5.5)	N/A	N/A
Smoking status				
Current smoker	522 (18.5%)	1289 (22.8%)	1178 (11.32%)	2102 (10.10%)
Ex-smoker	525 (18.6%)	845 (14.9%)	528 (5.07%)	904 (4.34%)
Non-smoker	1525 (53.9%)	2825 (50.0%)	2751 (26.44%)	5427 (26.08%)
Not available	256 (9.05%)	696 (12.31%)	5,948 (57.16%)	12,377 (59.48%)
Hypertension	523 (18.5%)	734 (13.0%)	185 (1.78%)	236 (1.13%)
Diabetes mellitus	149 (5.3%)	210(3.7%)	66 (0.63%)	133 (0.64%)
VTE	52 (1.8%)	61 (1.1%)	29 (0.28%)	37 (0.18%)
IHD	139 (4.9%)	220 (3.9%)	N/A	N/A
Stroke and TIA	68 (2.4%)	107 (1.9%)	N/A	N/A
eGFR category				
>90 mL/min per 1.73 m^2	639 (22.60%)	856 (15.14%)	116 (0.56%)	100 (0.96%)
60–90 mL/min per 1.73 m^2	697 (24.65%)	1160 (20.51%)	121 (0.58%)	157 (1.51%)
30–59 mL/min per 1.73 m^2	164 (5.80%)	209 (3.70%)	1339 (6.43%)	707 (6.79%)
<30 mL/min per 1.73 m^2	30 (1.06	20 (0.35%)	1358 (6.53%)	642 (6.17%)
N/A	1298 (45.90%)	3410 (60.30%)	17 876 (85.90%)	8799 (84.57%)
Lipid-regulating medication use	313 (11.1%)	506 (5.0%)	N/A	N/A
Current contraceptive use†	336 (23.1%)	572 (19.6%)	799 (16.44%)	1,432 (14.73%)
Townsend deprivation quintile				
(Least deprived) 1	622 (22.0%)	1231 (21.8%)	2216 (21.30%)	4529 (21.76%)
2	542 (19.2%)	1161 (20.5%)	1927 (18.52%)	3863 (18.56%)
3	557 (19.7%)	1080 (19.1%)	1968 (18.91%)	4055 (19.49%)
4	476 (16.8%)	955 (16.9%)	1856 (17.84%)	3633 (17.46%)
5	334 (11.8%)	670 (11.9%)	1293 (12.43%)	2614 (12.56%)
NA	297 (10.5%)	558 (9.9%)	1145 (11.0%)	2116 (10.17%)
Charlson comorbidity index				
0	1806 (63.86%)	4176 (73.85%)	N/A	N/A
1	590 (20.86%)	1063 (18.80%)	N/A	N/A
2	259 (9.16%)	258 (4.56%)	N/A	N/A
> 2	173 (6.12%)	158 (2.79%)	N/A	N/A

Childhood-onset patients include all incident and prevalent IgAV cases with a date of diagnosis before the age of 16 years. Controls were age-matched and sex-matched in a 2:1 ratio.

*Note that many patients had a IgAV diagnosis prior to study entry—prevalent cases. eGFR,estimated glomerular filtration rate; IHD, ischaemic heart disease; N/A, not available; VTE, venousthromboembolism.

†Current contraceptive use percentage reported for females only.

Table 2 Summary of primary outcomes in adult-onset IgA vasculitis (IgAV) cases and corresponding controls

	Hypertension		Ischaemic heart disease		Stroke/TIA		Venous thromboembolism		Chronic kidney disease		All-cause mortality	
	IgAV	Control	IgAV	Control	IgAV	Control	IgAV	Control	IgAV	Control	IgAV	Control
Patients, n	2305	4921	2689	5435	2760	5548	2776	5594	2487	5225	2828	5655
Numbers of outcomes	196	315	53	104	68	107	28	46	134	185	238	348
Person-years	12 847.60	28 174.46	15 974	32 542.38	16 552.39	33 468.58	16 771.07	33 813.95	15 359.92	32 167.31	17 085.13	34 391.52
Incidence rate (per 1000 person-years)	15.26	11.18	3.32	3.20	3.14	3.26	1.67	1.36	8.72	5.75	13.93	10.12
Crude HR (95% CI)	1.36 (1.14–1.63)		1.04 (0.75–1.44)		0.96 (0.69–1.34)		1.22 (0.76–1.96)		1.52 (1.22–1.90)		1.37 (1.17–1.62)	
P values	0.001		0.829		0.814		0.401		<0.001		<0.001	
Adjusted HR (95% CI)	1.42 (1.19–1.70)		1.08 (0.77–1.52)		0.95 (0.68–1.32)		1.21 (0.76–1.95)		1.54 (1.23–1.93)		1.27 (1.07–1.50)	
P values	<0.001		0.637		0.758		0.424		<0.001		0.006	

Hypertension

In total, 196 IgAV patients (6.93%) received a diagnosis of hypertension compared with 315 (5.57%) controls (table 2); incidence was 15.26 and 11.18 per 1000 person-years, respectively: aHR 1.42 (95% CI 1.19 to 1.70). Cumulative hazard curves are shown in figure 2. Results were robust in two sensitivity analyses restricting outcome to hypertension diagnosed at least 1 year after the index date and restricting to incident IgAV cases and their matched controls (online supplementary table S2).

Ischaemic heart disease and cerebrovascular disease

In total, 53 patients (1.87%) with adult-onset IgAV and 104 (1.84%) controls were diagnosed with IHD, corresponding to IRs of 3.32 and 3.20 per 1000 person-years, respectively. There was no evidence of association between IgAV and risk of IHD (aHR 1.08, 95% CI 0.77 to 1.52). Also, 52 patients with IgAV (1.84%) and 109 controls (1.93%) experienced a stroke/TIA, with IRs of 3.14 and 3.26 per 1000 person-years, respectively. There was no evidence of association between IgAV and risk of stroke/TIA (aHR 0.95, 95% CI 0.68 to 1.32).

Venous thromboembolism

In total, 28 patients with adult-onset IgAV (0.99%) and 46 controls (0.81%) were coded with a VTE event; incidence was 1.67 and 1.36 per 1000 person-years, respectively. Crude and adjusted HRs were not statistically significant: aHR 1.21 (95% CI 0.76 to 1.95).

Chronic kidney disease

There were 134 incident cases of CKD stages G3–5 (5.11%) in the adult-onset IgAV cohort compared with 185 (3.42%) in controls; incidence was 8.72 and 5.75 per 1000 person-years, respectively: aHR 1.54 (95% CI 1.23 to 1.93). This association remained significant in all sensitivity analyses, including adjustment for baseline eGFR (online supplementary table S2).

All-cause mortality

There were 238 deaths (8.42%) in the adult-onset IgAV cohort and 348 (6.15%) in the control cohort, corresponding to mortality rates of 13.93 and 10.12 per 1000 person-years, respectively. In the primary analysis, all-cause mortality was significantly increased in the adult-onset IgAV cohort compared with controls: aHR 1.27 (95% CI 1.07 to 1.50). However, in a sensitivity analysis using incident cases and their controls only, the effect was not statistically significant (aHR 1.09, 95% CI 0.83 to 1.42).

Adjusting for baseline eGFR did not affect the results for any outcome (online supplementary table S3).

Outcomes in childhood-onset IgAV

Baseline characteristics

In total, 10 405 patients with incident or prevalent childhood-onset IgAV were identified and matched to 20 810 controls without IgAV. Mean (SD) age at diagnosis for the IgAV cohort was 6.68 (3.4); mean age at study entry was 17.6 (13.1) years and 53% were male in both cohorts. Median (IQR) follow-up was similar: 4.86 2.06–9.08 years in the IgAV cohort and 4.98 2.17–9.86 years in controls. At baseline, more patients with IgAV had hypertension 1.78% versus 1.13% and VTE 0.28% versus 0.18% compared with controls (table 1). The cohorts were also similar with respect to baseline renal function, Townsend deprivation quintile and smoking status.

Hypertension

In total, 139 patients with IgAV (1.34%) and 193 controls (0.93%) had hypertension (table 3); incidence was 2.29 and 1.57 per 1000 person-years, respectively: aHR 1.52 (95% CI 1.22 to 1.89). This association remained significant in all of the sensitivity analyses performed (online supplementary table S3). Cumulative hazard curves are shown in figure 3.

Venous thromboembolism

In the childhood-onset IgAV cohort, 25 patients (0.24%) experienced VTE compared with 46 controls (0.22%); incidence was 0.40 and 0.37 per 1000 person-years, respectively. There was no evidence of association between childhood-onset IgAV and VTE (aHR 1.10, 95% CI 0.68 to 1.79).

Chronic kidney disease

There were 32 incident cases of CKD (0.31%) in patients with IgAV compared with 34 (0.16%) in the controls; incidence was 0.51 and 0.27 per 1000 person-years, respectively: aHR 1.89 (95%CI 1.16 to 3.07; see online supplementary table S3).

DISCUSSION

In this population-based study, we show that, compared with an age-matched and sex-matched control population, childhood-onset and adult-onset IgAV is associated with increased risk of hypertension and CKD. Adult-onset IgAV was not associated with IHD, cerebrovascular disease or VTE.

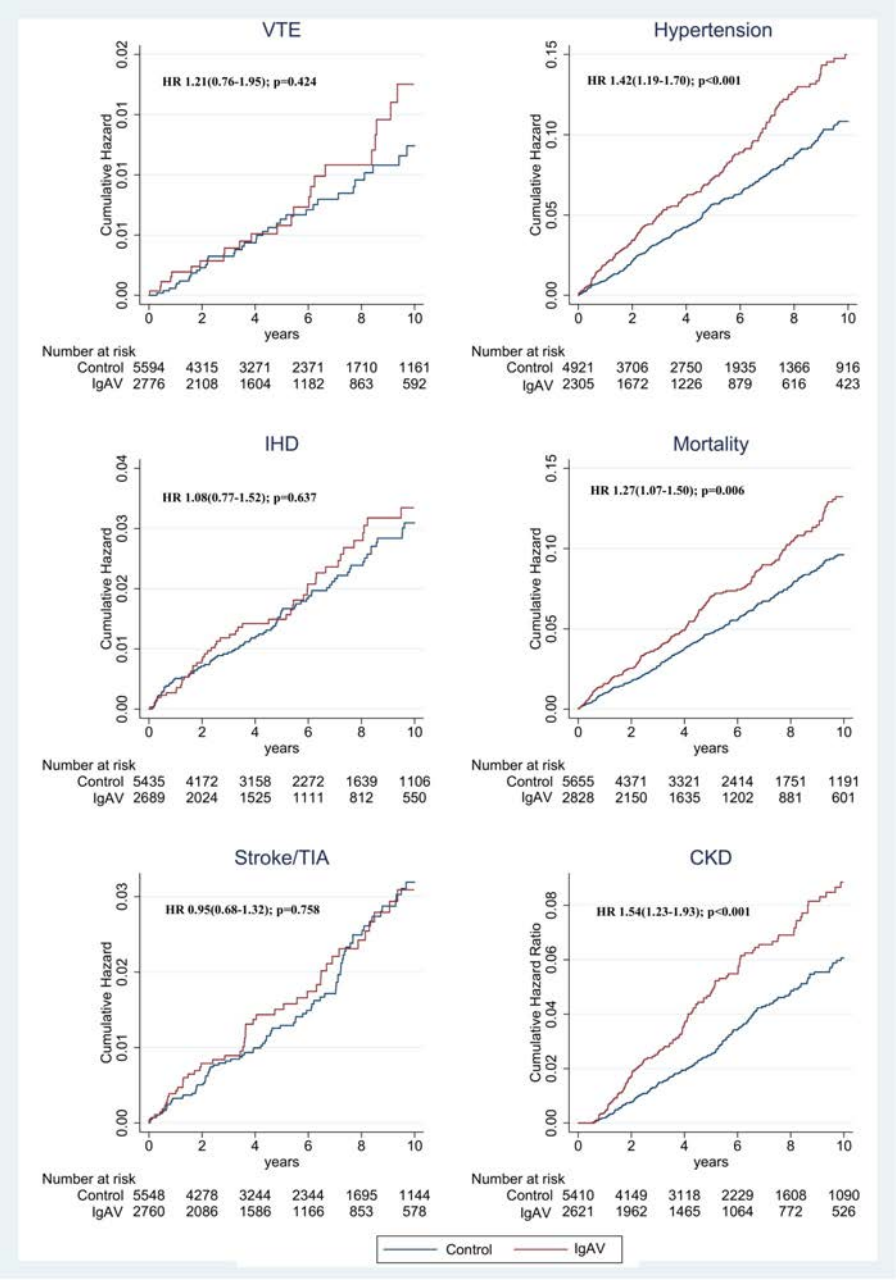

Figure 2 Cumulative hazard curves for outcomes in adult-onset IgA vasculitis (IgAV). Cumulative hazard curves are displayed for each of the six outcomes under study in the adult-onset IgAV cohort. IHD, ischaemic heart disease; VTE, venous thromboembolism.

Table 3 Summary of primary outcomes in childhood-onset IgA vasculitis (IgAV) cases and corresponding controls

	Hypertension		Venous thromboembolism		Chronic kidney disease	
	IgAV	Control	IgAV	Control	IgAV	Control
Patients, n	10 220	20 574	10 376	20 773	10 370	20 763
Numbers of outcomes	139	193	25	46	32	34
Person-years	60 753.91	123 318.1	62 333.31	125 359.3	62 185.31	125 362.3
Incidence rate (per 1000 person-years)	2.29	1.57	0.40	0.37	0.51	0.27
Crude HR (95% CI)	1.46 (1.17–1.81)		1.08 (0.66–1.75)		1.90 (1.17–3.08)	
Pvalues	0.001		0.762		0.009	
Adjusted HR (95% CI)	1.52 (1.22–1.89)		1.10 (0.68–1.79)		1.89 (1.16–3.07)	
P values	<0.001		0.697		0.010	

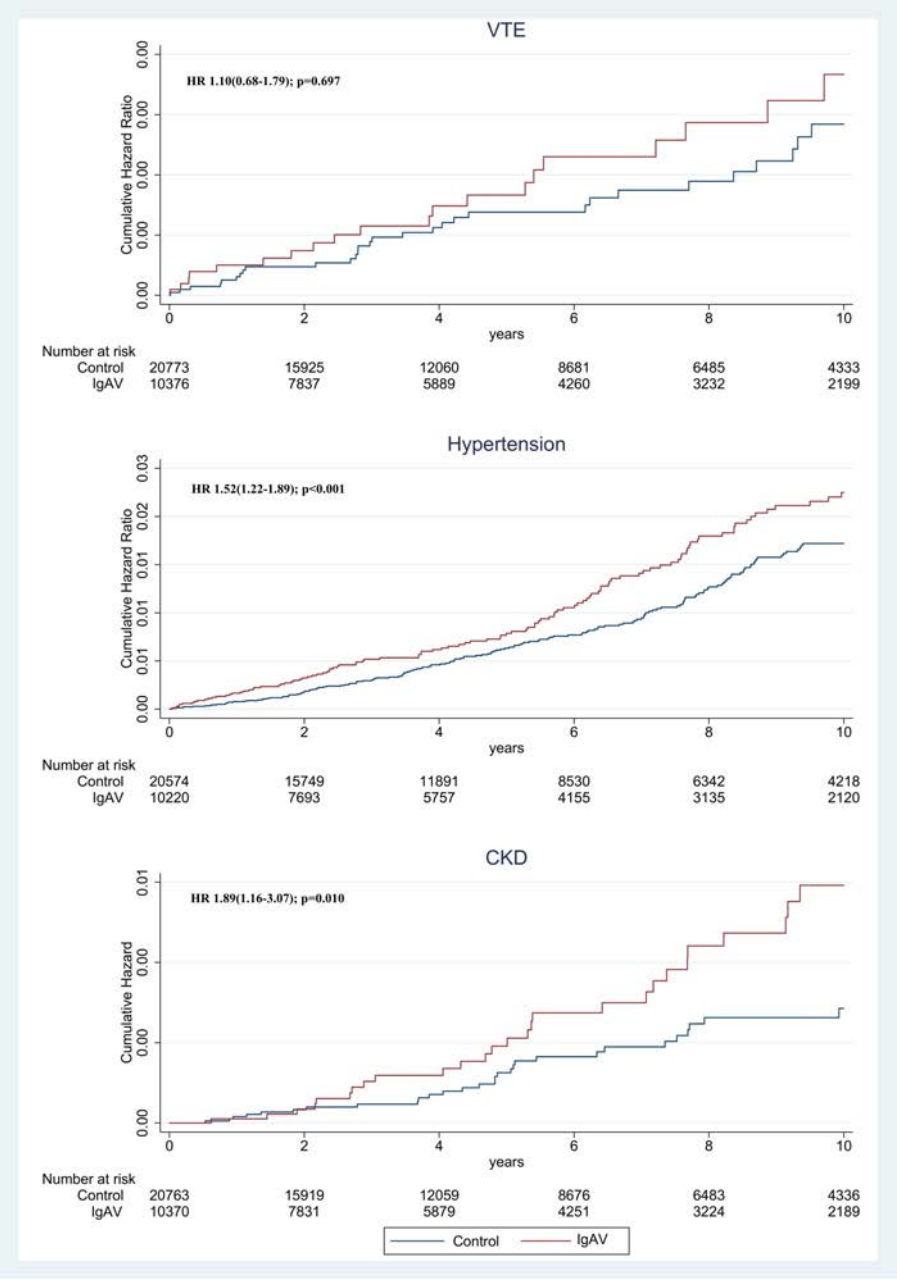

Figure 3 Cumulative hazard curves for outcomes in childhood-onset IgA vasculitis (IgAV). Cumulative hazard curves are displayed for each of the three outcomes under study in the childhood-onset IgAV cohort. VTE, venous thromboembolism.

Annual IgAV incidence from 2005 to 2016 in children (27.22 per 100 000 person-years) and adults (2.20 per 100 000 person-years) was marginally higher than previously reported (20 and 0.8–1.8 per 100 000 person-years in children and adults, respectively), likely due to the different case-finding strategies employed.[2 3] Previous studies likely underestimated IgAV incidence as they had access to less comprehensive datasets. While incidence remained stable over the study period, prevalence of adult-onset and childhood-onset IgAV increased. This could be explained by improved documentation of existing IgAV diagnoses or may reflect increased patient survival. The former explanation is more likely given the short time period over which this increase occurred.

To our knowledge, this is the first controlled study examining incidence of IHD, stroke/TIA and VTE in adult patients with IgAV. Despite evidence from case series suggesting that IgAV confers increased risk of these outcomes,[8–19] we found no evidence of association between IgAV and IHD, stroke/TIA or VTE. We note that incidence of VTE was low in this cohort (1.67 per 1000 person-years). VTE may be an isolated acute event unrecorded in general practice records, and therefore this result should be interpreted with caution.

Although hypertension has been associated with poor outcomes in some patients with IgAV,[35] incidence of hypertension in childhood-onset and adult-onset IgAV was previously unknown. This study revealed similar risk for children and adults. The effect could be explained by renal manifestations of IgAV or by use of medications such as non-steroidal anti-inflammatory drugs and corticosteroids. The association remained significant in a sensitivity analysis using only hypertension recorded at least 1 year post-index date, suggesting that it is not solely an acute feature of the disease.

IgAV is thought to have a poorer renal prognosis in adult patients than in children.[5 6 36] In patients with adult-onset IgAV, incidence of stage G3–5 CKD was 8.72 per 1000 person-years, with a 54% increase in risk compared with controls. In patients with childhood-onset IgAV, incidence of CKD was much lower (0.51 per 1000 person-years) but with a similar HR. The increased incidence of CKD in adults compared with children may be explained by a higher burden of comorbidity and renal impairment at baseline.

A 35% increase in all-cause mortality was observed in adult-onset IgAV patients compared with controls. However, this effect was no longer statistically significant in a sensitivity analysis including only incident cases of IgAV. This may be explained by reduced length of follow-up when only incident cases are considered.

Strengths and limitations

Definition of exposure status depended on accurate coding of IgAV diagnosis in primary care medical records. These records do not include information on whether IgAV was diagnosed based solely on clinical criteria, or whether biopsy findings were used. We are unable to identify patients with renal involvement at diagnosis and recognise that there may be stronger associations with the outcomes if the cohort is restricted to patients with biopsy-proven IgAV, as previously shown in patients with biopsy-proven Henoch-Schönlein nephritis.[37]

Alternatively, other vasculitides, such as microscopic polyangiitis, may have been misdiagnosed as IgAV in the absence of histological investigation. Inclusion of such patients could increase the incidence of complications such as CKD. Although widespread testing for anti-neutrophil cytoplasm autoantibodies has been available since the 1990s, we have no data on anti-neutrophil cytoplasm antibody testing and cannot exclude that this diagnosis may have been missed in some cases.

It is possible that patients with greater disease severity were selectively coded in primary care, leading to overestimation of effect size. However, our incidence and prevalence estimates were similar, if not slightly higher than previously reported. Similarly, inaccurate coding of outcomes is a potential source of error. CKD is likely to be underdiagnosed in primary care,[38] so practice records may underestimate its incidence. To minimise this risk, CKD was defined by eGFR criteria not clinical codes.

A further caveat is uncertainty regarding classification of IgAV as adult onset. In the primary analysis, all adult IgAV patients with coded date of diagnosis after their 16th birthday were included. However, some cases may have been inappropriately defined as adult onset, for example, if coded when the patient moved to a new practice. Nevertheless, results were replicated in sensitivity analyses using incident adult IgAV cases only, showing that our findings are robust to stricter definitions of adult-onset IgAV.

When considering cardiovascular outcomes in patients with adult-onset IgAV, it should be noted that the affected population was relatively young (mean age 43.3 years at study entry). Therefore, it is possible that length of follow-up in this study was insufficient to detect increased risk of IHD.

Finally, this study could be influenced by surveillance bias. Patients with IgAV may receive closer monitoring of blood pressure and renal function in primary care. This may contribute to the higher CCI scores observed in patients with adult-onset IgAV compared with controls.

Despite these limitations, a strength of this population-based study design is its external validity. Patients with IgAV and control participants were included from a primary care database which is broadly representative of the UK population in terms of ethnicity, chronic disease prevalence and mortality rates.[23]

CONCLUSION

This retrospective cohort study demonstrates associations between IgAV and hypertension and CKD. These findings emphasise the importance of blood pressure and renal function monitoring in patients with IgAV. Our data also suggest that IgAV should not be considered a 'single hit' disease, but that clinicians should monitor for long-term sequelae. Further research is required to clarify the cause of hypertension in patients with IgAV, and to investigate whether such patients suffer from additional long-term sequelae than that are currently unrecognised.

Contributors AT, LH and KN conceived the work. AT contributed to the analysis and interpretation of data, drafting the manuscript and revising for intellectual content. AS and KN contributed to the acquisition, analysis and interpretation of data, and drafting the manuscript. NJA contributed to data analysis and interpretation, and writing the manuscript. LH contributed to drafting the manuscript and revising for intellectual content. PC, CF and SB contributed to revising the manuscript for intellectual content. All authors approved the final version to be submitted for review.

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Competing interests None declared.

Patient consent Not required.

Ethics approval The THIN data collection scheme and research carried out using THIN data were approved by the NHS South-East Multicentre Research Ethics Committee in 2003; under the terms of this approval, studies must undergo independent scientific review. Approval for this analysis was obtained from the Scientific Review Committee (for the use of THIN data) in April 2018 (SRC reference number 18THIN016).

Provenance and peer review Not commissioned; externally peer reviewed.

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TRANSLATIONAL SCIENCE

RNA sequencing data integration reveals an miRNA interactome of osteoarthritis cartilage

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ABSTRACT

Objective To uncover the microRNA (miRNA) interactome of the osteoarthritis (OA) pathophysiological process in the cartilage.

Methods We performed RNA sequencing in 130 samples (n=35 and n=30 pairs for messenger RNA (mRNA) and miRNA, respectively) on macroscopically preserved and lesioned OA cartilage from the same patient and performed differential expression (DE) analysis of miRNA and mRNAs. To build an OA-specific miRNA interactome, a prioritisation scheme was applied based on inverse Pearson's correlations and inverse DE of miRNAs and mRNAs. Subsequently, these were filtered by those present in predicted (TargetScan/microT-CDS) and/or experimentally validated (miRTarBase/TarBase) public databases. Pathway enrichment analysis was applied to elucidate OA-related pathways likely mediated by miRNA regulatory mechanisms.

Results We found 142 miRNAs and 2387 mRNAs to be differentially expressed between lesioned and preserved OA articular cartilage. After applying prioritisation towards likely miRNA-mRNA targets, a regulatory network of 62 miRNAs targeting 238 mRNAs was created. Subsequent pathway enrichment analysis of these mRNAs (or genes) elucidated that genes within the 'nervous system development' are likely mediated by miRNA regulatory mechanisms (familywise error=8.4×10^{-5}). Herein *NTF3* encodes neurotrophin-3, which controls survival and differentiation of neurons and which is closely related to the nerve growth factor.

Conclusions By an integrated approach of miRNA and mRNA sequencing data of OA cartilage, an OA miRNA interactome and related pathways were elucidated. Our functional data demonstrated interacting levels at which miRNA affects expression of genes in the cartilage and exemplified the complexity of functionally validating a network of genes that may be targeted by multiple miRNAs.

Key messages

What is already known about this subject?
- Dysfunctional microRNA–messenger RNA (miRNA–mRNA) interactions have been demonstrated to mark osteoarthritis (OA) pathophysiology.
- Targeting dysfunctional miRNA–mRNA interactions by miR mimics or antagomirs fulfil an important therapeutic promise.

What does this study add?
- A data integration framework to systematically identify miRNAs involved in OA pathophysiology.
- A first comprehensive miRNA interactome of OA pathophysiology.

How might this impact on clinical practice or future developments?
- The OA miRNA interactome provides a roadmap to pinpoint candidates for future miRNA-based therapies.

INTRODUCTION

Osteoarthritis (OA) is an age-related, disabling joint disease characterised by progressive heterogeneous changes in articular cartilage and subchondral bone. OA is the most common arthritic disease causing serious restrictions in major daily life activities, yet without an effective treatment.[1] At the tissue level, it has been demonstrated that OA pathophysiology is marked by altered gene expression regulation.[2–5] This may be triggered by dysfunctional adaptation processes of the bone and/or cartilage on inevitable challenges occurring during life, due to ageing,[6] genetic make-up[7] or mechanical (over)loading.[8]

A substantial number of mechanisms, commonly referred to as epigenetics, are known to dynamically regulate changes in gene expression, particularly relevant in postmitotic cells such as chondrocytes. Epigenetic variation includes DNA methylation at CpG sites, histone modifications, and expression of non-coding RNAs such as microRNAs (<22 base pairs; miRNA) and long non-coding RNAs (<200 base pairs).[9–11] miRNAs are known to play an important role in post-translational regulation of gene expression via antisense binding to messenger RNA (mRNA), whereas their dysfunction has been demonstrated to mark many complex diseases including OA.[12] Notably, targeting dysfunctional miRNA–mRNA interactions has emerged as an important therapeutic promise for preclinical development as exemplified by successfully applied miRNA mimics or anti-miRs in cancer.[13]

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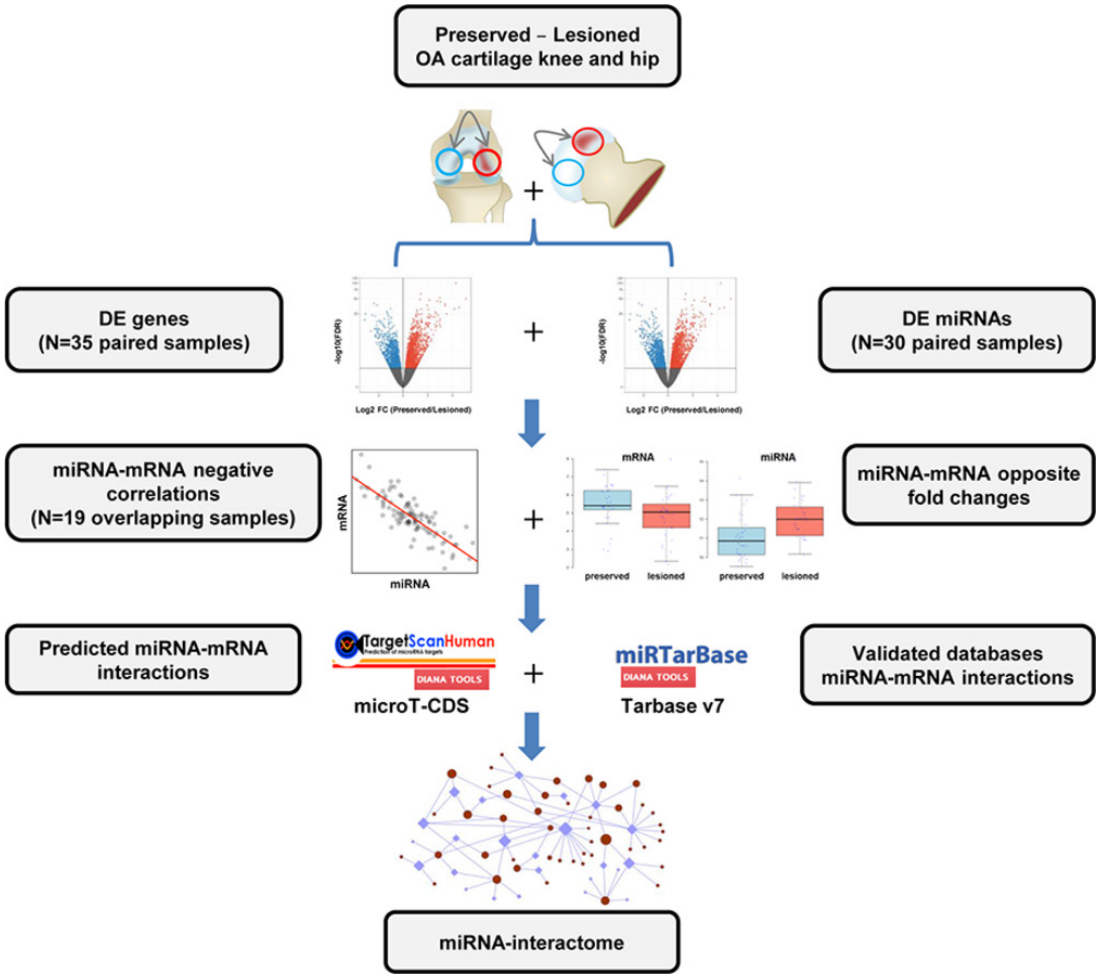

Figure 1 Data integration approach. Relative log normalised miRNA and mRNA expression matrices were concatenated. Next, differentially expressed miRNAs and genes were correlated and integrated according to the opposite direction of its FC. Further, miRNA–mRNA interactions from prediction tools and experimentally validated databases were integrated. Finally, target genes that followed these criteria were considered to build an OA miRNA–mRNA network. DE, differential expression; FC, fold change; miRNA, microRNA; mRNA, messenger RNA; OA, osteoarthritis.

With respect to OA, an increasing number of studies report on differential expression (DE) of miRNAs with ongoing OA pathophysiology.[12 14] Nevertheless, the majority of these studies report on candidate miRNA and mRNA interactions, such as miR-140 with *ADAMTS5*, *MMP13* and *IGFBP5*.[15 16] To explore the full miRNA interactome of OA pathophysiology and explore its full potential to dynamically regulate gene expression in articular cartilage, we performed genome-wide miRNA and mRNA sequencing in preserved and OA-affected articular cartilage samples. To prioritise the most likely genes sensitive to the OA process that are targeted by miRNAs, we applied a stepwise integrative approach to our partly overlapping miRNA and mRNA sequencing data sets of preserved and lesioned OA cartilage, integrated with data from publicly available databases such as those with target predictions as well as those with experimentally validated data.

METHODS

Sample description

Preserved and lesioned cartilage samples from the same donor were obtained from the Research in Articular osteoArthritis Cartilage (RAAK) study consisting of patients with OA who underwent joint replacement surgery due to an end-stage disease.[4] In the current study, cartilage samples of 63 patients were included (online supplementary table-S1).

Small RNA and mRNA sequencing

Sequencing of high-quality miRNA and mRNA was performed on, respectively, the Illumina HiSeq 2500 and the HiSeq 2000/4000. A detailed description on alignment, mapping and normalisation is available in online supplementary materials.

DE analysis

DE analysis was performed on paired samples of both data sets, that is, 30 paired samples (14 knees and 16 hips) for miRNA and 35 paired samples for mRNA (28 knees and 7 hips; figure 1). miRNA DE analysis was also performed while stratifying for joint (14 paired knees and 16 paired hips) (online supplementary materials). Benjamini-Hochberg multiple testing corrected-p values with a significance cut-off of 0.05 are reported as false discovery rate (FDR).

Prioritisation of miRNA–mRNA targeting pairs

To generate an OA-specific miRNA interactome, we applied an integrated stepwise prioritisation approach (figure 1) to the identified set of DE miRNAs and mRNAs based on (1) significant negative Pearson's correlation ($|r|>0.5$ and $p<0.05$) between miRNA and mRNA levels (19 overlapping samples, 15 patients); (2) opposite direction of fold change (FC) of mRNA and miRNA between the paired samples; (3) miRNA–mRNA

target prediction from TargetScan[17] and microT-CDS[18]; and (4) experimentally validated miRNA–mRNA target pairs downloaded from miRTarBase V.7.0[19] and TarBase V.7.[20] Prioritised miRNA–mRNA target pairs were integrated into an miRNA–mRNA network based on their correlation and FCs (online supplementary materials).

Pathway enrichment

Pathway enrichment analysis was performed using the online tool DAVID[21] while selecting Gene Ontology terms for biological processes (GOTERM_BP_DIRECT). Bonferroni multiple testing-corrected p values with a significance cut-off of 0.05 are reported as familywise error rate (FWER). Enrichment analyses of the DE genes with FC ≥2 were performed separately for further comparison. To specifically identify the miRNA regulated pathways in OA cartilage, enrichments of miRNA-target genes were performed using all significant DE genes (FDR<0.05) as background.

Functional validation

Primary chondrocytes isolated from three independent donors were transfected with antagomirs and miR mimics for miR-143–3 p, miR-329–3 p and miR-99a-3p using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcriptase-quantitative PCR (RT-qPCR) was performed adjusting for the housekeeping genes *GAPDH* and *ARP* (for further details see online supplementary materials).

Data availability

FASTQ files are available on ArrayExpress E-MTAB-7313. The code to reproduce the analysis is available on https://git.lumc.nl/rcoutinhodealmeida/miRNAmRNA.

RESULTS

To identify the miRNA–mRNA regulatory landscape in OA cartilage, we performed a stepwise approach to integrate the miRNA (n=30 pairs) and mRNA (n=35 pairs) sequencing data sets of preserved and lesioned OA cartilage, samples containing n=19 overlapping samples (figure 1).

Differentially expressed miRNAs between lesioned and preserved OA cartilage

We found 142 DE miRNAs (FDR<0.05) between lesioned and preserved OA cartilage with absolute FCs ranging from 1.2 to 4.9 (figure 2A, online supplementary table-S2). The most significant DE miRNAs were miR-127–3 p (FC=0.5, FDR=1.1×10^{-6}) and miR-451a (FC=2.3, FDR=1.2×10^{-6}). As shown in figure 2A, the majority of DE miRNAs were upregulated in lesioned as compared with preserved OA cartilage (91 out of 142 miRNAs) with miR-206, recently reported in relation to OA,[22] displaying the largest FC (FC=4.9, FDR=3.5×10^{-6}). Conversely miR-504–5 p (FC=0.4, FDR=2×10^{-5}) showed the largest fold decrease in lesioned OA cartilage (figure 2A, online supplementary table-S2). Next to miR-206, we found 40 other DE miRNAs that have consistently been associated with OA in functional follow-up studies, for example miR-140–5 p (FC=1.4, FDR=0.04), miR-143–3 p (FC=2.1, FDR=0.0001), miR-146a (FC=0.5, FDR=0.01) and miR-155–5 p (FC=1.8, FDR=0.005).[16 23–25] Additionally, we identified 102 DE miRNAs not previously reported in OA, such as miR-95–3 p (FC=4.3, FDR=2.8×10^{-8}), miR-3934–5 p (FC=1.9, FDR=1.8×10^{-6}) and miR-99a-3p (FC=0.6, FDR=0.004). Moreover, several members of particular miRNA families are found to be differentially expressed, such as the miR-320 and let-7 family (online supplementary table-S2). DE expression was confirmed for four out of four miRNAs by applying RT-qPCR in independent paired preserved and lesioned OA cartilage samples (n=21): miR-127–3 p (FC=0.8, p=0.012), miR-451a (FC=2.3, p=0.024), miR-99a-3p (FC=0.8, p=0.0007) and miR143-3p (FC=1.8, p=0.07) (online supplementary figure-S1).

To explore whether we could detect joint site-specific miRNAs, stratified analyses for hip (n=16 pairs) and knee joints (n=15 pairs) were performed. Despite the relative equal number of samples, we found in the hip 117 (online supplementary table-S3) and in the knee 22 (online supplementary table-S4) significant DE miRNAs (FDR<0.05) between lesioned and preserved OA cartilage. Of these, 14 DE miRNAs were specific for hip cartilage (eg, miR-122–5 p: FC=5.14, FDR=6.6×10^{-5}) and five for knee cartilage (online supplementary figure-S2). Notably miR-451a was the most significantly differentially expressed

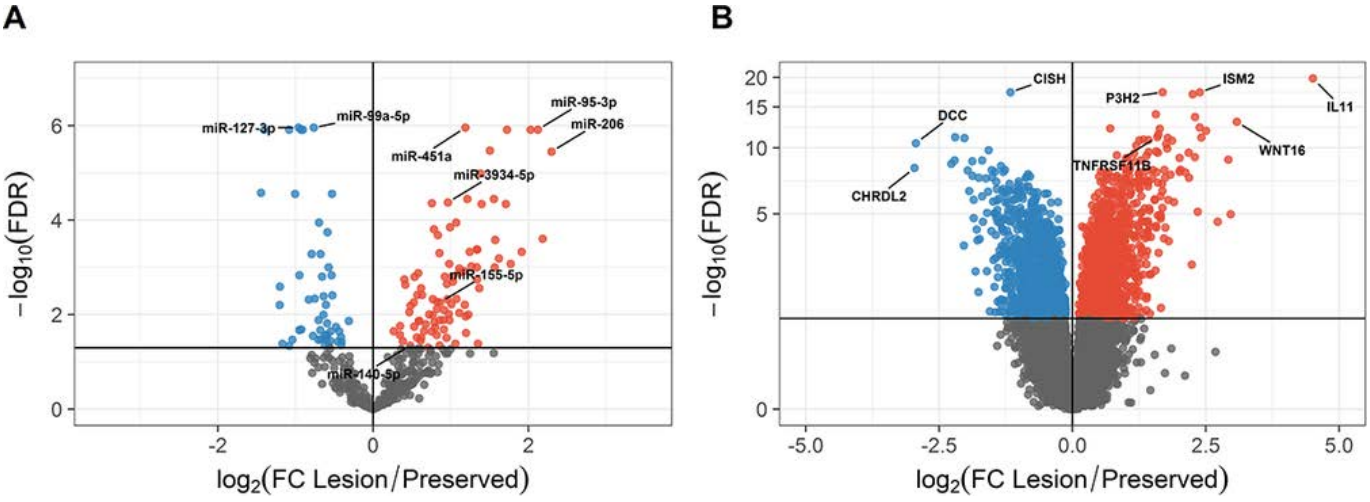

Figure 2 Paired differential expression analyses between preserved and lesioned OA cartilage. (A) Volcano plot with the differentially expressed miRNAs. (B) Volcano plot with the differentially expressed genes. Blue circles represent downregulated miRNAs (A) or genes (B); circles are red when they are upregulated. Labelled are the top differentially expressed genes and miRNAs, as well as the known and novel discovered ones. FC, fold change; FDR, false discovery rate; miRNA, microRNA; OA, osteoarthritis.

Table 1 Pathways enrichment analysis of differentially expressed genes

Term	P values	FWER	Fold enrichment	Genes
Extracellular matrix organisation	1.82E-07	5.99E-05	5.20	MATN4, POSTN, VIT, COL9A1, LAMB3, TNFRSF11B, FBLN1, COL19A1, COL7A1, FOXF1, TNR, SERPINE1, TGFBI, LAMC2, VCAN, SPP1, FN1
Skeletal system development	3.07E-06	1.01E-03	5.69	BMP3, NOG, SOX11, POSTN, NPR3, FRZB, PAX1, TNFRSF11B, COL19A1, GDF10, VCAN, BMPR1B, BMP6
Cell adhesion	8.94E-05	2.93E-02	2.74	AMTN, POSTN, AJAP1, CDH6, TNFAIP6, LAMB3, WISP2, COL19A1, COL7A1, LSAMP, TNR, MSLN, TGFBI, DSC3, RELN, LAMC2, VCAN, CHL1, SPP1, FN1, AOC3
Positive regulation of gene expression	1.30E-04	4.25E-02	3.43	ODAM, WNT16, NOG, TESC, SOX11, IQGAP3, KIT, HMGA2, RIMS1, NTRK3, INHBA, FBLN1, ANK3, NGF, FN1

FWER, familywise error rate.

in the hip (FC=3.3, FDR=5.0×10^{-8}) and total (FC=2.3, FDR=1.2×10^{-6}) data set, yet not differentially expressed in the knee data set.

Genes differentially expressed between lesioned and preserved OA cartilage

We identified 2387 DE mRNAs or genes (FDR<0.05) between lesioned and preserved OA cartilage (figure 2B, online supplementary table-S5). Of these, 1188 genes were downregulated in lesioned compared with preserved OA cartilage, and 1199 upregulated (online supplementary table-S5). As shown in figure 2B, the most significantly downregulated gene was *CISH* (FC=0.5, FDR=4.7×10^{-18}), encoding the cytokine inducible SH2 containing protein which is known as an important suppressor of cytokine signalling through the JAK-STAT5 pathway. The most significantly upregulated gene was *IL11* encoding interleukin-11, which also showed the largest FC in lesioned as compared with preserved OA cartilage (FC=22.7, FDR=1.5×10^{-20}). The genes *DCC* (FC=0.1, FDR=3.3×10^{-11}) and *CHRDL2* (FC=0.1, FDR=7×10^{-9}) showed the largest fold decrease in lesioned as compared with preserved OA cartilage (figure 2B, online supplementary table-S5). We found several previously reported OA-related genes, such as *WNT16* (FC=8.4, FDR=1.1×10^{-13}) and *TNFRSF11B* (FC=3.0, FDR=7.1×10^{-12}),[26] but also revealed new DE genes with OA, such as *P3H2* (FC=3.2, FDR=4.7×10^{-18}) and *ISM2* (FC=5.2, FDR=4.7×10^{-18}). As shown in table 1, enrichment analyses of the DE genes with FC≥2 (n=372) revealed significant enrichment (FWER <0.05) for pathways earlier reported in relation to OA pathophysiology (eg, 'extracellular matrix organization', 'skeletal system development', 'cell adhesion' and 'positive regulation of gene expression').

OA-specific miRNA interactome

To generate an OA-specific miRNA interactome of the most likely miRNA–mRNA target pairs, the integrated stepwise prioritisation approach outlined in figure 1 was applied using 19 samples for which we had both miRNA and mRNA sequencing data. In online supplementary table-S6 we provided the 331 prioritised miRNA–mRNA target pairs, including their target predictions and/or experimental validations from respective databases. Prioritised miRNA–mRNA target pairs were integrated into an miRNA–mRNA network based on their correlation and FCs as such generating the OA-specific miRNA interactome (figure 3). The fact that 62 DE miRNAs were interacting with 238 DE target genes reflects that miRNAs are bound to target many genes in a complex structure. As shown in figure 3, the network consists of two large clusters of connected miRNA–mRNA pairs, one in which the miRNAs are downregulated (blue miRNA nodes) and another in which the miRNAs are upregulated (red miRNA nodes). Notably within the 'downregulated miRNA cluster' is the previously unknown OA-related miR-99a-3p that targets 36 DE genes, with 3 of them showing a strong correlation: *FZD1* (r=−0.73, p=0.0001), *ITGB5* (r=−0.70, p=0.00031) and *GDF6* (r=−0.70, p=0.00039) (online supplementary table-S6). Furthermore, these three genes each correlates with at least one other targeting miRNA (figure 3). A notable example in the 'upregulated miRNA cluster' is the previously identified miR-143–3 p that targets 16 DE genes. Among these, at least three genes, *DCAKD* (r=−0.71, p=0.0002), *AMIGO1* (r=−0.70, p=0.0003) and *SMAD3* (r=−0.68, p=0.0008), show a strong correlation to miR-143–3 p, which additionally shares target genes with miR-10a-5p and miR-21–5 p, being *TNS3*, *THRA* and *GID8*. Of note in the 'upregulated miRNA cluster' is also the miRNA family miR-320, targeting the mRNA of 30 genes including *MANF* and *CISD2*, which are correlated with all miRNAs from this respective family. Besides these two large clusters, there are 15 small clusters, mostly with one miRNA targeting few mRNAs (figure 3). For example, miR-493–3 p forms a separate cluster with its 10 target DE genes. By applying RT-qPCR in n=21 independent preserved OA cartilage samples, we confirmed correlation between the miRNA–mRNA expression for miR-99a-3p with *FZD1* (r=−0.54, p=0.02) and with *GDF6* (r=−0.58, p=0.01) (online supplementary figure-S3).

Functional validation of miRNA–mRNA target pairs

To study the downstream effects of highlighted miRNAs, we studied the effects of miR-143–3 p antagomir and mimic. This miRNA shows singular connections to the *GHR*, *SMAD3*, *AMIGO1* and *DCAKD* genes (figure 4A). As shown in figure 4A, transfection with the miR-143–3 p mimic resulted in consistent downregulation of its singular target genes *AMIGO1* (p=0.071), *SMAD3* (p=0.032), *GHR* (p=0.115) and *DCAKD* (p=0.089). The antagomir of miR-143–3 p did not result in a clear response to the respective target genes. Furthermore, the effects of antagomirs and miR-mimics of miR-99a-3p and miR-329–3 p, both correlating to the *WNT9A*, *FZD1* and *GDF6* genes (figure 4B), were analysed. As shown in figure 4B, the miR-99a and miR-329–3 p mimics and antagomirs resulted in consistent changes in the *WNT9A*, *FZD1* and *GDF6* gene expression; however, the direction of effects was variable. Most notable is the inverse effect of the miR mimics on *GDF6* expression (FC=1.8, p=0.036 and FC=0.6, p=0.059 for miR-329–3 p and miR-99a-3p, respectively). The strongest effect was observed for miR-329–3 p mimic on *WNT9A* expression (FC=0.25, p=0.036). To further explore these interactions, we correlated the expression levels of *WNT9A*, *GDF6* and *FZD1* on transfections with mock controls, antagomirs and mimics of miR-329–3

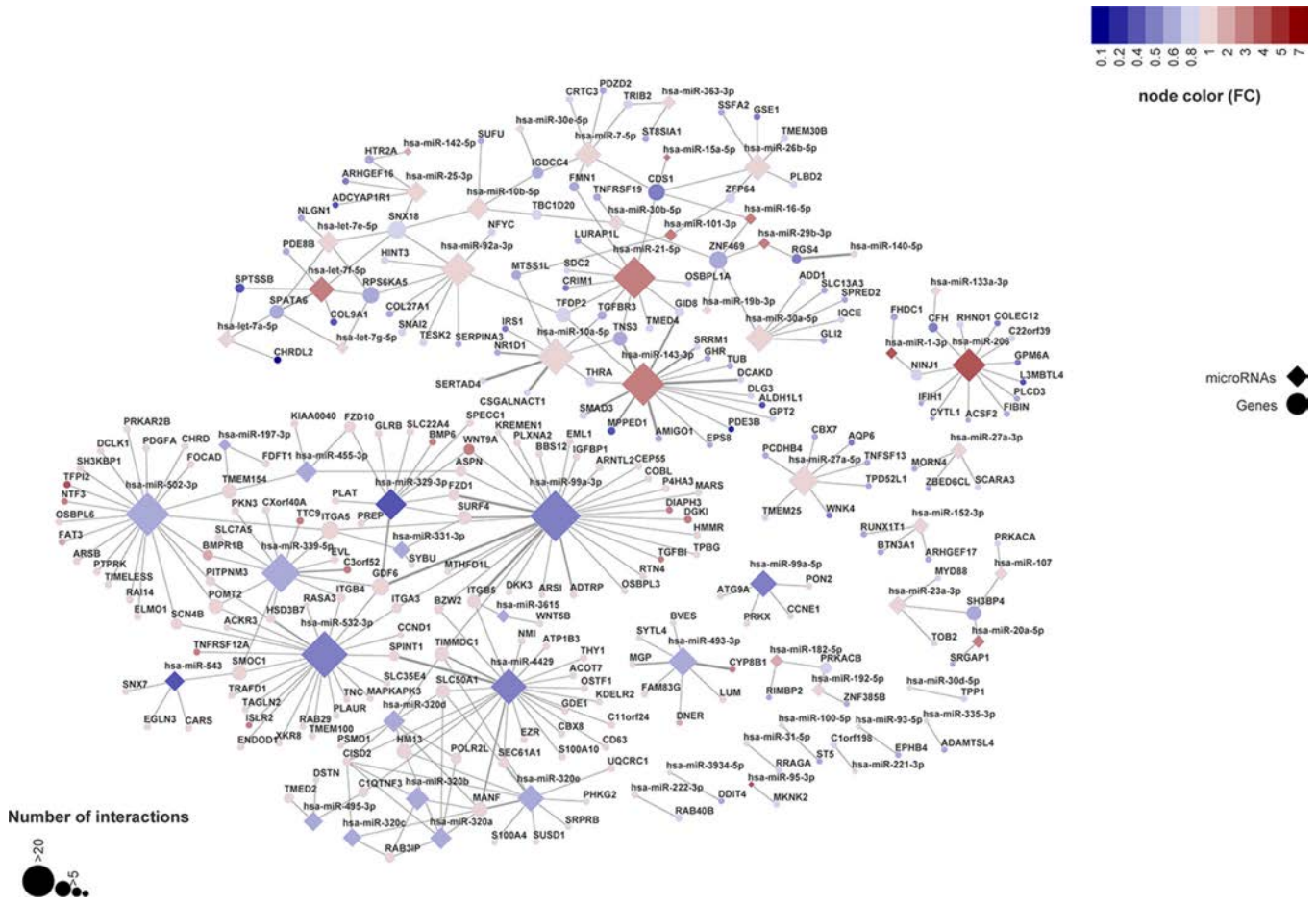

Figure 3 OA miRNA–mRNA interactome. Network of differentially expressed miRNAs targeting differentially expressed genes between unaffected (preserved) and lesioned OA cartilage. Diamonds are miRNAs and circles genes; edges denote that an miRNA targets the connected gene. The size of the nodes is proportional to the number of edges (interactions). Node colour characterises the strength and the direction of the expression change between unaffected (preserved) and lesioned OA cartilage. Edge thickness corresponds to Pearson's correlation between the miRNA and gene across all samples. miRNA, microRNA; mRNA, messenger RNA; OA, osteoarthritis.

p and miR-99a-3p (online supplementary figure-S4). Notable is the reduced correlation between *WNT9A* and *FZD1* particularly on transfection with, respectively, the miR-329–3 p mimic and the miR-99a-3p antagomir illustrating the different interacting levels at which expression of these genes is controlled by miR-99a-3p and miR-329–3 p.

miRNA-regulated gene pathways

To find specific miRNA-regulated gene pathways involved in OA pathophysiology, we analysed the 238 prioritised mRNAs likely targeted by DE miRNAs in OA cartilage for enrichment in biological processes using the 2387 DE genes as background. As shown in table 2, 10 pathways were significantly enriched, including 'positive regulation of GTPase activity' (FWER=9.8×10^{-6}) and 'nervous system development' (FWER=8.4×10^{-5}). Notable genes involved in the latter were *NLGN1* (FC=0.61, FDR=0.014), which plays a role in synapse function, and *NTF3* (FC=2.7, FDR=6.6×10^{-6}), which controls survival and differentiation of neurons.

DISCUSSION

By integrating overlapping RNA sequencing of mRNA and miRNA in paired preserved and lesioned OA cartilage samples, we presented the first comprehensive, OA-specific, miRNA interactome. Hereto, we identified 142 miRNAs and 2387 genes with significant DE between lesioned and preserved cartilage. By a strict prioritisation scheme, we created a novel OA-associated miRNA interactome of 62 miRNAs and their 238 likely target mRNAs. Subsequent pathway analyses of the miRNA targeted genes showed significant enrichment for genes that act, among others, within 'positive regulation of GTPase-activity' and 'nervous system development'. To allow biological interpretation of some of the highlighted clusters, functional validation experiments were performed with antagomirs and mimics. We observed that mimics of miR-143–3 p, with singular correlation to the *GHR*, *SMAD3*, *AMIGO1* and *DCAKD* genes, had consistent inverse effects on gene expression. On the other hand, antagomirs and mimics of miR-99a-3p and miR-329–3 p, both paired with *WNT9A*, *FZD1* and *GDF6* genes (figure 4B), had consistent effects on gene expression but with variable directions. Together, our data suggest that interacting levels of miRNAs collectively affect gene expression in the cartilage, yet exemplifies the complexity of functional validation of miRNA–mRNA networks.

Among the enriched miRNA targeted genes in the nervous system development pathway were *NLGN1* and *NTF3*. *NLGN1* encodes neuroligin 1, which is a postsynaptic adhesion molecule involved in the regulation of glutamatergic transmission. More recently it was shown that *NLGN1* is expressed during chondrogenesis and marks cellular identity of articular chondrocytes.[27]

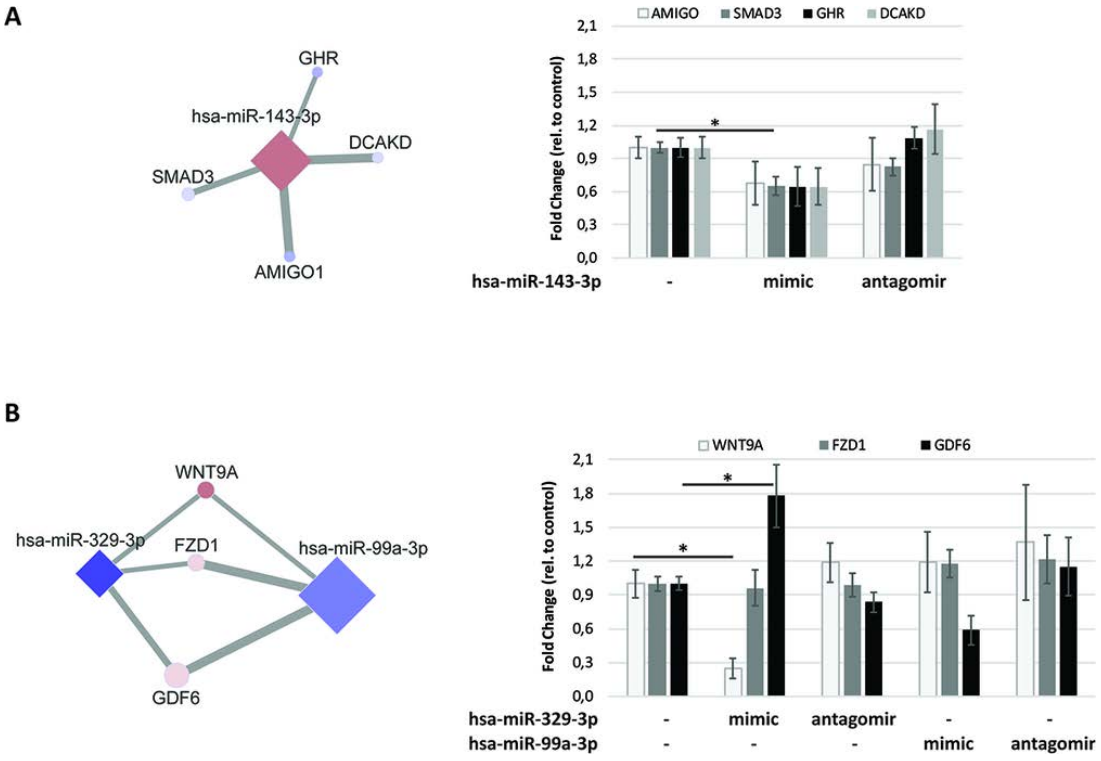

Figure 4 Functional validation miRNA–mRNA interactions. (A) Expression of *AMIGO1*, *SMAD3*, *GHR* and *DCAKD* in primary chondrocytes transfected with miR-143–3 p mimic or antagomir compared with control as determined by RT-qPCR. (B) Expression of *WNT9A*, *FZD1* and *GDF6* in primary chondrocytes transfected with miR-329–3 p or miR-99a-3p mimic or antagomir compared with control as determined by RT-qPCR. Data shown are the average±SE of the mean for three independent donors (*p<0.05). miRNA, microRNA; mRNA, messenger RNA; RT-qPCR, reverse transcriptase-quantitative PCR.

NTF3 encodes neurotrophin-3, a member of the neurotrophin family that controls survival and differentiation of mammalian neurons. The protein is closely related to both nerve growth factor and brain-derived neurotrophic factor. In our data set we prioritised the *NTF3* gene as a likely target of miR-502–3 p and involved in OA pathophysiology. This since *NTF3* is highly significantly upregulated (FC=2.7, FDR=6.6×10^{-6}) and miR-502–3 p is significantly downregulated (FC=0.8, FDR=0.04) in lesioned OA cartilage, the expression of *NTF3* and miR-502–3 p was inversely correlated (r=−0.57, p=0.007), and they are a predicted mRNA–miRNA target pair (miTG score=0.473) (online supplementary table-S6). Targeting such a dysfunctional miRNA–mRNA interaction may represent a therapeutic promise for preclinical development, for example, by applying miRNA mimics of miR-502–3 p. As exemplified by our functional validation, the direct miRNA–mRNA target interaction should, however, be carefully assessed, for example, by luciferase assays. Moreover, we advocate that a systems

Table 2 Pathway analysis of differentially expressed target genes

Term	P values	FWER	Fold enrichment	Genes
Positive regulation of GTPase activity	4.60E-08	9.80E-06	7.68	SNX18, PDGFA, ARHGEF17, S100A10, IRS1, RAB3IP, ELMO1, THY1, FZD10, RGS4, RASA3, ST5, TBC1D20, SRGAP1
Nervous system development	3.93E-07	8.37E-05	7.07	GLRB, NTF3, PCDHB4, NLGN1, NINJ1, EVL, SLC7A5, CSGALNACT1, BZW2, TPP1, DLG3, CRIM1, DCLK1
Protein phosphorylation	5.66E-06	1.20E-03	5.46	RPS6KA5, CCNE1, CCND1, PKN3, WNK4, PHKG2, MKNK2, TESK2, PRKACA, PRKACB, BMPR1B, DCLK1, TRIB2
IRE1-mediated unfolded protein response	6.84E-06	1.46E-03	102.27	TPP1, SRPRB, SEC61A1, ADD1
Stimulatory C-type lectin receptor signalling pathway	6.84E-06	1.46E-03	102.27	RPS6KA5, PSMD1, PRKACA, PRKACB
Extracellular matrix organisation	9.93E-06	2.11E-03	5.16	PDGFA, LUM, ADAMTSL4, TNC, ITGB4, SPINT1, ITGB5, ITGA3, CSGALNACT1, COL9A1, ITGA5, COL27A1, TGFBI
Signal transduction	1.96E-05	4.17E-03	3.06	PTPRK, OSTF1, NTF3, CYTL1, MAPKAPK3, PDE3B, TNFSF13, CDS1, IRS1, SUFU, PLAUR, TMED4, MYD88, EPS8, PKN3, SMOC1, PDE8B, IGFBP1, PRKACB, RASA3, SRGAP1
Tumour necrosis factor-mediated signalling pathway	1.11E-04	2.36E-02	42.61	TNFRSF12A, PSMD1, TNFRSF19, TNFSF13
Activation of protein kinase A activity	1.50E-04	3.19E-02	153.41	PRKAR2B, PRKACA, PRKACB
Cellular response to BMP stimulus	2.18E-04	4.65E-02	34.09	SPINT1, TMEM100, BMPR1B, BMP6

*p<0.05.
BMP, bone morphogenic protein; FWER, familywise error rate.

medicines approach of antagomirs or miR mimics transfections followed by RNA sequencing is preferably taken to obtain a thorough understanding of all biological mechanisms involved. Finally, bioinformatics tools need to be developed that take into account, or take advantage of, the fact that the miRNA 'seed' sequence (nucleotides 2 and 7) can target the 3'UTR region of multiple mRNAs[28] or may bind to other parts of the gene.[29] Together, our miRNA interactome represents a comprehensive legacy to directly probe miRNAs of interest with their likely downstream signalling pathways, target predictions and/or experimental validations from respective databases. The fact that some of these tools use publication criteria as experimental validation of miRNA–mRNA target pairs should raise awareness that this could confound complex miRA–mRNA target interactions rather than illuminating them.

Within the interactome, miR-99a-3p, not previously associated with OA, targets the highest number of genes (n=36), with 20 of those genes targeted only by miR-99a-3p, while 16 genes were also targeted by other DE miRNAs. Of the latter, *GDF6* is targeted by three other miRNAs (miR-329–3 p, miR-339–5 p, miR-532–3 p), with miR329-3p having the strongest inverse correlation (r=−0.67). *GDF6* is a member of the transforming growth factor-beta super family whose members are essential for normal formation of the bones and joints in the limbs, skull and axial skeleton.[30] Also notable is that *GDF6* is an important paralogue of *GDF5*, the most consistently OA susceptibility gene found to date.[31] Moreover, *GDF5* has recently been identified as one of the key genes able to stratify two OA subgroups of knee articular cartilage based on expression levels.[32]

In our DE miRNA data set, we identified many of the previously reported, OA-associated, miRNAs such as miR-206[22] and miR-140.[15 16] However, in our miRNA interactome, these miRNAs did not necessarily correlate to their previously reported target genes. For example, miR-140–5 p in our miRNA interactome is only connected to *RGS4* (figure 3) and not to *ADAMTS5*, *MMP13* and *IGFBP5*, as reported previously by Tardif *et al*.[15 16] To explore this further, we report in online supplementary table-S7 our miRNA and mRNA expression data of the most consistently reported miRNA–mRNA target pairs, for example, as reported in a recent review.[33] As shown in online supplementary table-S7, miR-140–5 p has only very modest correlations to these previous reported target genes, likely reflecting their indirect effects. Moreover, some of the previously reported miRNAs are not among the ones presented in the miRNA interactome due to our strict prioritisation approach (figure 3). For example, miR-27a-3p is highly upregulated in lesioned OA cartilage (FC=1.8, FDR=5.0×10^{-4}), but is not present in our miRNA interactome because it has significant positive correlation to *MMP13* (r=0.5, p=3.6×10^{-2}), and this gene does not show significant DE in preserved versus lesioned OA cartilage (FC=0.9, FDR=8.34×10^{-1}).

Another example of earlier reported miRNA associated to OA pathophysiology is miR-206. In a recent study, it was shown that increased expression of miR-206 significantly inhibited proliferation of chondrocytes while promoting expression of catabolic enzymes and apoptosis-inducing factors, suggesting that inhibition of miR-206 may control cartilage degradation in OA.[22] In our data set miR-206 indeed exhibits a high and significant upregulation in lesioned compared with preserved OA cartilage (FC=4.9, FDR=2.6×10^{-6}) and, based on the here identified interactions with *CFH*, *IFIH1*, *NINJ1*, *GPM6A*, *L3MBTL4*, *COLEC12*, *PLCD3*, *ACSF2*, *CYTL1*, *RHNO1*, *FIBIN* and *C22orf39*, we advocate that these genes may be involved in this process (figure 3). Taken together, the field of miRNA biology has demonstrated that miRNAs are bound to target multiple mRNAs in a network and, via dysregulation, causal to complex diseases,[34] including OA.[35] Moreover, targeting dysfunctional miRNA–mRNA interactions has emerged as an important therapeutic promise for preclinical development. As such, the here identified miRNA interactome of OA articular cartilage may represent a first important step to fulfil this promise. Nevertheless, our functional validation experiments highlighted that additional high-throughput (ie, high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP)) functional analyses towards systems medicines approaches are necessary to demonstrate direct binding of miRNAs to specific target genes and concurrent downstream changes in all mRNA expression levels.

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How to reduce the waiting time for the first consultation with the rheumatologist as a first step for a timely treatment

In Chile, the guaranteed therapy for rheumatoid arthritis did not consider biological drugs until 2015, when Law 20850 was promulgated.[1] The criteria to start biological treatment and the follow-up periods differ from validated treat to target (T2T) strategies,[2] allowing access only to patients who remain with a disease activity Score-28 with erythrocite sedimentation rate (DAS28-ESR) >5.1 despite 6 months of treatment with three non-biological disease-modifying antirheumatic drugs (DMARDs).[3] Our results of the application of this law during the first year were recently published.[4]

The implementation of this law allowed us to identify difficulties to implement a successful T2T strategy: (1) access to the rheumatologist; (2) impossibility of carrying out an early control because of absence of medical hours; (3) limitation in the therapeutic arsenal in the first stage of application of the law (only two biologics available).

Taken into account previous experiences,[5–7] our group decided to intervene in facilitating access to the rheumatologist, to achieve an early diagnosis and start adequate treatment within the window of opportunity.

Our hospital has four rheumatologists and is in charge of three communes in the southwest sector of the capital, which together represent a population of approximately 376 806 inhabitants[8] (1 rheumatologist per 94 201.5 inhabitants). Only referred patients are allowed to attend a rheumatologist in the Chilean public health system. In November 2017, there were 503 referrals waiting for a first visit with the rheumatologist. Given the impossibility of covering this number of waiting patients through normal operation, it was decided to implement a rapid access polyclinic that started in December of 2017. This intervention was approved by the ethics committee of the east-metropolitan health service on 12 June 2017.

After an informative meeting with the general practitioners, all referred patients for rheumatology consultation were evaluated by a senior rheumatologist in a 10 min consultation using a predefined interrogation, expanded case by case based on the criteria of each rheumatologist. According to the results of the interview, the situation of the patient was categorised into urgent (any suspected active inflammatory rheumatic disease), habitual control (suspected inflammatory rheumatic disease but not active) or control in primary care (no inflammatory rheumatic disease). For urgent consultations, an early control polyclinic was created to evaluate these patients within the following 15 days. The usual consultations entered into the usual scheduling system. The pathologies that were considered to require control and management in primary care were assigned to a coordination polyclinic where the patients were evaluated by an internist, in charge of confirming the diagnosis, educating the patient, and, if applicable, refer to primary care. No patient was discharged immediately after the triage.

A total of 328 referrals were evaluated in the first 4 months. The waiting time was reduced from a median of 275 days (IQR 66–591) to 60.5 days (IQR 30–228). This reduction was statistically significant ($p<0.001$, Mann-Whitney U test). Ninety-one patients (27.7%) were sent to the internist and 45 (13.7%) to usual control. The rest (58.6%) were sent to the early control polyclinic.

We consider this strategy as successful in reducing care times and identifying patients who require an early start of treatment and close control. The waiting time to access the rheumatologist is only one of the variables necessary to implement a timely treatment. It would be interesting to validate composite indicators (time to first visit, time to diagnosis, time to treatment start) to evaluate the capacity of a health system to implement management for rheumatic diseases according to the current state of the art.

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Chronic hydroxychloroquine/chloroquine exposure for connective tissue diseases and risk of Alzheimer's disease: a population-based cohort study

Autophagy is an intracellular pathway by which cells generate energy and metabolites by recycling their own non-essential, redundant or damaged components.[1] Pathophysiological

studies have demonstrated that the impairment of autophagy contributes to protein aggregate accumulation that occurs during Alzheimer's disease and experiments have shown that autophagy inhibitors, such as chloroquine and hydroxychloroquine, block amyloid plaque degradation.[1 2] Further, a recent case–control study found that patients with rheumatoid arthritis who used hydroxychloroquine were at increased risk of dementia.[3] We investigated whether chronic exposure to chloroquine/hydroxychloroquine increases the risk of Alzheimer's disease.

Data from The Health Improvement Network (THIN) were used (January 1990–December 2016). THIN is a UK primary care database on >12 million people. Participating general practitioners prospectively enter clinical information on individuals so that the database provides a longitudinal medical record for each individual. THIN is representative of the UK population. The diagnostic and prescribing data compare favourably with external statistics.[4] Individuals were included in the exposed sample if they had been prescribed hydroxychloroquine/chloroquine for connective tissue diseases (CTD), for ≥1 year, at a mean dosage ≥50 mg/day for chloroquine and ≥100 mg/day for hydroxychloroquine. The first control group was made of individuals who received hydroxychloroquine for <1 year for the same underlying condition as the exposed individuals. For these groups, the start of at-risk period was defined as the first day of the first prescription of hydroxychloroquine/chloroquine. The second control group included individuals who had never been exposed to chloroquine, hydroxychloroquine, quinine, quinacrine or mefloquine, but were suffering from the same CTD as the exposed individuals. For each unexposed individual, a uniformly randomly selected start of the at-risk period was defined. Up to three unexposed and shortly exposed individuals were selected for every chronically exposed. For all individuals included in our

Table 1 Characteristics of the study population

	Long-term exposed N=11 550	Short-term exposed N=4873	Unexposed N=30 930
Age (years)	56 (46–669)	57 (45–67)	57 (46–67)
Female, n (%)	8970 (77.7)	4023 (82.6)	23 251 (75.2)
Duration of follow-up after 'start date' (days)	1630 (935–2737)	1658 (923–2806)	1521 (1097–2377)
Dosage (mg/day)			
Hydroxychloroquine	261 (200–356)	391 (255–400)	–
Chloroquine	162 (102–224)	–	–
Underlying diseases, n (%)			
Rheumatoid arthritis	7866 (68.1)	3411 (70.0)	22 274 (72.0)
Lupus erythematosus	2.032 (17.6)	616 (12.6)	3105 (10.0)
Sjogren syndrome	781 (6.8)	412 (8.5)	2653 (8.6)
Other connective tissue diseases	782 (6.8)	401 (8.2)	2632 (8.5)
Light eruption	89 (0.7)	33 (0.7)	266 (0.9)
Number of prescriptions of*			
Methotrexate	0 (0–69)	0 (0–63)	0 (0–46)
Azathioprine	0 (0–2)	0 (0–2)	0 (0–0)
Glucocorticoids	1 (0–64)	1 (0–74)	0 (0–35)
NSAIDs	8 (0–100)	9 (0–104)	4 (0–82)
Vitamin D	0 (0–34)	0 (0–33)	0 (0–20)
Smoking status, n (%)			
Non-smokers	5350 (46.3)	2286 (46.9)	15 221 (49.2)
Ex-smokers	3694 (32.0)	1406 (28.9)	8842 (28.6)
Smokers	2481 (21.5)	1170 (24.0)	6702 (21.7)
Missing	25 (0.2)	11 (0.2)	165 (0.5)
Townsend deprivation index, n (%)			
0 (less deprived)	459 (4.0)	223 (4.6)	1104 (3.6)
1	2961 (25.6)	1205 (24.7)	8111 (26.2)
2	2547 (22.1)	1066 (21.9)	6729 (21.8)
3	2301 (19.9)	1002 (20.6)	6137 (19.8)
4	1916 (16.6)	785 (16.1)	5253 (17.0)
5 (more deprived)	1229 (10.6)	527 (10.8)	3228 (10.4)
Missing	137 (1.2)	65 (1.3)	368 (1.2)
BMI (kg/m^2)	26.6 (23.3–30.9)	26.0 (22.9–30.2)	26.5 (23.3–30.6)
Past history of, n (%)			
Diabetes	1269 (11.0)	510 (10.5)	3347 (10.8)
Hypertension	5603 (48.5)	2346 (48.1)	13.805 (44.6)
Hypercholesterolaemia	3099 (26.8)	1289 (26.4)	8040 (26.0)

Continuous variables are reported as medians and IQR except for number of medication prescriptions that are reported as medians and (5th–95th percentile range). Categorical variables are reported as counts (percentages) for categorical variables.
*Before date of dementia or a randomly selected date for those without dementia.
BMI, body mass index;NSAID, non-steroidal anti-inflammatory drug.

Table 2 Risk of dementia and death

	Long-term exposed (n=11 550) compared with short-term exposed (n=4873)				Long-term exposed (n=11 550) compared with unexposed (n=30 930)			
	Crude sHR	P values	Adjusted sHR	P values	Crude sHR	P values	Adjusted sHR	P values
Risk of dementia								
AD (AD medical codes)	0.95 (0.58–1.53)	0.82	1.03 (0.63–1.69)*	0.89	0.79 (0.58–1.09)	0.15	0.81 (0.58–1.12)*	0.20
AD (AD / 'senile dementia' medical codes)	0.90 (0.60–1.34)	0.60	0.97 (0.65–1.45)†	0.87	0.76 (0.58–0.99)	0.04	0.79 (0.60–1.04)†	0.09
AD (AD / 'senile dementia' medical codes or specific medications‡)	0.86 (0.60–1.24)	0.42	0.97 (0.67–1.39)†	0.85	0.75 (0.59–0.95)	0.02	0.78 (0.61–1.00)†	0.05
Vascular dementia§	1.00 (0.52–1.93)	0.98	1.05 (0.54–2.04)*	0.88	0.81 (0.53–1.22)	0.32	0.84 (0.55–1.28)*	0.41
Other or unspecified dementia§	0.83 (0.47–1.45)	0.51	0.83 (0.47–1.47)†	0.53	0.78 (0.53–1.13)	0.19	0.78 (0.53–1.14)¶	0.20
Symptoms	1.03 (0.81–1.30)	0.83	1.03 (0.81–1.31)**	0.82	1.08 (0.92–1.27)	0.33	1.14 (0.97–1.33)†	0.12
Risk of death	Crude HR	P values	Adjusted HR	P values	Crude HR	P values	Adjusted HR	P values
Overall population	0.87 (0.77–0.98)	0.02	0.93 (0.83–1.05)¶	0.25	0.78 (0.72–0.84)	<0.001	0.79 (0.72–0.85)¶	<0.001
≤70 years old	0.78 (0.66–0.91)	0.002	0.84 (0.72–0.99)¶	0.04	0.71 (0.64–0.79)	<0.001	0.66 (0.59–0.75) ¶	<0.001
>70 years old	1.10 (0.92–1.31)	0.30	1.05 (0.88–1.27)¶	0.57	0.96 (0.86–1.08)	0.49	0.94 (0.83–1.05)¶	0.28
Those diagnosed with AD	0.72 (0.35–1.50)	0.38	1.10 (0.44–2.74)††	0.84	1.04 (0.62–1.75)	0.88	1.08 (0.60–1.92)††	0.80

*Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, and vitamin D prescriptions, history of hypertension or hypercholesterolaemia, smoking status and body mass index.
†Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, vitamin D prescriptions, history of hypertension, hypercholesterolaemia or diabetes, smoking status and body mass index.
‡That is, donepezil, galantamine, rivastigmine or memantine.
§Without any record of AD, senile dementia or specific medications.
¶Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, vitamin D prescriptions and history of hypertension.
**Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, vitamin D prescriptions, history of hypertension, hypercholesterolaemia, or diabetes, smoking status and Townsend index.
††Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, vitamin D prescriptions and history of hypertension.
AD, Alzheimer's diseases; HR, hazard ratio; NSAID, non-steroidal anti-inflammatory drug; ; sHR, sub-distribution HR.

study population, we identified cases of Alzheimer's disease, vascular dementia and other/unspecified dementias, symptoms that can be linked to dementia and death. Competing risk regression with death as a competing event and multivariable Cox proportional hazard models were used.

A total of 11 550 individuals exposed to hydroxychloroquine/chloroquine for ≥1 year, 4873 individuals exposed to hydroxychloroquine for <1 year and 30 930 individuals unexposed to the drugs were included in the study. The patients' characteristics are reported in table 1. On comparison with the control groups, people who had been chronically exposed to hydroxychloroquine/chloroquine were not at higher risk of Alzheimer's disease (table 2). In those chronically exposed, neither the duration of exposure (adjusted sHR: 1.03 (0.98–1.09) per each year of exposure, p=0.24) nor the mean hydroxychloroquine/chloroquine dosage (adjusted sHR: 1.01 (0.90–1.13), per each 50 mg/day increase, p=0.90) were associated with the risk of Alzheimer's disease. The risks of vascular dementia or other forms of dementia were not significantly different between groups. Eight per cent (n=3779) out of the 47 353 individuals died. Those ≤70 year old and chronically exposed to hydroxychloroquine/chloroquine were at lower risk of death compared with those shortly exposed or those unexposed.

In a recent case–control study, it was found that patients who used hydroxychloroquine were at increased risk of dementia (OR: 1.91 (1.39–2.64) for exposure ≥305 days on comparison with no exposure).[3] However, a significant risk increase was also evidenced in people using methotrexate or sulfasalazine. The effect of hydroxychloroquine on progression of dementia in early Alzheimer's disease was also investigated in 2001 in an 18-month randomised, placebo-controlled trial.[5] The results of the study showed no effect of treatment against placebo. We found similar results. We also found that a chronic exposure to hydroxychloroquine/chloroquine noticeably lowered risk of death in people with CTD. Whether this can be explained by metabolic and cardiovascular profile improvement[6] remains to be determined.

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Interaction between B-cell activation factor and methotrexate impacts immunogenicity of seasonal influenza vaccination in patients with rheumatoid arthritis

Methotrexate (MTX) with its proven efficacy and safety profile remains as the anchor drug for the treatment of rheumatoid arthritis (RA).[1 2] However, the impact of MTX alone or in conjunction with antitumour necrosis factor (anti-TNF) on humoral immune system and infection risk varies markedly among patients with RA, suggesting that other host factors influence the therapeutic response to MTX and/or anti-TNF treatment.[3] A possible candidate is B-cell activating factor (BAFF), which promotes B-cell activation and differentiation for antibody production.[4] When patients with RA received anti-TNF treatment, a high BAFF serum level prevented formation of antidrug antibody in patients taking MTX but not those who did not.[5] Thus, in the presence of MTX, BAFF may exert a paradoxical anti-inflammatory effect. Here, we investigated whether high BAFF levels negatively impact vaccine response via the inhibitory BAFF–MTX interaction in patients with RA taking MTX.

Patients with RA according to the revised 1987 American College of Rheumatology from the randomised controlled trial (ClinicalTrials.gov identifier: NCT02897011) that aimed to investigate the effects of a 2-week MTX discontinuation on vaccine response to seasonal influenza vaccination were included in this study.[6] Patients with RA were randomised to continue MTX or to hold MTX for 2 weeks after vaccination with 2016–2017 seasonal quadrivalent influenza vaccine that contained H1N1, H3N2, B-Yamagata and Victoria (GC Influenza, GC Pharma, South Korea). BAFF levels at vaccination and antibody titres to influenza antigens at baseline and 4 weeks after vaccination were measured (online methods and supplementary figure S1). A positive vaccine response was defined as a ≥4-fold increase in haemagglutination inhibition antibody titre.

Baseline characteristics of 316 patients (156 in the MTX-continue group and 160 in the MTX-hold group) were summarised in table 1. Baseline BAFF levels did not differ between the MTX-continue group and the MTX-hold group (866.1 (703.4–1036.2) vs 841.6 (688.4–108.9) pg/mL, p=0.741). The BAFF levels correlated with patient's age, prednisolone dose and absolute lymphocyte counts but not with disease activity, rheumatoid factor titre, anticyclic citrullinated peptide-antibody titre or MTX dose (online supplementary table S1). In the MTX-continue group, vaccine responders had significantly lower BAFF levels than the non-responders except in response in ≥1/4 antigens (figure 1A, left panel). However, BAFF levels did not differ between the vaccine responders and the non-responders in the MTX-hold group (figure 1A, right panel). Similarly, the antibody titre changes relative to the baseline against individual antigen (except against H3N2) correlated inversely with the respective serum BAFF levels in the MTX-continue group but not in the MTX-hold group (figure 1B). The impact of the MTX and BAFF interaction on antibody formation was significant for H1N1 (p=0.047), Yamagata (p=0.019) and Victoria (p=0.045) but not for H3N2 (p=0.177). The inverse correlation between BAFF levels and antibody production seemed to be more robust in patients taking MTX>15 mg/week than those taking MTX<7.5 mg/week (online supplementary table S2). Use of biologics and corticosteroids did not influence antibody formation (online supplementary table S3).

MTX in the presence of higher (and not lower) BAFF levels negatively impacted vaccine response to seasonal influenza vaccination, further supporting the counter-intuitive, paradoxical immune suppressive effect of BAFF in the presence of MTX. This hypothesis-driven study is a first proof of concept to confirm the recent basic-translational finding by Bitoun *et al* that provides a biological explanation of MTX–BAFF interaction that induces a tolerance to biological disease modifying antirheumatic drugs (ie, TNF inhibitor) and new antigens such as vaccination by generating immune suppressive adenosine and regulatory B cells.[5] This study supports that BAFF–MTX interaction at the time of antigen challenge is critical and that immune modulation by DMARDs depends on host immune factors. A soluble BAFF might serve as a surrogate marker of vaccination response in patients with RA taking MTX. Targeting

Table 1 Baseline characteristics of patients with RA

	MTX continue (n=156)	MTX hold (n=160)
Female (%)	129 (82.7%)	140 (87.5%)
Age, years	52.2±9.5	53.7±10.3
Duration of RA, years	6.8±6.5	6.9±6.2
RF positivity	120/154 (77.9%)	132/157 (84.1%)
ACPA positivity	105/121 (86.8%)	111/135 (82.2%)
DAS28-CRP	2.2±0.9	2.3±1.1
Treatment		
GC	82 (52.6%)	74 (46.3%)
Mean GC dose, mg/day	1.8±2.1	1.7±2.1
MTX	156 (100%)	160 (100%)
MTX dose, mg/week	13.3±3.4	13.1±3.2
Sulfasalazine	8 (5.1%)	10 (6.3%)
Hydroxychloroquine	35 (22.4%)	31 (19.4%)
Leflunomide	33 (21.2%)	37 (23.1%)
Tacrolimus	2 (1.3%)	2 (1.3%)
Biological DMARDs		
TNF inhibitor	11 (7.1%)	13 (8.1%)
Abatacept	1 (0.6%)	6 (3.8%)
Tocilizumab	4 (2.6%)	7 (4.4%)
Rituximab	1 (0.6%)	1 (0.6%)
Tofacitinib	0 (0%)	1 (0.6%)

Numbers are in n (%) or mean±SD.
ACPA, anticyclic citrullinated peptide-antibody; BAFF, B-cell activation factor; CRP, C reactive protein; DAS28, Disease Activity Score in 28 joints; DMARD, disease modifying antirheumatic drugs; GC, glucocorticoids; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; TNF, tumour necrosis factor.

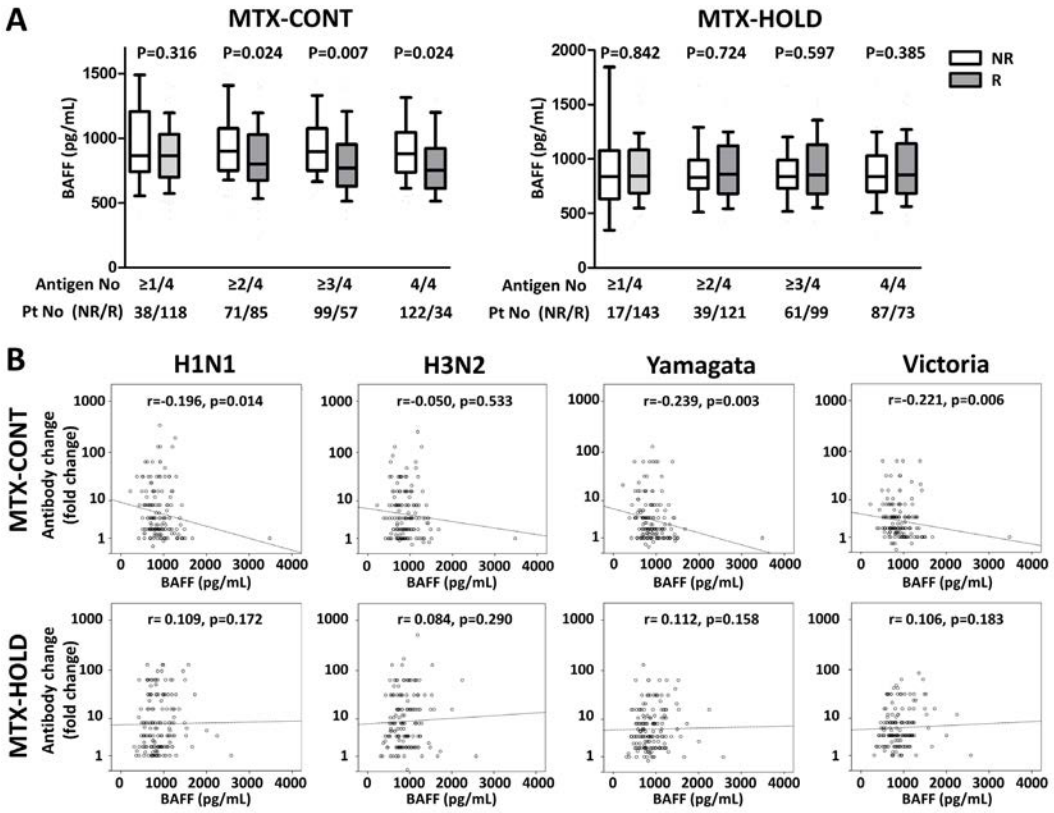

Figure 1 (A) Vaccine response depends on the MTX–BAFF interaction. BAFF levels were lower in the responders than the non-responders (according to number of antigen combination) in the MTX-continue group (left panel) but not when MTX was discontinued during peri-vaccination period (right panel). Box plot indicates the median and IQR, while whiskers indicate 10th and 90th percentile. P values were generated by Mann-Whitney test. (B) Correlation between BAFF levels and antibody formation against individual influenza strain. Fold changes in antibody titres relative to the baseline were plotted against their respective baseline BAFF levels in the MTX-continue group (upper panels) and the MTX-hold group (lower panels). Correlation was examined by using Spearman correlation. BAFF, B-cell activation factor; MTX, methotrexate; Pt No (NR/R), number of patients who responded and who did not respond to influenza antigen combination from ≥1/4 to 4/4 (responder/non-responder); r, Spearman rho.

the BAFF–MTX interaction might offer novel therapeutic approaches in RA treatment.

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Patient consent Obtained.

Ethics approval The study was approved by the Institutional Review Board of the Seoul National University Hospital (IRB 1608-158-787) and was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. The study was registered with http://www.clinicaltrials.gov, protocol number: NCT02897011. The protocol allows to use the stored sera for additional testing.

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Significant joint-destructive association of HLA-DRB1*04:05 independent of DAS28 in rheumatoid arthritis

Preventing joint destruction is one of the challenges in rheumatoid arthritis (RA).[1] Presence of two antibodies, namely, rheumatoid factor (RF) and cyclic citrullinated peptide antibodies (CCPs), is one of the major correlates of joint destruction.[2] We recently showed the association between the progression of joint destruction and HLA-DRB1*04:05, which is independent from CCP positivity.[3] HLA-DRB1*04:05 is one of shared epitope (SE) allele carrying common amino acid sequences at position 70–74 frequently found in Japanese and rarely observed in Europeans. Importantly, we showed that SE alleles other than DRB1*04:05 did not show independent associations from CCP.[3]

Based on the unique characteristics of HLA-DRB1*04:05, we hypothesised that HLA-DRB1*04:05 might lead to high disease activity not fully captured by Disease Activity Acore 28 (DAS28) and that it independently of DAS28 determines radiographic progression in patients with anti-CCP-positive RA (figure 1A).

We analysed the data set of our previous study[3] composed of 572 patients with CCP-positive RA all of whom fulfilled 2010 American College of Rheumatology/European League Against Rheumatism RA classification criteria.[4] All patients had data of modified Sharp/van der Heijde score (SHS), consecutive DAS28 to allow us to calculate time-averaged DAS28, which was shown to fit joint destruction better than one-time DAS28,[5] HLA-DRB1 genotypes, RF and disease duration.

Subjects carrying HLA-DRB1*04:05 had higher time-averaged DAS28 than subjects without as expected (3.64±1.03 and 3.49±1.02). However, we found that HLA-DRB1*04:05 was significantly associated with SHS in condition with RF, disease duration, cohort information and time-averaged DAS28 (p=0.00034, figure 1B, crude and with adjustment association results are shown in online supplementary material 1), indicating that the association between HLA-DRB1*04:05 and SHS could not be explained by difference in DAS28.

These results suggest that DAS28 is not enough to estimate disease activity to predict future joint destruction in patients carrying HLA-DRB1*04:05. Thus, we assumed the influence of HLA-DRB1*04:05 on SHS even in patients who had been in low disease activities or remission. We classified the patients according to time-averaged DAS28 and found that with the same covariates as the aforementioned analyses, HLA-DRB1*04:05 demonstrated a significant association with higher SHS in the group of high or moderate disease activities and also in low disease activities or remission (p=0.0077 and 0.013, respectively, figure 2). This trend was observed in both cohorts (data not shown).

Since we did not have full data of DAS28 in all the disease course for the participants, this limitation would lead to overestimation of the association of HLA-DRB1*04:05.[3] On this point, when we picked up 97 participants whose observation

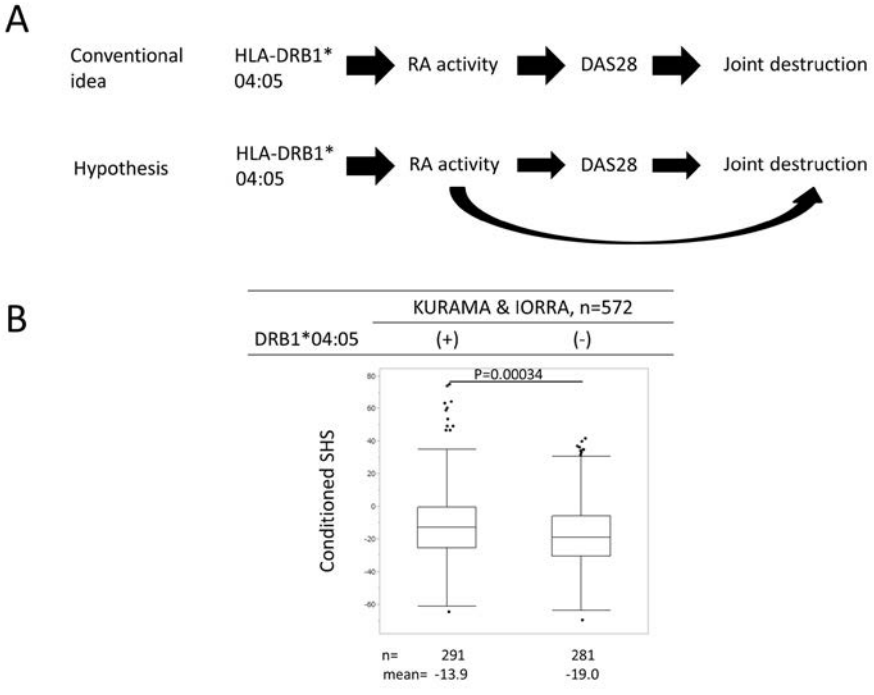

Figure 1 HLA-DRB1*04:05 showed a significant association with SHS independently of DAS28. (A) Schematic hypothesis of the association of HLA-DRB1*04:05 with SHS, independently of DAS28. (B) SHS is conditioned on time-averaged DAS28, disease duration, RF and cohort information and compared between subjects with HLA-DRB1*04:05 and without. The p value in linear regression analysis is indicated. RA, rheumatoid arthritis; SHS, Sharp/van der Heijde score.

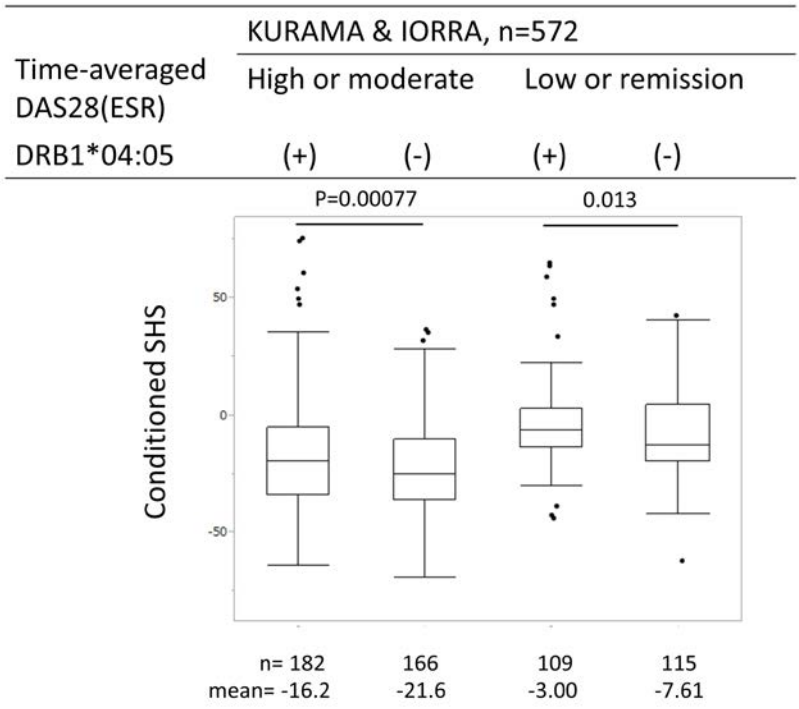

Figure 2 HLA-DRB1*04:05 carriers show higher Sharp/van der Heijde score (SHS) even in low disease activity or remission. Subjects with rheumatoid arthritis are classified into two groups based on their time-averaged DAS28 and the SHS conditioned on covariates are compared between the two groups. The p values in linear regression analyses are indicated.

time is approximately the same as their disease duration, we observed a similar association result with a comparable effect size (data not shown).

In conclusion, we showed that HLA-DRB1*04:05 has a joint-destructive association independently of CCP and DAS28. It would be interesting to identify unknown markers reflecting the remaining disease activity in subjects carrying HLA-DRB1*04:05, which cannot be fully evaluated by DAS28. It would be interesting to evaluate the association of HLA-DRB1*04:05 in large European RA cohorts.

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Contributors CT conceived the study design. HT and CT analysed the data. HT and CT wrote the main manuscript text. KY, KI, MH, MF, HI, TF, KO, AT, HY and TM contributed to collection of samples and/or data. KY and MF counted SHS score for the IORRA and KURAMA, respectively. WY aggregated the KURAMA database. All authors reviewed the manuscript.

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Prevalence and incidence of psoriasis and psoriatic arthritis

Psoriasis (Pso) and psoriatic arthritis (PsA) are inflammatory disorders which can severely impair health and quality of life. For both Pso and PsA, an increasing prevalence has been reported.[1 2] Comprehensive data on the prevalence and incidence of Pso and PsA are important in order to adequately allocate specialist care and financial resources. These data are incomplete for Pso and especially PsA. In particular, no population-based study has estimated their prevalence or incidence in Germany. We obtained the statutory health insurance data of approximately 65 million people from 2009 to 2012, covering 80% of the German population. Pso and PsA age-standardised prevalence based on the International Classification of Diseases (ICD) codes was obtained and age-standardised incidence rates calculated as described previously.[3] Briefly, cross-sectional prevalence data of consecutive years were used in conjunction with different assumed mortality rates of 1.1–1.5, with assumed reductions in annual mortality rates of 0%–5% in order to estimate incidence ranges.

Depending on the year, approximately 65 million individuals were assessed. There were 1.4–1.6 million cases of Pso and 127 000–156 000 cases of PsA identified. The age-standardised prevalence for Pso was 22.2–22.9 and 21.3–22.1 per 100 000 individuals in men and women, respectively (online supplementary figure 1). The prevalence for PsA was 1.8–2.1 and 2.1–2.5 per 100 000 individuals in men and women, respectively (online supplementary figure 2). A steady increase in prevalence was observed for both Pso and PsA. The incidence of Pso in 2009 ranged from 35.4 to 50.3 and from 46.3 to 58.2 in men and women, respectively, and declined thereafter. The incidence of PsA in 2009 ranged from 13.8 to 14.9 and from 18.1 to 19.1 in men and women, respectively, and declined thereafter. All data are detailed in table 1. Based on these data we used two different scenarios to estimate the number of patients living in Germany in 2018: (1) German age pyramid of 2018 applied to prevalence in 2012, or (2) projection of prevalence extrapolated by annual per cent change, then application of the 2018 age pyramid. Concerning Pso, 959 362–1 012 167 male and 956 822–1 030 847 female patients are expected to be living in Germany in 2018. Concerning PsA, 75 376–102 320 male and 90 473–127 349 female patients are expected to be living in Germany in 2018. This calculation may serve to project future mortality in other European countries.

Thus, we summarise that roughly 2 million patients with Pso and at least 200 000 patients with PsA are currently living in Germany. The age-standardised prevalence and incidence of PsA are in line with estimates from other European countries or the USA,[4] although higher incidences have been reported.[2] The ratio of PsA/Pso prevalence in the current study was approximately 10%, which is well within the range of previous reports. The ICD-based case definition is a limitation to the study as it may result in reduced precision as opposed to diagnostic criteria.[5] Most recent observational studies report an increase[6] or at least stable incidences[2] for Pso or PsA. In the current study, we calculated incidences based on cross-sectionally observed prevalence and different assumed mortality ratios in reference to the mathematical relation between incidence, prevalence and mortality.[3] These analyses consistently resulted in a decline in the incidence of both diseases over the observed study period. However, we suggest a careful interpretation of these incidences since changed awareness for the respective diagnoses or changed coding behaviour of ICD codes may account for the differences. Thus, the results should be interpreted as possible trends in incidences.

The epidemiological data reported herein cover a substantial portion of the German population and thereby improve our understanding of the prevalence and incidence of Pso and PsA in Europe.

Table 1 Prevalence of Pso and PsA from 2009 to 2012 in Germany

Year	2009	2010	2011	2012
Population (n)	64 637 752	63 962 071	64 988 016	65 792 296
Female (%)	53.5	53.4	53.3	53.2
Pso (n)	1 419 537	1 440 807	1 477 333	1 512 769
Pso prevalence (n/1000)				
Male	22.22	22.59	22.69	22.86
Female	21.27	21.76	21.93	22.12
Pso incidence (n/100 000)				
Male	35.38–50.27	26.44–39.36	17.32–29.31	17.14–26.31
Female	46.30–58.17	35.30–45.63	21.67–30.47	19.05–26.39
PsA (n)	127 334	137 763	146 463	156 182
PsA prevalence (n/1000)				
Male	1.81	1.96	2.03	2.13
Female	2.07	2.26	2.37	2.49
PsA incidence (n/100 000)				
Male	13.81–14.88	11.59–12.54	9.59–10.39	9.84–10.49
Female	18.12–19.14	15.23–16.14	12.03–12.80	11.76–12.38

Data from the German statutory health insurance system of approximately 64 million people (population) were employed to assess age-standardised prevalence of psoriasis (Pso) and psoriatic arthritis (PsA) for the male and female German population (mean values). Age-standardised incidence was calculated based on prevalence data and different assumed mortality scenarios resulting in the given ranges.

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Evaluation of retinal microvascular density in patients affected by systemic lupus erythematosus: an optical coherence tomography angiography study

Retinopathy in systemic lupus erythematosus (SLE) has an incidence of 7%–29% and is suggestive of high disease activity being a marker of poor visual outcome and prognosis for survival.[1] Recently, we demonstrated a subclinical retinal involvement in patients with SLE that seems to be related to kidney involvement where hydroxychloroquine had a protective role.[2] The pathogenesis of lupus retinopathy is attributed to a vasculopathy most commonly immune complex-mediated microangiopathy.[1] Optical coherence tomography angiography (OCTA) is a non-invasive technique for imaging the microvasculature of the retina and choroid that may quantify foveal avascular zone, non-perfused or low-perfused areas. Quantitative measurements based on OCTA may have value in managing retinopathy but also correlate with visual outcome and mirror vascular involvement in systemic diseases.[3] The aim of this study was to evaluate retinal microvasculature using OCTA in patients with SLE without signs of retinopathy according to standard lupus retinopathy

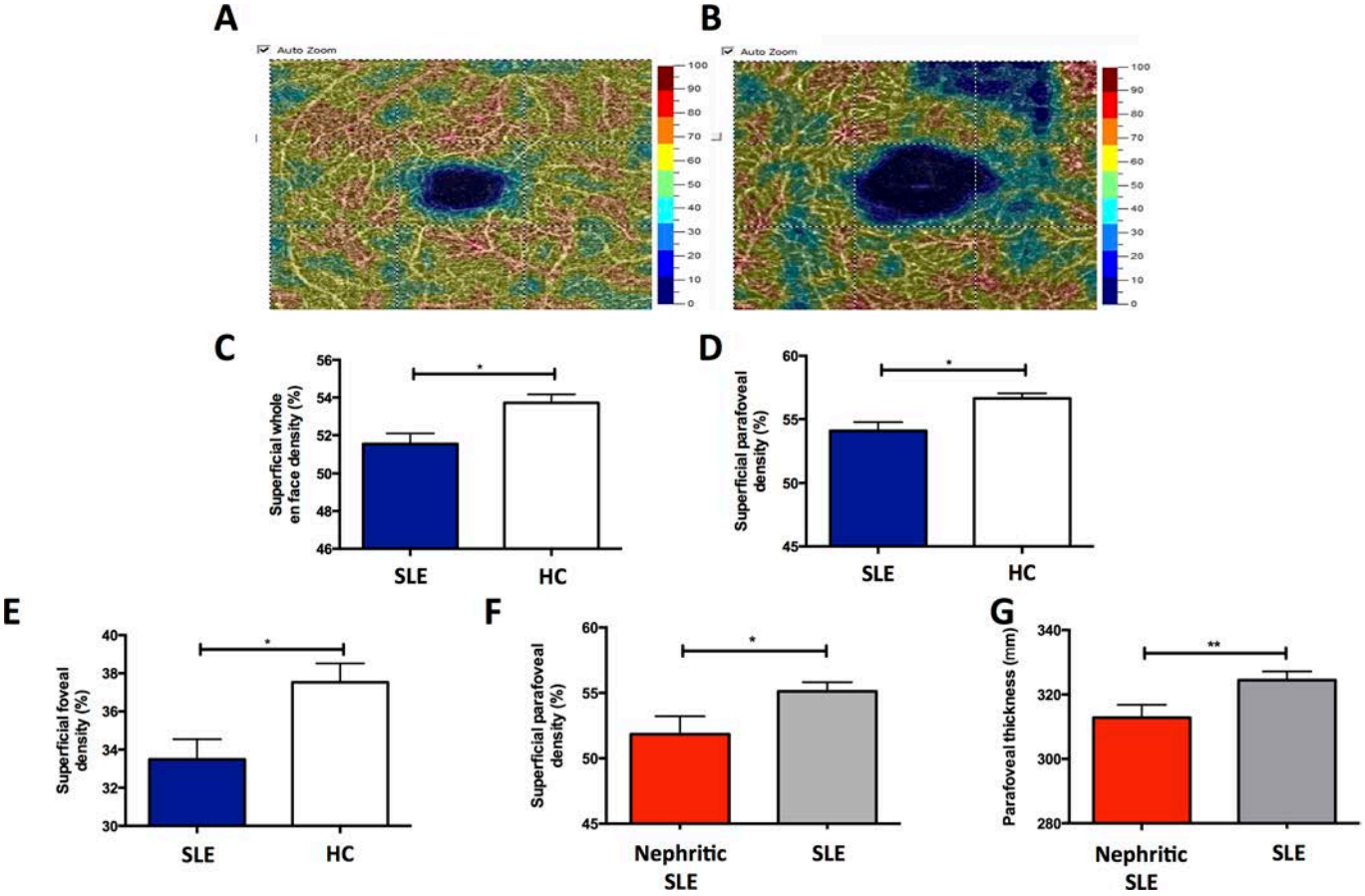

Figure 1 Superficial whole en face vessel density (%, colour-coded) maps in a (A) healthy control (HC) and a (B) patient with systemic lupus erythematosus (SLE). (B) Enlargement of the foveal avascular zone (central dark blue area) and parafoveal areas of reduced perfusion (light and dark blue areas). Patients with SLE displayed reduced superficial whole en face (C), parafoveal (D) and foveal (E) vessel density (%) compared with those in healthy eyes. Patients with SLE with lupus nephritis showed superficial parafoveal vessel density (%) compared with those in patients without nephritis (F). Parafoveal thickness in patients with SLE with nephritis was reduced than that in patients without kidney involvement (G). *P<0.05; **P<0.01.

classification[4] and correlate abnormal vascular density with disease activity, damage accrual, treatment and visual outcome. From 20 November 2015 to 31 December 2017, a total of 52 eyes of patients with SLE, diagnosed according to the American College of Rheumatology classification criteria,[5] and 40 eyes of healthy controls (HC) were examined by means of a 6 mm OCTA scan (Optovue XR Avanti, Fremont, CA). Split-spectrum amplitude-decorrelation angiography generated optical coherence tomography angiograms of both superficial and deep retinal capillaries referred to the whole en face, foveal and parafoveal zone from patients with SLE and HC (figure 1A,B). Capillary density values were compared with clinical data by Spearman's rank correlation coefficient, and groups were compared using analysis of variance and Kruskal-Wallis analyses. Values of $p<0.05$ were considered statistically significant. Demographic and clinical features of enrolled subjects are summarised in table 1. The eyes from patients with SLE had a lower mean superficial whole en face density, superficial parafoveal density and superficial foveal density ($p=0.02$ for all comparisons) compared with healthy eyes (figure 1C–E). Patients with SLE with nephritis displayed reduced parafoveal vessel density and parafoveal thickness compared with those of patients without nephritis ($p=0.02$ and $p=0.008$, figure 1F,G). A negative correlation was demonstrated in patients with SLE between age and superficial whole en face density ($p=0.0005$, $r=-0.5$), superficial foveal density ($p=0.006$, $r=-0.4$), superficial parafoveal density ($p=0.004$, $r=-0.4$), deep whole en face density ($p=0.003$, $r=-0.4$) and deep parafoveal density ($p=0.001$, $r=-0.4$). Systemic Lupus Erythematosus Disease Activity Index correlated inversely with superficial en face density ($p=0.002$, $r=-0.4$), superficial parafoveal density ($p=0.0003$, $r=-0.5$ and $p=0.002$), deep whole en face density ($p=0.01$, $r=-0.4$) and deep parafoveal density ($p=0.002$, $r=-0.4$). A negative correlation was also found between Systemic Lupus International Collaborating Clinics (SLICC) and superficial whole en face density ($p=0.0001$, $r=-0.5$), superficial parafoveal density ($p<0.0001$, $r=-0.6$), deep whole en face density ($p<0.0001$, $r=-0.6$) and deep parafoveal density ($p<0.0001$, $r=-0.7$). A positive correlation was found between hydroxychloroquine cumulative dose and both superficial and deep parafoveal density ($p=0.009$, $r=0.4$ and $p=0.04$, $r=0.3$). Best corrected visual acuity in SLE positively correlated with superficial whole en face density, superficial parafoveal density, deep whole en face density and deep parafoveal density ($p<0.0001$, $r=0.7$ for all correlations).

Patients with SLE displayed a reduced retinal microvascular density compared with normal subjects, in particular those with kidney involvement. Vessel density provides a quantitative metric of capillary network that correlated with age, best corrected visual acuity and clinical features as SLE disease activity and damage accrual. Hydroxychloroquine might have a protective role preserving the microvascular structures.

Table 1 Demographic and clinical characteristics of enrolled subjects

	HC (n=20)	SLE (n=26)
Age (years)	46±8.9	49.6±13.6
Female, n (%)	16 (80)	23 (88.5)
Disease duration (years)	NA	15.1±7.7
Anti-dsDNA positive Abs, n (%)	NA	13 (50)
aPL positive Abs, n (%)	NA	10 (40)
C3 (mg/L)	NA	98.9±21.7
C4 (mg/L)	NA	19.6±5.8
SLEDAI-2K	NA	4.3±4.4
SLICC	NA	1.9±1.5
HCQ, n (%) HCQ cumulative dose (g)	NA	16 (61.5) 738.8±486.8
BCVA (logMAR)	0.0±0.1	0.0±0.1
Kidney involvement*, n (%)	NA	10 (40)

*Kidney involvement was defined as the presence of biopsy-proven glomerulonephritis class III, IV or V according to the International Society of Nephrology/Renal Pathology Society glomerulonephritis classification criteria.[6]
Continuous variables were shown using mean and SD.
aPL, antiphospholipid; BCVA, best corrected visual acuity; HC, healthy controls; HCQ, hydroxychloroquine; NA, not applicable; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; SLICC, SLICC/ACR damage index score.

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Kawasaki disease in infants 3 months of age and younger: a multicentre Spanish study

Kawasaki disease (KD) is a multisystem vasculitis of small and medium vessels typical of childhood. Timely treatment with intravenous immunoglobulin (IVIG) has reduced the incidence of coronary artery abnormalities (CAAs) from 25% to approximately 4%.[1] Asian studies have focused on infants under 3 months of age, but there are no published data about these patients from Western countries.[2 3]

We reviewed 621 patients under 16 years old with a diagnosis of KD between 2011 and 2016 from a multicentre study in Spain (KAWA-RACE study); 84 hospitals participated throughout the country.

We found seven children under 90 days (1.13%), with a male predominance (6 of 7). Five presented irritability, but only two fulfilled the criteria for complete KD (table 1).[1] The following were the main laboratory findings (median, IQR): highest C reactive protein (CRP) 24 mg/L (8.48–31.4), highest erythrocyte sedimentation rate 79 mm (70–105.5), maximum and minimum platelet count 900 ×10^9/L (682–1 117) and 506 ×10^9/L (449–612), minimum haemoglobin 10 g/dL (9–10.8), maximum leucocytes 21 ×10^9/L (16.45–23.37), minimum sodium 135.5 mEq/L (133–137.5), and minimum albumin 2.9 mg/dL (2.6–3.4).

In three cases, a viral infection was diagnosed and four patients presented with CAA, but no other echocardiographic findings were detected (table 1).

The median time interval since fever onset to IVIG administration was 8 days. All patients responded well to the first dose of IVIG, and only one received concomitant intravenous steroids because he was considered to be at high risk for IVIG resistance. All CAAs were transient and resolved during follow-up (table 1).

Epidemiology is different in Western countries when compared with Asian countries, where the incidence can reach up to 264.8 cases/100 000 children <5 years of age, as in Japan 2012. In USA there is also a relatively high incidence of around 25/100 000 when compared with European countries.[1] Incidence in Spain is only known in the Catalonia region and was estimated to be 8/100 000 <5 years old (2004–2014).[4]

KD in younger children is more difficult to diagnose as it presents more frequently as incomplete KD. A study from Korea with 24 patients younger than 3 months of age describes an 87.5% of incomplete KD forms, and a mean number of major diagnostic criteria of 2.8±1.4: rash was the most common (50%) and conjunctival injection was the least common (12.5%).[3] In our population non-complete KD cases represented 71.4% of the total, rash was present in 85.7%, but cervical lymphadenopathy was the least common finding (14.3%).

When we looked at laboratory tests, our case series showed less CRP increment when compared with Asian studies, 24 mg/L (median), vs 79±52 or 78.4±69 (mean), respectively, but no other relevant differences were found.[3 5] Infections were not documented in any children from the studies of Lee *et al*, Bae *et al* or Satoh *et al*.[2 3 5] In our population, 42.8% of patients presented with a confirmed infection, but were treated for KD regardless as the role of these pathogens is unclear and the consequences of not treating KD in time could be devastating.

The incidence of CAA in our series is considerably higher when compared with others, and may be due to late diagnosis: three had aneurysms (42.8% of patients), and one had dilation, according to McCrindle Z-score classification[1] (57.14% of the total had an abnormal echo). A large Korean study with 609 patients <3 months old showed an incidence of CAA of 19.9% (116 of 583), 18% dilation and 3.4% aneurysms.[2] Echocardiographic abnormalities were detected in 25% of the Bae *et al*[3] population (three cases of valve dysfunctioning without coronary involvement), and only 12.5% were CAA. All our cases with CAA recovered completely compared with the Japanese series from Satoh *et al*,[5] where 7 of 24 patients presented CAA, but in only 2 cases these alterations persisted for 1 year (8.3%).

This multicentre study let us study an uncommon condition from a large series. Despite the small number of patients, we have observed more frequent CAA, but good response to IVIG and no long-term sequelae.

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Table 1 Diagnosis, symptoms, cardiological and microbiological findings

Age (months)	Complete Kawasaki disease	Irritability	Extremity changes	Rash	Conjunctivitis	Oral changes	Cervical lymph nodes	Microbiological findings	Cardiological findings	Z-score* (SD)	Vessels (n)	Time to resolution (weeks)
3	No	No	Yes	Yes	Yes	No	No	*Enterovirus* (CSF)	CA	2.9	1	18
1.6	No	Yes	No	Yes	No	Yes	No	No	Unremarkable	–	–	–
2.6	Yes	Yes	Yes	Yes	Yes	No	Yes	No	CA	NA	1	5
2	No	No	No	Yes	Yes	Yes	No	No	Unremarkable	–	–	–
2.2	No	Yes	No	Yes	No	No	No	No	CA	3.2	2	13
2.9	Yes	Yes	Yes	Yes	Yes	Yes	No	Adenovirus (PS)	Unremarkable	–	–	–
2.3	No	Yes	No	No	Yes	Yes	No	Coryzal symptoms	CD	2–2.5	1	–

In three cases, a viral infection was diagnosed and four patients presented CAA, but no other echocardiographic findings were detected (table 1).
*Maximum Z-score[1 6] all measured at acute phase.
CA, coronary aneurysm; CD, coronary dilation; CSF, cerebrospinal fluid; NA, not available; PS, pharyngeal swab.

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Chronic hydroxychloroquine/chloroquine exposure for connective tissue diseases and risk of Alzheimer's disease: a population-based cohort study

Autophagy is an intracellular pathway by which cells generate energy and metabolites by recycling their own non-essential, redundant or damaged components.[1] Pathophysiological

studies have demonstrated that the impairment of autophagy contributes to protein aggregate accumulation that occurs during Alzheimer's disease and experiments have shown that autophagy inhibitors, such as chloroquine and hydroxychloroquine, block amyloid plaque degradation.[1 2] Further, a recent case–control study found that patients with rheumatoid arthritis who used hydroxychloroquine were at increased risk of dementia.[3] We investigated whether chronic exposure to chloroquine/hydroxychloroquine increases the risk of Alzheimer's disease.

Data from The Health Improvement Network (THIN) were used (January 1990–December 2016). THIN is a UK primary care database on >12 million people. Participating general practitioners prospectively enter clinical information on individuals so that the database provides a longitudinal medical record for each individual. THIN is representative of the UK population. The diagnostic and prescribing data compare favourably with external statistics.[4] Individuals were included in the exposed sample if they had been prescribed hydroxychloroquine/chloroquine for connective tissue diseases (CTD), for ≥1 year, at a mean dosage ≥50 mg/day for chloroquine and ≥100 mg/day for hydroxychloroquine. The first control group was made of individuals who received hydroxychloroquine for <1 year for the same underlying condition as the exposed individuals. For these groups, the start of at-risk period was defined as the first day of the first prescription of hydroxychloroquine/chloroquine. The second control group included individuals who had never been exposed to chloroquine, hydroxychloroquine, quinine, quinacrine or mefloquine, but were suffering from the same CTD as the exposed individuals. For each unexposed individual, a uniformly randomly selected start of the at-risk period was defined. Up to three unexposed and shortly exposed individuals were selected for every chronically exposed. For all individuals included in our

Table 1 Characteristics of the study population

	Long-term exposed N=11 550	Short-term exposed N=4873	Unexposed N=30 930
Age (years)	56 (46–669)	57 (45–67)	57 (46–67)
Female, n (%)	8970 (77.7)	4023 (82.6)	23 251 (75.2)
Duration of follow-up after 'start date' (days)	1630 (935–2737)	1658 (923–2806)	1521 (1097–2377)
Dosage (mg/day)			
Hydroxychloroquine	261 (200–356)	391 (255–400)	–
Chloroquine	162 (102–224)	–	–
Underlying diseases, n (%)			
Rheumatoid arthritis	7866 (68.1)	3411 (70.0)	22 274 (72.0)
Lupus erythematosus	2.032 (17.6)	616 (12.6)	3105 (10.0)
Sjogren syndrome	781 (6.8)	412 (8.5)	2653 (8.6)
Other connective tissue diseases	782 (6.8)	401 (8.2)	2632 (8.5)
Light eruption	89 (0.7)	33 (0.7)	266 (0.9)
Number of prescriptions of*			
Methotrexate	0 (0–69)	0 (0–63)	0 (0–46)
Azathioprine	0 (0–2)	0 (0–2)	0 (0–0)
Glucocorticoids	1 (0–64)	1 (0–74)	0 (0–35)
NSAIDs	8 (0–100)	9 (0–104)	4 (0–82)
Vitamin D	0 (0–34)	0 (0–33)	0 (0–20)
Smoking status, n (%)			
Non-smokers	5350 (46.3)	2286 (46.9)	15 221 (49.2)
Ex-smokers	3694 (32.0)	1406 (28.9)	8842 (28.6)
Smokers	2481 (21.5)	1170 (24.0)	6702 (21.7)
Missing	25 (0.2)	11 (0.2)	165 (0.5)
Townsend deprivation index, n (%)			
0 (less deprived)	459 (4.0)	223 (4.6)	1104 (3.6)
1	2961 (25.6)	1205 (24.7)	8111 (26.2)
2	2547 (22.1)	1066 (21.9)	6729 (21.8)
3	2301 (19.9)	1002 (20.6)	6137 (19.8)
4	1916 (16.6)	785 (16.1)	5253 (17.0)
5 (more deprived)	1229 (10.6)	527 (10.8)	3228 (10.4)
Missing	137 (1.2)	65 (1.3)	368 (1.2)
BMI (kg/m^2)	26.6 (23.3–30.9)	26.0 (22.9–30.2)	26.5 (23.3–30.6)
Past history of, n (%)			
Diabetes	1269 (11.0)	510 (10.5)	3347 (10.8)
Hypertension	5603 (48.5)	2346 (48.1)	13.805 (44.6)
Hypercholesterolaemia	3099 (26.8)	1289 (26.4)	8040 (26.0)

Continuous variables are reported as medians and IQR except for number of medication prescriptions that are reported as medians and (5th–95th percentile range). Categorical variables are reported as counts (percentages) for categorical variables.
*Before date of dementia or a randomly selected date for those without dementia.
BMI, body mass index;NSAID, non-steroidal anti-inflammatory drug.

Table 2 Risk of dementia and death

	Long-term exposed (n=11 550) compared with short-term exposed (n=4873)				Long-term exposed (n=11 550) compared with unexposed (n=30 930)			
	Crude sHR	P values	Adjusted sHR	P values	Crude sHR	P values	Adjusted sHR	P values
Risk of dementia								
AD (AD medical codes)	0.95 (0.58–1.53)	0.82	1.03 (0.63–1.69)*	0.89	0.79 (0.58–1.09)	0.15	0.81 (0.58–1.12)*	0.20
AD (AD / 'senile dementia' medical codes)	0.90 (0.60–1.34)	0.60	0.97 (0.65–1.45)†	0.87	0.76 (0.58–0.99)	0.04	0.79 (0.60–1.04)†	0.09
AD (AD / 'senile dementia' medical codes or specific medications‡)	0.86 (0.60–1.24)	0.42	0.97 (0.67–1.39)†	0.85	0.75 (0.59–0.95)	0.02	0.78 (0.61–1.00)†	0.05
Vascular dementia§	1.00 (0.52–1.93)	0.98	1.05 (0.54–2.04)*	0.88	0.81 (0.53–1.22)	0.32	0.84 (0.55–1.28)*	0.41
Other or unspecified dementia§	0.83 (0.47–1.45)	0.51	0.83 (0.47–1.47)†	0.53	0.78 (0.53–1.13)	0.19	0.78 (0.53–1.14)¶	0.20
Symptoms	1.03 (0.81–1.30)	0.83	1.03 (0.81–1.31)**	0.82	1.08 (0.92–1.27)	0.33	1.14 (0.97–1.33)†	0.12
Risk of death	Crude HR	P values	Adjusted HR	P values	Crude HR	P values	Adjusted HR	P values
Overall population	0.87 (0.77–0.98)	0.02	0.93 (0.83–1.05)¶	0.25	0.78 (0.72–0.84)	<0.001	0.79 (0.72–0.85)¶	<0.001
≤70 years old	0.78 (0.66–0.91)	0.002	0.84 (0.72–0.99)¶	0.04	0.71 (0.64–0.79)	<0.001	0.66 (0.59–0.75) ¶	<0.001
>70 years old	1.10 (0.92–1.31)	0.30	1.05 (0.88–1.27)¶	0.57	0.96 (0.86–1.08)	0.49	0.94 (0.83–1.05)¶	0.28
Those diagnosed with AD	0.72 (0.35–1.50)	0.38	1.10 (0.44–2.74)††	0.84	1.04 (0.62–1.75)	0.88	1.08 (0.60–1.92)††	0.80

*Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, and vitamin D prescriptions, history of hypertension or hypercholesterolaemia, smoking status and body mass index.
†Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, vitamin D prescriptions, history of hypertension, hypercholesterolaemia or diabetes, smoking status and body mass index.
‡That is, donepezil, galantamine, rivastigmine or memantine.
§Without any record of AD, senile dementia or specific medications.
¶Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, vitamin D prescriptions and history of hypertension.
**Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, vitamin D prescriptions, history of hypertension, hypercholesterolaemia, or diabetes, smoking status and Townsend index.
††Adjusted on sex, age, underlying condition, number of glucocorticoids, NSAIDs, immunosuppressive therapies, vitamin D prescriptions and history of hypertension.
AD, Alzheimer's diseases; HR, hazard ratio; NSAID, non-steroidal anti-inflammatory drug; ; sHR, sub-distribution HR.

study population, we identified cases of Alzheimer's disease, vascular dementia and other/unspecified dementias, symptoms that can be linked to dementia and death. Competing risk regression with death as a competing event and multivariable Cox proportional hazard models were used.

A total of 11 550 individuals exposed to hydroxychloroquine/chloroquine for ≥1 year, 4873 individuals exposed to hydroxychloroquine for <1 year and 30 930 individuals unexposed to the drugs were included in the study. The patients' characteristics are reported in table 1. On comparison with the control groups, people who had been chronically exposed to hydroxychloroquine/chloroquine were not at higher risk of Alzheimer's disease (table 2). In those chronically exposed, neither the duration of exposure (adjusted sHR: 1.03 (0.98–1.09) per each year of exposure, p=0.24) nor the mean hydroxychloroquine/chloroquine dosage (adjusted sHR: 1.01 (0.90–1.13), per each 50 mg/day increase, p=0.90) were associated with the risk of Alzheimer's disease. The risks of vascular dementia or other forms of dementia were not significantly different between groups. Eight per cent (n=3779) out of the 47 353 individuals died. Those ≤70 year old and chronically exposed to hydroxychloroquine/chloroquine were at lower risk of death compared with those shortly exposed or those unexposed.

In a recent case–control study, it was found that patients who used hydroxychloroquine were at increased risk of dementia (OR: 1.91 (1.39–2.64) for exposure ≥305 days on comparison with no exposure).[3] However, a significant risk increase was also evidenced in people using methotrexate or sulfasalazine. The effect of hydroxychloroquine on progression of dementia in early Alzheimer's disease was also investigated in 2001 in an 18-month randomised, placebo-controlled trial.[5] The results of the study showed no effect of treatment against placebo. We found similar results. We also found that a chronic exposure to hydroxychloroquine/chloroquine noticeably lowered risk of death in people with CTD. Whether this can be explained by metabolic and cardiovascular profile improvement[6] remains to be determined.

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Interaction between B-cell activation factor and methotrexate impacts immunogenicity of seasonal influenza vaccination in patients with rheumatoid arthritis

Methotrexate (MTX) with its proven efficacy and safety profile remains as the anchor drug for the treatment of rheumatoid arthritis (RA).[1 2] However, the impact of MTX alone or in conjunction with antitumour necrosis factor (anti-TNF) on humoral immune system and infection risk varies markedly among patients with RA, suggesting that other host factors influence the therapeutic response to MTX and/or anti-TNF treatment.[3] A possible candidate is B-cell activating factor (BAFF), which promotes B-cell activation and differentiation for antibody production.[4] When patients with RA received anti-TNF treatment, a high BAFF serum level prevented formation of antidrug antibody in patients taking MTX but not those who did not.[5] Thus, in the presence of MTX, BAFF may exert a paradoxical anti-inflammatory effect. Here, we investigated whether high BAFF levels negatively impact vaccine response via the inhibitory BAFF–MTX interaction in patients with RA taking MTX.

Patients with RA according to the revised 1987 American College of Rheumatology from the randomised controlled trial (ClinicalTrials.gov identifier: NCT02897011) that aimed to investigate the effects of a 2-week MTX discontinuation on vaccine response to seasonal influenza vaccination were included in this study.[6] Patients with RA were randomised to continue MTX or to hold MTX for 2 weeks after vaccination with 2016–2017 seasonal quadrivalent influenza vaccine that contained H1N1, H3N2, B-Yamagata and Victoria (GC Influenza, GC Pharma, South Korea). BAFF levels at vaccination and antibody titres to influenza antigens at baseline and 4 weeks after vaccination were measured (online methods and supplementary figure S1). A positive vaccine response was defined as a ≥4-fold increase in haemagglutination inhibition antibody titre.

Baseline characteristics of 316 patients (156 in the MTX-continue group and 160 in the MTX-hold group) were summarised in table 1. Baseline BAFF levels did not differ between the MTX-continue group and the MTX-hold group (866.1 (703.4–1036.2) vs 841.6 (688.4–108.9) pg/mL, p=0.741). The BAFF levels correlated with patient's age, prednisolone dose and absolute lymphocyte counts but not with disease activity, rheumatoid factor titre, anticyclic citrullinated peptide-antibody titre or MTX dose (online supplementary table S1). In the MTX-continue group, vaccine responders had significantly lower BAFF levels than the non-responders except in response in ≥1/4 antigens (figure 1A, left panel). However, BAFF levels did not differ between the vaccine responders and the non-responders in the MTX-hold group (figure 1A, right panel). Similarly, the antibody titre changes relative to the baseline against individual antigen (except against H3N2) correlated inversely with the respective serum BAFF levels in the MTX-continue group but not in the MTX-hold group (figure 1B). The impact of the MTX and BAFF interaction on antibody formation was significant for H1N1 (p=0.047), Yamagata (p=0.019) and Victoria (p=0.045) but not for H3N2 (p=0.177). The inverse correlation between BAFF levels and antibody production seemed to be more robust in patients taking MTX>15 mg/week than those taking MTX<7.5 mg/week (online supplementary table S2). Use of biologics and corticosteroids did not influence antibody formation (online supplementary table S3).

MTX in the presence of higher (and not lower) BAFF levels negatively impacted vaccine response to seasonal influenza vaccination, further supporting the counter-intuitive, paradoxical immune suppressive effect of BAFF in the presence of MTX. This hypothesis-driven study is a first proof of concept to confirm the recent basic-translational finding by Bitoun *et al* that provides a biological explanation of MTX–BAFF interaction that induces a tolerance to biological disease modifying antirheumatic drugs (ie, TNF inhibitor) and new antigens such as vaccination by generating immune suppressive adenosine and regulatory B cells.[5] This study supports that BAFF–MTX interaction at the time of antigen challenge is critical and that immune modulation by DMARDs depends on host immune factors. A soluble BAFF might serve as a surrogate marker of vaccination response in patients with RA taking MTX. Targeting

Table 1 Baseline characteristics of patients with RA

	MTX continue (n=156)	MTX hold (n=160)
Female (%)	129 (82.7%)	140 (87.5%)
Age, years	52.2±9.5	53.7±10.3
Duration of RA, years	6.8±6.5	6.9±6.2
RF positivity	120/154 (77.9%)	132/157 (84.1%)
ACPA positivity	105/121 (86.8%)	111/135 (82.2%)
DAS28-CRP	2.2±0.9	2.3±1.1
Treatment		
GC	82 (52.6%)	74 (46.3%)
Mean GC dose, mg/day	1.8±2.1	1.7±2.1
MTX	156 (100%)	160 (100%)
MTX dose, mg/week	13.3±3.4	13.1±3.2
Sulfasalazine	8 (5.1%)	10 (6.3%)
Hydroxychloroquine	35 (22.4%)	31 (19.4%)
Leflunomide	33 (21.2%)	37 (23.1%)
Tacrolimus	2 (1.3%)	2 (1.3%)
Biological DMARDs		
TNF inhibitor	11 (7.1%)	13 (8.1%)
Abatacept	1 (0.6%)	6 (3.8%)
Tocilizumab	4 (2.6%)	7 (4.4%)
Rituximab	1 (0.6%)	1 (0.6%)
Tofacitinib	0 (0%)	1 (0.6%)

Numbers are in n (%) or mean±SD.
ACPA, anticyclic citrullinated peptide-antibody; BAFF, B-cell activation factor; CRP, C reactive protein; DAS28, Disease Activity Score in 28 joints; DMARD, disease modifying antirheumatic drugs; GC, glucocorticoids; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; TNF, tumour necrosis factor.

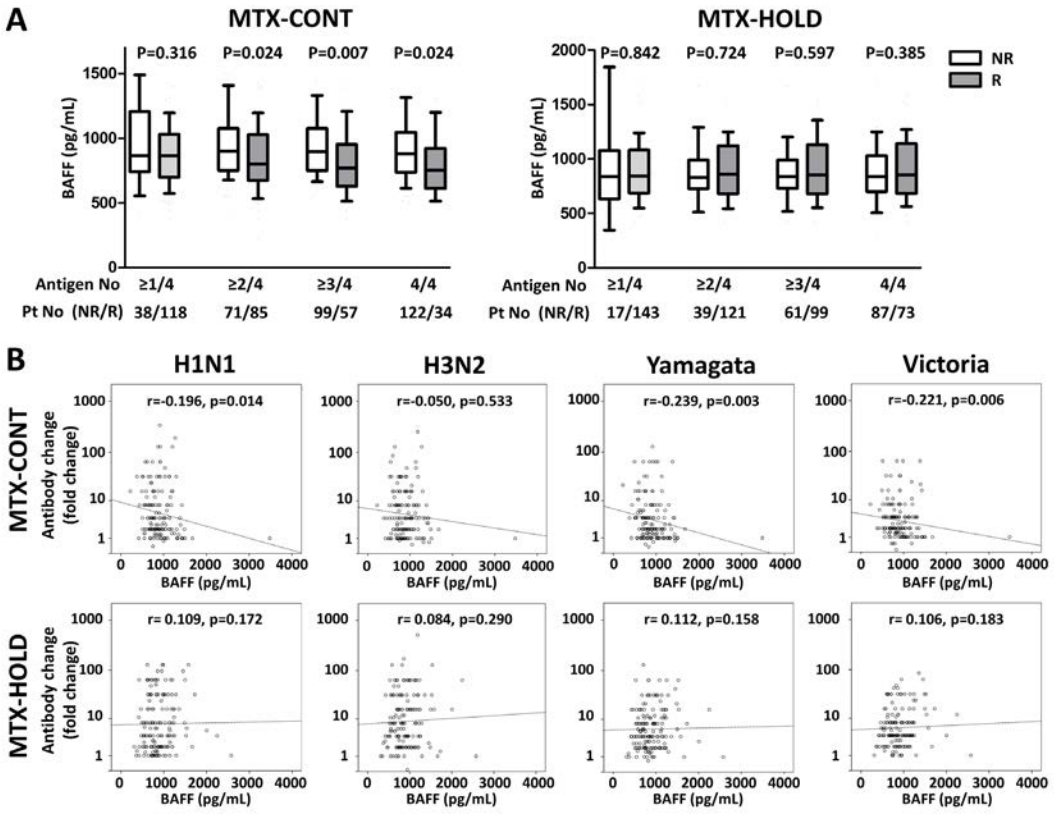

Figure 1 (A) Vaccine response depends on the MTX–BAFF interaction. BAFF levels were lower in the responders than the non-responders (according to number of antigen combination) in the MTX-continue group (left panel) but not when MTX was discontinued during peri-vaccination period (right panel). Box plot indicates the median and IQR, while whiskers indicate 10th and 90th percentile. P values were generated by Mann-Whitney test. (B) Correlation between BAFF levels and antibody formation against individual influenza strain. Fold changes in antibody titres relative to the baseline were plotted against their respective baseline BAFF levels in the MTX-continue group (upper panels) and the MTX-hold group (lower panels). Correlation was examined by using Spearman correlation. BAFF, B-cell activation factor; MTX, methotrexate; Pt No (NR/R), number of patients who responded and who did not respond to influenza antigen combination from ≥1/4 to 4/4 (responder/non-responder); r, Spearman rho.

the BAFF–MTX interaction might offer novel therapeutic approaches in RA treatment.

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Patient consent Obtained.

Ethics approval The study was approved by the Institutional Review Board of the Seoul National University Hospital (IRB 1608-158-787) and was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines. The study was registered with http://www.clinicaltrials.gov, protocol number: NCT02897011. The protocol allows to use the stored sera for additional testing.

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Significant joint-destructive association of HLA-DRB1*04:05 independent of DAS28 in rheumatoid arthritis

Preventing joint destruction is one of the challenges in rheumatoid arthritis (RA).[1] Presence of two antibodies, namely, rheumatoid factor (RF) and cyclic citrullinated peptide antibodies (CCPs), is one of the major correlates of joint destruction.[2] We recently showed the association between the progression of joint destruction and HLA-DRB1*04:05, which is independent from CCP positivity.[3] HLA-DRB1*04:05 is one of shared epitope (SE) allele carrying common amino acid sequences at position 70–74 frequently found in Japanese and rarely observed in Europeans. Importantly, we showed that SE alleles other than DRB1*04:05 did not show independent associations from CCP.[3]

Based on the unique characteristics of HLA-DRB1*04:05, we hypothesised that HLA-DRB1*04:05 might lead to high disease activity not fully captured by Disease Activity Acore 28 (DAS28) and that it independently of DAS28 determines radiographic progression in patients with anti-CCP-positive RA (figure 1A).

We analysed the data set of our previous study[3] composed of 572 patients with CCP-positive RA all of whom fulfilled 2010 American College of Rheumatology/European League Against Rheumatism RA classification criteria.[4] All patients had data of modified Sharp/van der Heijde score (SHS), consecutive DAS28 to allow us to calculate time-averaged DAS28, which was shown to fit joint destruction better than one-time DAS28,[5] HLA-DRB1 genotypes, RF and disease duration.

Subjects carrying HLA-DRB1*04:05 had higher time-averaged DAS28 than subjects without as expected (3.64±1.03 and 3.49±1.02). However, we found that HLA-DRB1*04:05 was significantly associated with SHS in condition with RF, disease duration, cohort information and time-averaged DAS28 (p=0.00034, figure 1B, crude and with adjustment association results are shown in online supplementary material 1), indicating that the association between HLA-DRB1*04:05 and SHS could not be explained by difference in DAS28.

These results suggest that DAS28 is not enough to estimate disease activity to predict future joint destruction in patients carrying HLA-DRB1*04:05. Thus, we assumed the influence of HLA-DRB1*04:05 on SHS even in patients who had been in low disease activities or remission. We classified the patients according to time-averaged DAS28 and found that with the same covariates as the aforementioned analyses, HLA-DRB1*04:05 demonstrated a significant association with higher SHS in the group of high or moderate disease activities and also in low disease activities or remission (p=0.0077 and 0.013, respectively, figure 2). This trend was observed in both cohorts (data not shown).

Since we did not have full data of DAS28 in all the disease course for the participants, this limitation would lead to overestimation of the association of HLA-DRB1*04:05.[3] On this point, when we picked up 97 participants whose observation

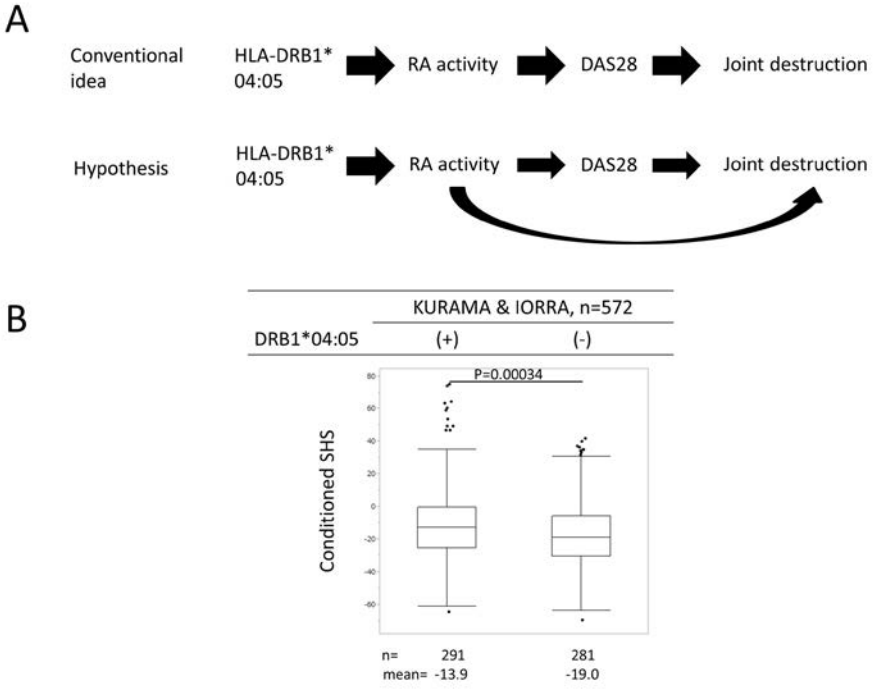

Figure 1 HLA-DRB1*04:05 showed a significant association with SHS independently of DAS28. (A) Schematic hypothesis of the association of HLA-DRB1*04:05 with SHS, independently of DAS28. (B) SHS is conditioned on time-averaged DAS28, disease duration, RF and cohort information and compared between subjects with HLA-DRB1*04:05 and without. The p value in linear regression analysis is indicated. RA, rheumatoid arthritis; SHS, Sharp/van der Heijde score.

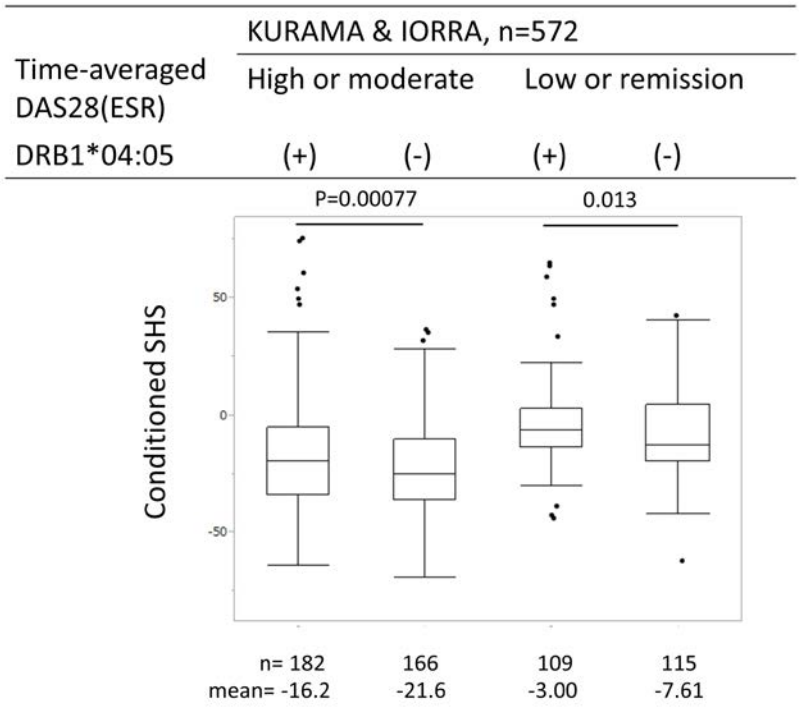

Figure 2 HLA-DRB1*04:05 carriers show higher Sharp/van der Heijde score (SHS) even in low disease activity or remission. Subjects with rheumatoid arthritis are classified into two groups based on their time-averaged DAS28 and the SHS conditioned on covariates are compared between the two groups. The p values in linear regression analyses are indicated.

time is approximately the same as their disease duration, we observed a similar association result with a comparable effect size (data not shown).

In conclusion, we showed that HLA-DRB1*04:05 has a joint-destructive association independently of CCP and DAS28. It would be interesting to identify unknown markers reflecting the remaining disease activity in subjects carrying HLA-DRB1*04:05, which cannot be fully evaluated by DAS28. It would be interesting to evaluate the association of HLA-DRB1*04:05 in large European RA cohorts.

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Prevalence and incidence of psoriasis and psoriatic arthritis

Psoriasis (Pso) and psoriatic arthritis (PsA) are inflammatory disorders which can severely impair health and quality of life. For both Pso and PsA, an increasing prevalence has been reported.[1 2] Comprehensive data on the prevalence and incidence of Pso and PsA are important in order to adequately allocate specialist care and financial resources. These data are incomplete for Pso and especially PsA. In particular, no population-based study has estimated their prevalence or incidence in Germany. We obtained the statutory health insurance data of approximately 65 million people from 2009 to 2012, covering 80% of the German population. Pso and PsA age-standardised prevalence based on the International Classification of Diseases (ICD) codes was obtained and age-standardised incidence rates calculated as described previously.[3] Briefly, cross-sectional prevalence data of consecutive years were used in conjunction with different assumed mortality rates of 1.1–1.5, with assumed reductions in annual mortality rates of 0%–5% in order to estimate incidence ranges.

Depending on the year, approximately 65 million individuals were assessed. There were 1.4–1.6 million cases of Pso and 127 000–156 000 cases of PsA identified. The age-standardised prevalence for Pso was 22.2–22.9 and 21.3–22.1 per 100 000 individuals in men and women, respectively (online supplementary figure 1). The prevalence for PsA was 1.8–2.1 and 2.1–2.5 per 100 000 individuals in men and women, respectively (online supplementary figure 2). A steady increase in prevalence was observed for both Pso and PsA. The incidence of Pso in 2009 ranged from 35.4 to 50.3 and from 46.3 to 58.2 in men and women, respectively, and declined thereafter. The incidence of PsA in 2009 ranged from 13.8 to 14.9 and from 18.1 to 19.1 in men and women, respectively, and declined thereafter. All data are detailed in table 1. Based on these data we used two different scenarios to estimate the number of patients living in Germany in 2018: (1) German age pyramid of 2018 applied to prevalence in 2012, or (2) projection of prevalence extrapolated by annual per cent change, then application of the 2018 age pyramid. Concerning Pso, 959 362–1 012 167 male and 956 822–1 030 847 female patients are expected to be living in Germany in 2018. Concerning PsA, 75 376–102 320 male and 90 473–127 349 female patients are expected to be living in Germany in 2018. This calculation may serve to project future mortality in other European countries.

Thus, we summarise that roughly 2 million patients with Pso and at least 200 000 patients with PsA are currently living in Germany. The age-standardised prevalence and incidence of PsA are in line with estimates from other European countries or the USA,[4] although higher incidences have been reported.[2] The ratio of PsA/Pso prevalence in the current study was approximately 10%, which is well within the range of previous reports. The ICD-based case definition is a limitation to the study as it may result in reduced precision as opposed to diagnostic criteria.[5] Most recent observational studies report an increase[6] or at least stable incidences[2] for Pso or PsA. In the current study, we calculated incidences based on cross-sectionally observed prevalence and different assumed mortality ratios in reference to the mathematical relation between incidence, prevalence and mortality.[3] These analyses consistently resulted in a decline in the incidence of both diseases over the observed study period. However, we suggest a careful interpretation of these incidences since changed awareness for the respective diagnoses or changed coding behaviour of ICD codes may account for the differences. Thus, the results should be interpreted as possible trends in incidences.

The epidemiological data reported herein cover a substantial portion of the German population and thereby improve our understanding of the prevalence and incidence of Pso and PsA in Europe.

Table 1 Prevalence of Pso and PsA from 2009 to 2012 in Germany

Year	2009	2010	2011	2012
Population (n)	64 637 752	63 962 071	64 988 016	65 792 296
Female (%)	53.5	53.4	53.3	53.2
Pso (n)	1 419 537	1 440 807	1 477 333	1 512 769
Pso prevalence (n/1000)				
Male	22.22	22.59	22.69	22.86
Female	21.27	21.76	21.93	22.12
Pso incidence (n/100 000)				
Male	35.38–50.27	26.44–39.36	17.32–29.31	17.14–26.31
Female	46.30–58.17	35.30–45.63	21.67–30.47	19.05–26.39
PsA (n)	127 334	137 763	146 463	156 182
PsA prevalence (n/1000)				
Male	1.81	1.96	2.03	2.13
Female	2.07	2.26	2.37	2.49
PsA incidence (n/100 000)				
Male	13.81–14.88	11.59–12.54	9.59–10.39	9.84–10.49
Female	18.12–19.14	15.23–16.14	12.03–12.80	11.76–12.38

Data from the German statutory health insurance system of approximately 64 million people (population) were employed to assess age-standardised prevalence of psoriasis (Pso) and psoriatic arthritis (PsA) for the male and female German population (mean values). Age-standardised incidence was calculated based on prevalence data and different assumed mortality scenarios resulting in the given ranges.

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Evaluation of retinal microvascular density in patients affected by systemic lupus erythematosus: an optical coherence tomography angiography study

Retinopathy in systemic lupus erythematosus (SLE) has an incidence of 7%–29% and is suggestive of high disease activity being a marker of poor visual outcome and prognosis for survival.[1] Recently, we demonstrated a subclinical retinal involvement in patients with SLE that seems to be related to kidney involvement where hydroxychloroquine had a protective role.[2] The pathogenesis of lupus retinopathy is attributed to a vasculopathy most commonly immune complex-mediated microangiopathy.[1] Optical coherence tomography angiography (OCTA) is a non-invasive technique for imaging the microvasculature of the retina and choroid that may quantify foveal avascular zone, non-perfused or low-perfused areas. Quantitative measurements based on OCTA may have value in managing retinopathy but also correlate with visual outcome and mirror vascular involvement in systemic diseases.[3] The aim of this study was to evaluate retinal microvasculature using OCTA in patients with SLE without signs of retinopathy according to standard lupus retinopathy

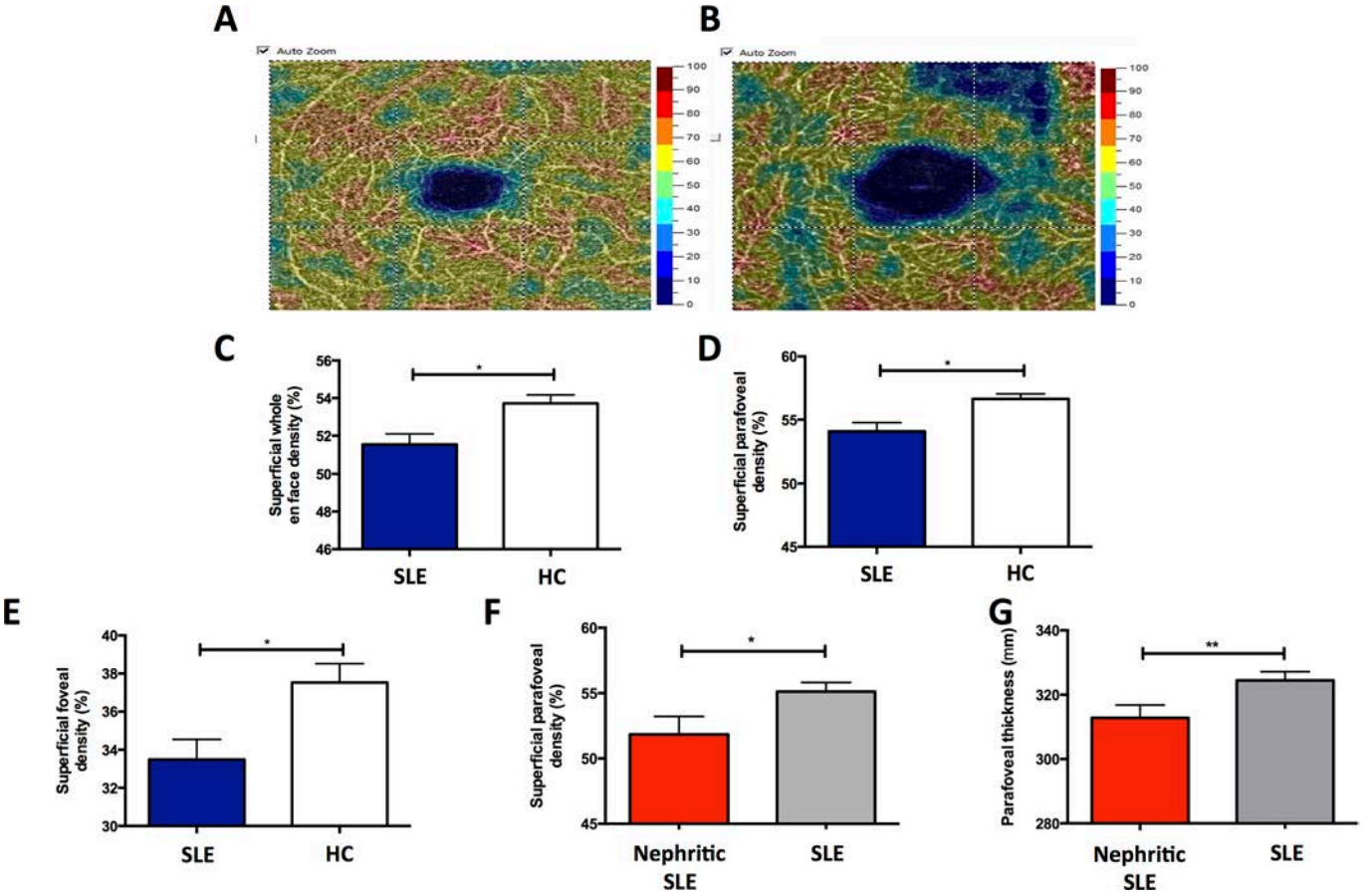

Figure 1 Superficial whole en face vessel density (%, colour-coded) maps in a (A) healthy control (HC) and a (B) patient with systemic lupus erythematosus (SLE). (B) Enlargement of the foveal avascular zone (central dark blue area) and parafoveal areas of reduced perfusion (light and dark blue areas). Patients with SLE displayed reduced superficial whole en face (C), parafoveal (D) and foveal (E) vessel density (%) compared with those in healthy eyes. Patients with SLE with lupus nephritis showed superficial parafoveal vessel density (%) compared with those in patients without nephritis (F). Parafoveal thickness in patients with SLE with nephritis was reduced than that in patients without kidney involvement (G). *P<0.05; **P<0.01.

classification[4] and correlate abnormal vascular density with disease activity, damage accrual, treatment and visual outcome. From 20 November 2015 to 31 December 2017, a total of 52 eyes of patients with SLE, diagnosed according to the American College of Rheumatology classification criteria,[5] and 40 eyes of healthy controls (HC) were examined by means of a 6 mm OCTA scan (Optovue XR Avanti, Fremont, CA). Split-spectrum amplitude-decorrelation angiography generated optical coherence tomography angiograms of both superficial and deep retinal capillaries referred to the whole en face, foveal and parafoveal zone from patients with SLE and HC (figure 1A,B). Capillary density values were compared with clinical data by Spearman's rank correlation coefficient, and groups were compared using analysis of variance and Kruskal-Wallis analyses. Values of $p<0.05$ were considered statistically significant. Demographic and clinical features of enrolled subjects are summarised in table 1. The eyes from patients with SLE had a lower mean superficial whole en face density, superficial parafoveal density and superficial foveal density ($p=0.02$ for all comparisons) compared with healthy eyes (figure 1C–E). Patients with SLE with nephritis displayed reduced parafoveal vessel density and parafoveal thickness compared with those of patients without nephritis ($p=0.02$ and $p=0.008$, figure 1F,G). A negative correlation was demonstrated in patients with SLE between age and superficial whole en face density ($p=0.0005$, $r=-0.5$), superficial foveal density ($p=0.006$, $r=-0.4$), superficial parafoveal density ($p=0.004$, $r=-0.4$), deep whole en face density ($p=0.003$, $r=-0.4$) and deep parafoveal density ($p=0.001$, $r=-0.4$). Systemic Lupus Erythematosus Disease Activity Index correlated inversely with superficial en face density ($p=0.002$, $r=-0.4$), superficial parafoveal density ($p=0.0003$, $r=-0.5$ and $p=0.002$), deep whole en face density ($p=0.01$, $r=-0.4$) and deep parafoveal density ($p=0.002$, $r=-0.4$). A negative correlation was also found between Systemic Lupus International Collaborating Clinics (SLICC) and superficial whole en face density ($p=0.0001$, $r=-0.5$), superficial parafoveal density ($p<0.0001$, $r=-0.6$), deep whole en face density ($p<0.0001$, $r=-0.6$) and deep parafoveal density ($p<0.0001$, $r=-0.7$). A positive correlation was found between hydroxychloroquine cumulative dose and both superficial and deep parafoveal density ($p=0.009$, $r=0.4$ and $p=0.04$, $r=0.3$). Best corrected visual acuity in SLE positively correlated with superficial whole en face density, superficial parafoveal density, deep whole en face density and deep parafoveal density ($p<0.0001$, $r=0.7$ for all correlations).

Patients with SLE displayed a reduced retinal microvascular density compared with normal subjects, in particular those with kidney involvement. Vessel density provides a quantitative metric of capillary network that correlated with age, best corrected visual acuity and clinical features as SLE disease activity and damage accrual. Hydroxychloroquine might have a protective role preserving the microvascular structures.

Table 1 Demographic and clinical characteristics of enrolled subjects

	HC (n=20)	SLE (n=26)
Age (years)	46±8.9	49.6±13.6
Female, n (%)	16 (80)	23 (88.5)
Disease duration (years)	NA	15.1±7.7
Anti-dsDNA positive Abs, n (%)	NA	13 (50)
aPL positive Abs, n (%)	NA	10 (40)
C3 (mg/L)	NA	98.9±21.7
C4 (mg/L)	NA	19.6±5.8
SLEDAI-2K	NA	4.3±4.4
SLICC	NA	1.9±1.5
HCQ, n (%) HCQ cumulative dose (g)	NA	16 (61.5) 738.8±486.8
BCVA (logMAR)	0.0±0.1	0.0±0.1
Kidney involvement*, n (%)	NA	10 (40)

*Kidney involvement was defined as the presence of biopsy-proven glomerulonephritis class III, IV or V according to the International Society of Nephrology/Renal Pathology Society glomerulonephritis classification criteria.[6]
Continuous variables were shown using mean and SD.
aPL, antiphospholipid; BCVA, best corrected visual acuity; HC, healthy controls; HCQ, hydroxychloroquine; NA, not applicable; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; SLICC, SLICC/ACR damage index score.

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Kawasaki disease in infants 3 months of age and younger: a multicentre Spanish study

Kawasaki disease (KD) is a multisystem vasculitis of small and medium vessels typical of childhood. Timely treatment with intravenous immunoglobulin (IVIG) has reduced the incidence of coronary artery abnormalities (CAAs) from 25% to approximately 4%.[1] Asian studies have focused on infants under 3 months of age, but there are no published data about these patients from Western countries.[2 3]

We reviewed 621 patients under 16 years old with a diagnosis of KD between 2011 and 2016 from a multicentre study in Spain (KAWA-RACE study); 84 hospitals participated throughout the country.

We found seven children under 90 days (1.13%), with a male predominance (6 of 7). Five presented irritability, but only two fulfilled the criteria for complete KD (table 1).[1] The following were the main laboratory findings (median, IQR): highest C reactive protein (CRP) 24 mg/L (8.48–31.4), highest erythrocyte sedimentation rate 79 mm (70–105.5), maximum and minimum platelet count 900 ×10^9/L (682–1 117) and 506 ×10^9/L (449–612), minimum haemoglobin 10 g/dL (9–10.8), maximum leucocytes 21 ×10^9/L (16.45–23.37), minimum sodium 135.5 mEq/L (133–137.5), and minimum albumin 2.9 mg/dL (2.6–3.4).

In three cases, a viral infection was diagnosed and four patients presented with CAA, but no other echocardiographic findings were detected (table 1).

The median time interval since fever onset to IVIG administration was 8 days. All patients responded well to the first dose of IVIG, and only one received concomitant intravenous steroids because he was considered to be at high risk for IVIG resistance. All CAAs were transient and resolved during follow-up (table 1).

Epidemiology is different in Western countries when compared with Asian countries, where the incidence can reach up to 264.8 cases/100 000 children <5 years of age, as in Japan 2012. In USA there is also a relatively high incidence of around 25/100 000 when compared with European countries.[1] Incidence in Spain is only known in the Catalonia region and was estimated to be 8/100 000 <5 years old (2004–2014).[4]

KD in younger children is more difficult to diagnose as it presents more frequently as incomplete KD. A study from Korea with 24 patients younger than 3 months of age describes an 87.5% of incomplete KD forms, and a mean number of major diagnostic criteria of 2.8±1.4: rash was the most common (50%) and conjunctival injection was the least common (12.5%).[3] In our population non-complete KD cases represented 71.4% of the total, rash was present in 85.7%, but cervical lymphadenopathy was the least common finding (14.3%).

When we looked at laboratory tests, our case series showed less CRP increment when compared with Asian studies, 24 mg/L (median), vs 79±52 or 78.4±69 (mean), respectively, but no other relevant differences were found.[3 5] Infections were not documented in any children from the studies of Lee *et al*, Bae *et al* or Satoh *et al*.[2 3 5] In our population, 42.8% of patients presented with a confirmed infection, but were treated for KD regardless as the role of these pathogens is unclear and the consequences of not treating KD in time could be devastating.

The incidence of CAA in our series is considerably higher when compared with others, and may be due to late diagnosis: three had aneurysms (42.8% of patients), and one had dilation, according to McCrindle Z-score classification[1] (57.14% of the total had an abnormal echo). A large Korean study with 609 patients <3 months old showed an incidence of CAA of 19.9% (116 of 583), 18% dilation and 3.4% aneurysms.[2] Echocardiographic abnormalities were detected in 25% of the Bae *et al*[3] population (three cases of valve dysfunctioning without coronary involvement), and only 12.5% were CAA. All our cases with CAA recovered completely compared with the Japanese series from Satoh *et al*,[5] where 7 of 24 patients presented CAA, but in only 2 cases these alterations persisted for 1 year (8.3%).

This multicentre study let us study an uncommon condition from a large series. Despite the small number of patients, we have observed more frequent CAA, but good response to IVIG and no long-term sequelae.

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Table 1 Diagnosis, symptoms, cardiological and microbiological findings

Age (months)	Complete Kawasaki disease	Irritability	Extremity changes	Rash	Conjunctivitis	Oral changes	Cervical lymph nodes	Microbiological findings	Cardiological findings	Z-score* (SD)	Vessels (n)	Time to resolution (weeks)
3	No	No	Yes	Yes	Yes	No	No	*Enterovirus* (CSF)	CA	2.9	1	18
1.6	No	Yes	No	Yes	No	Yes	No	No	Unremarkable	–	–	–
2.6	Yes	Yes	Yes	Yes	Yes	No	Yes	No	CA	NA	1	5
2	No	No	No	Yes	Yes	Yes	No	No	Unremarkable	–	–	–
2.2	No	Yes	No	Yes	No	No	No	No	CA	3.2	2	13
2.9	Yes	Yes	Yes	Yes	Yes	Yes	No	Adenovirus (PS)	Unremarkable	–	–	–
2.3	No	Yes	No	No	Yes	Yes	No	Coryzal symptoms	CD	2–2.5	1	–

In three cases, a viral infection was diagnosed and four patients presented CAA, but no other echocardiographic findings were detected (table 1).
*Maximum Z-score[1 6] all measured at acute phase.
CA, coronary aneurysm; CD, coronary dilation; CSF, cerebrospinal fluid; NA, not available; PS, pharyngeal swab.

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Elephant in the room

We read with interest the article by Aggarwal *et al* on repository corticotropin injection (RCI) in the treatment of refractory polymyositis and dermatomyositis (PM and DM) published in the *Annals of the Rheumatic Diseases*.[1]

The authors, who are well-respected researchers in the field of myositis, have done a good job in conducting a small open-label trial of RCI in the treatment of PM/DM using validated disease activity measures and outcome measures. Whether these patients were truly 'resistant' is debatable, since only 3 of the 11 patients were treated with intravenous immunoglobulin (IVIG) before entering the study. Also, three serious adverse events related to the study drug among 10 patients in a 6-month period (all requiring hospitalisation, one being disseminated zoster with pneumonitis and not counting patients with incident hypertension and hyperglycaemia) should be a major cause of concern and would invalidate their assertive statement 'RCI was generally well tolerated with a reasonable safety profile'. They have mostly toned down the efficacy conclusions drawn from their case series, although 'promotional' language creeps in at some places, such as '….support the concept of RCI as a novel immunomodulatory therapy for myositis beyond the steroidogenesis effect.'

It is telling that we are still publishing and reading 'case reports' on the only Food and Drug Administration (FDA)-approved therapy for the treatment of PM and DM. It is also a sad reflection on an 'approved drug' that was never held to the same standards of showing efficacy and safety in prospective, double-blind, placebo-controlled trials that other drugs have to go through. The authors call it a 'proof-of-concept' study, which is an odd statement given RCI is FDA approved and marketed to treat PM/DM and then state that this is 'the first clinical trial of RCI in adult DM & PM using rigorous methodology', which nullifies previously published case studies, which form the so-called 'evidence' for the use of RCI for myositis.

Unfortunately, the authors do not mention the 'elephant in the room', which is the outrageous price of this treatment. At its current wholesale acquisition cost of $36 382 per 400 unit 5 mL vial, a 24-week course of RCI therapy at 80 units twice a week as given in their study would require 10 vials and cost $363 820. Although the authors of this manuscript have nothing to do with the pricing decisions related to RCI, and they do suggest a cost–benefit analysis, we recommend readers to take into account the excessive costs of RCI before considering this treatment for their patients with inflammatory myositis.

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Response to: 'Elephant in the room' by Hartung *et al*

We read the letter to the editor 'Elephant in the room' by Hartung *et al* in response to our open-label pilot clinical trial report on the use of repository corticotropin injection (RCI) in refractory polymyositis (PM) and dermatomyositis (DM).[1 2]

First, the authors believe that the population studied may not be truly resistant given that only 3 of the 10 patients were treated with intravenous immunoglobulin (IVIg) before entering the trial. There are strong data to support that patients with myositis who have failed 40-60mg slow prednisolne taper and an average of 2.6 immunosuppressive drugs in addition to nearly 20 mg of prednisone at study entry are considered. In the largest international multicentre randomised clinical trial done on PM and DM—the Rituximab in Myositis (RIM) trial, which included many international myositis experts, subjects failed an average of 3.1 immunosuppressive drugs in addition to at least 20 mg of prednisone.[3] Our pilot cohort is clearly consistent with RIM trial entry criteria in terms of subject refractoriness. Further, there is no clear consensus definition of 'refractory myositis' and, in general, patients failing high dose glucocorticoids plus one additional immunosuppressive agent in adequate doses for a reasonable period of time can be considered 'refractory'. Regarding IVIg, although we agree that IVIg is considered a reasonable immunomodulatory agent in patients with refractory DM (and perhaps PM), it is not a Food and Drug Administration (FDA)-approved therapy. Further, there are significant barriers for patients to receive IVIg including insurance approval, the significant expense of the drug and the onerous nature of a 2 to 5-day long-duration intravenous infusion which is not practical for many working patients.

Second, the authors are questioning the safety and tolerability profile of the RCI in the study. We would like to point out that out of three serious adverse events (SAE), two were in the same patient (disseminated herpes zoster and avascular necrosis), and that patient was on mycophenolate, azathioprine and prednisone concomitantly at the time of the SAEs. We believe it was the combination immunosuppressive therapy that led to these unfortunate SAEs. Certainly, this is a lesson learnt for future clinical trials. Despite the SAEs and the patient being afforded the option of discontinuing study drug, this patient elected to continue the RCI (after temporary discontinuation) due to significant efficacy that she was experiencing for her severe refractory DM. Moreover, the frequency and severity of SAEs and adverse events (AE) in this open-label trial were consistent with other clinical trials in myositis where subjects received multiple concomitant immunosuppressive medications. The RIM trial had 67 SAEs and 308 AEs as well as infusion reactions (15%) on 200 patients with myositis, and yet was considered safe and tolerable therapy for refractory disease.

Third, the authors comment about 'promotional' language as absolutely not justified. Our goal in performing this trial was to provide data from a prospective open-label study that went beyond the case reports that were previously published. We specifically mentioned that despite FDA approval, there are limited data on its clinical utility in myositis, and categorically stated that ours was a 'proof of concept' study and further studies are required to prove the merit of the drug. We provided detailed mechanistic plausibility and preliminary data for proposed steroid-dependent and independent hypotheses of RCI.[4] Further, the blood sample repository that we concomitantly collected in the trial was done to allow for detailed mechanistic studies that would provide valuable data as to whether there was justification for a steroid-independent immunosuppressive effect for RCI. Moreover, we stated that despite the apparent favourable results of this pilot trial, the efficacy and safety of RCI need to be proven by a larger randomised clinical trial in myositis.

Fourth, we concur with the authors that RCI needs to be held by the same standards of efficacy and safety in prospective, double-blind, placebo controlled trials. However, our pilot clinical trial provided a much higher level of evidence than earlier 'case reports', given that it was prospective with predetermined inclusion and exclusion criteria as well as the incorporation of validated outcome measures. Further, no change in concomitant therapies including physical therapy was allowed throughout the trial period. AEs were monitored and reported in a standardised fashion throughout the study using the NIH's Common Terminology Criteria for Adverse Events v4.0. The 'proof of concept' terminology refers to the scientific methodology for the trial with no relationship to earlier FDA 'approval' of RCI in the 1950s.

Fifth, there are various levels of evidence-based support for any therapeutic agent for any disease from case reports or retrospective studies, to prospective open-label trial, and finally to a randomised controlled trial.[5] Each study collectively provides evidence in support or against a scientific hypothesis. Therefore, we fail to understand why the authors believe that a higher level of evidence in a clinical trial using rigorous methodology nullifies earlier evidence of efficacy in published case studies when the findings point towards the same direction.[6–8]

Finally, we strongly disagree that we failed to mention the 'elephant in the room', that is, cost of the drug. We, in fact, categorically stated that RCI is unlikely to be first-line therapy given the high cost of the drug. Moreover, we recommended that a cost benefit analysis should be done in the future to determine the role of RCI in the treatment algorithm of myositis. In a retrospective study, patients with DM/PM receiving RCI had a lower mean hospitalisation rate, emergency room visits or outpatient visits, as compared with propensity-matched patients with DM/PM receiving IVIg, rituximab or a combination of the latter agents.[9] Unfortunately, these investigators failed to compare the medication cost or differences in efficacy, which is necessary to do in such a comparison.

We agree with the authors' final conclusion that a cost benefit analysis of RCI should be considered before recommending this drug, which is generally true of any of the newer expensive therapies including biological therapies for various rheumatic diseases.

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Association between bisphosphonate use and risk of undergoing knee replacement in patients with osteoarthritis

Osteoarthritis (OA) is the most common joint disorder and the major cause of chronic musculoskeletal pain and mobility disability in elderly populations worldwide.[1] Currently there is no effective pharmacological treatment for OA, necessitating joint replacement to reduce joint pain and improve physical functions at advanced stages of the disease.[2] It has been reported that abnormal subchondral bone resorption and bone loss play an important role in both OA initiation and progression.[3–5] Therefore, antiresorptive drugs are suggested to be potential OA therapies.[6] We read with deep interest a recent article published in this journal by Neogi *et al*, who found that in elderly women with newly diagnosed knee OA, those who use bisphosphonates had lower risk of knee replacement than non-users, and suggested that treatment with bisphosphonates has a potential beneficial effect on knee OA.[7] We really appreciate the great work performed by the authors; nevertheless, some worthwhile issues need to be further explored.

First, the definition of knee OA at baseline is not clearly described in the study. Nowadays there is no consensus on the classification criteria of knee OA despite extensive epidemiological and clinical studies. The two criteria most frequently used are the American College of Rheumatology (ACR) classification criteria[8] and the Kellgren and Lawrence (K-L) system.[9] The ACR classification criteria depend on clinical (such as pain, aching or stiffness in joint), radiographic and laboratory aspects of OA. On the other hand, the K-L system identifies and grades OA based on radiographs. With this system, most subchondral bone changes in OA, such as osteophyte, bone sclerosis, bone cyst and joint space narrowing, can be observed on radiographs.[10] Furthermore, due to the heterogeneity of OA, there are subgroups of patients who have only radiographic but not symptomatic OA and vice versa.[11] For example, it was reported that the prevalence of radiographic knee OA was 35.3% in women and 31.2% in men, while self-reported knee pain was found in 62% of women and 35% of men in a sample of 170 men and 488 women.[12] It is likely that the effects of bisphosphonates on radiographic OA are different from that on symptomatic OA. Thus, differences in knee OA definition at baseline may lead to increased heterogeneity of the severity of the disease and result in bias of the results. It would be better to clarify the definition of knee OA in the study.

Second, the only outcome of this study is the incidence of knee replacement. The purpose of the study was to explore the potential beneficial effect of bisphosphonates on knee OA process.[7] To achieve this, the authors evaluated 'the relation of bisphosphonate use to knee replacement surgery'. We agree with the authors that knee replacement can serve as an indication for knee OA severity. But more precisely, utility of knee replacement does not indicate the 'end-stage' of OA. On the one hand, as knee replacement surgery develops and more and more patients demand for higher quality of life, the number of knee replacement has increased greatly.[13] For example, it was reported that low-grade OA (K-L grade <3) comprised 12% of the total sample of 176 patients with knee OA who underwent total knee arthroplasty in Denmark.[13] This condition may increase the heterogeneity of knee OA severity at baseline. On the other hand, studies have demonstrated that in K-L grade 4 OA knees, MRI-detected cartilage loss and fluctuation of bone marrow lesions, effusion and synovitis occurred frequently over a 30-month period,[14] suggesting that even K-L grade 4 knee OA does not represent the true 'end-stage' of the disease. Thus we have no idea if the use of knee replacement as the only outcome is enough. Furthermore, the information on the important characteristics of knee OA and direct indications for knee replacement, the level of knee pain (eg, Western Ontario McMasters Osteoarthritis Index pain score) and dysfunction (eg, knee society score)[1] were not demonstrated in the paper. If use of bisphosphonates did have beneficial effects on subchondral bone structure in OA, there should be significant relationships between bisphosphonate use and knee pain relief and improvement in function. Thus, knee pain and knee function as outcomes are worthy of expectation.

Third, the criteria for patient selection should be described with more details. Studies have shown that previous knee injuries such as fracture, anterior cruciate ligament injuries, meniscal tear and/or knee operation appeared to be important risk factors for the development of knee OA.[15] Hence, it is interesting to know whether patients with previous knee injuries or knee operation had been excluded. Additionally, some other confounders needed to be addressed, such as physical activity level, occupation, races and so on. Is it possible that non-users of bisphosphonates had lower social status and consequently higher physical work load and higher severity of OA than the users? It would be interesting to know more details of these confounders, which may influence the results.

Last but not the least, the information regarding the treatment of knee pain of these patients was not shown in detail in the paper. These treatments, especially the use of pain medication, such as non-steroidal anti-inflammatory drugs and glucosamine sulfate, may have affected the knee pain and knee function, and in turn the need for knee replacement. Furthermore, it has been reported that bisphosphonate users had higher rates of comedications compared with non-users.[16] It is likely that users of bisphosphonates in this study took more pain medication, got more pain relief, and thus had lower rate of knee replacement. The significant associations of bisphosphonate use and knee replacement, as shown in the paper, may probably be no longer significant after the adjustment by use of pain medication. In addition, it was reported that high adherence to bisphosphonate treatment during 24 months of follow-up was associated with a significantly decreased risk of knee replacement (propensity score-adjusted HR, 0.66 (95% CI 0.43 to 0.99); P=0.048).[16] As there was only one follow-up period (ie, '3.13 years' for bisphosphonate users and '2.91 years' for non-users) in the study, it is very important to analyse the bisphosphonate treatment adherence of the patients during this long period. And we are confused about the results of the mean follow-up time of the study, which has no SD or 95% CI. This needs to be clarified.

We respect the great contributions of the authors and we would also be very interested in the authors' response regarding the above issues.

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Response to: 'Association between bisphosphonate use and risk of undergoing knee replacement in osteoarthritis patients' by Chen *et al*

We thank Dr Chen and colleagues for their interest in our paper.[1] As outlined in our paper,[2] the definition of knee osteoarthritis (OA) was based on diagnosis by the patient's general practitioner (GP), which is recorded as a read code in The Health Improvement Network (THIN). Because these are patients who are being seen by their GP, the diagnosis of knee OA is typically for symptomatic knee OA. While it is true that knee replacement surgery is not the only relevant longer term knee OA outcome, we were unable to assess other facets of knee OA outcomes due to the nature of the database used; for example, The Western Ontario and McMaster Universitities Osteoarthritis Index (WOMAC) scores are not available in this GP database. Nonetheless, knee replacement surgery is considered an important symptomatic endpoint for knee OA. We excluded individuals who had prior knee replacement surgery but not prior knee injuries or other surgeries that were unlikely to be confounders. We were able to adjust for socioeconomic status (Townsend deprivation index). The SD for the mean follow-up time were 2.43 and 2.36 years for the bisphosphonate initiators and the comparator group, respectively. Any medications that were prescribed after the initiation of bisphosphonates and after these subjects' newly diagnosed knee OA would be considered intermediates along the causal pathway in the scenario proposed by Dr Chen and colleagues; adjustment for those types of medication use would induce bias. The potential mechanisms by which bisphosphonates may confer the noted effects was beyond the scope of this paper.

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Bisphosphonates reduce the risk of knee replacement: we need more analyses!

With great interest, we read the recent article by Neogi *et al* entitled, 'Effect of bisphosphonates on knee replacement (KR) surgery'.[1] In the study, the authors concluded that in this population-based cohort of older women with incident knee osteoarthritis (OA), those with incident bisphosphonate users had lower risk of KR than non-users of bisphosphonates, which was further supported by another large cohort study.[2] The strengths of this study include a propensity score-matched cohort design, Cox proportional hazards regression to control for potential confounders and sensitivity analyses focused on residual confounding. Meanwhile, the authors acknowledged the limitations of their work. We applaud and congratulate their important work for clinical practice. However, several important points should be further discussed.

Generally, determinants of patient preferences for KR are OA severity, the level of knee pain, disability, and the weakness of quality of life, and more importantly, their willingness and access to KR. In the current study, authors tended to agree that reduced risk of KR has resulted from weakened OA progression after bisphosphonates use. However, recent high-level evidence indicated that bisphosphonates neither provide pain relief and function improvement nor radiographic progression in knee OA.[3 4] Therefore, we wonder that bisphosphonates may affect the willingness and access to KR of patients with OA, thus reducing the risk of KR. Actually, many clinical and sociocultural factors, such as racial differences (African Americans and whites), social support and an educational intervention included a decision aid, have vital important influence on patient preferences for KR.[5–7] More importantly, a recent study revealed that patient preferences for KR were strongly associated with knee pain severity in patients with OA with health insurance, but their inverse relationship disappeared in patients without health insurance.[8] Also, another study suggested that despite worse baseline knee pain and function, black participants had much lower adjusted risk of having total KR (TKR) than white participants.[9] Thus, these indicated that sociocultural factors, but not OA severity, may have more significant influence on the willingness and access to KR. However, Neogi and colleagues seemed to ignore these important sociocultural factors in Cox proportional hazards regression, propensity score model and sensitivity analyses. We strongly recommend additional analyses based on aforementioned risk factors, which may further increase the robustness and credibility of the current study.

Additionally, the authors assessed the influence of bisphosphonates use on the risk of KR in patients with OA. However, there were no any data involving the baseline characteristics of OA severity, the level of knee pain, disability and the weakness of quality of life between both cohorts. Moreover, the influence of the dose, route, time point or measuring instrument of bisphosphonates administration on the risk of KR was unclear, so further subgroups should be warranted. Meanwhile, the primary outcome (the risk of KR) may be involved in many similar terms, such as TKR, revision KR, hemi-KR or KR in both knees. As with us, some readers may be confused about the notion. Therefore, the primary outcome should be clearly defined.

To sum up, we respect the great work done by the authors, but the study should be interpreted with the aforementioned limitations and further analyses should be performed.

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Response to: 'Bisphosphonates reduce the risk of knee replacement: we need more analyses!' by Li *et al*

We thank Dr Li and colleagues[1] for their interest in our paper. As outlined in our paper,[2] we used data from a large general practitioner electronic health records database for our study. These data are collected and recorded as part of routine clinical care with diagnostic codes. As such, factors such as willingness to undergo and access to knee replacement (KR), knee pain severity, disability, quality of life and others are not available in this database, and we were thus unable to examine these as potential confounders. We were, however, able to account for socioeconomic status (Townsend Deprivation Index), and performed sensitivity analyses demonstrating that substantial residual confounding is unlikely. The assessment of bisphosphonate exposure was based on prescription data, and alendronate was the most common bisphosphonate prescribed (84%), as described in the paper.[2] The potential mechanisms by which bisphosphonates may confer the noted effects were beyond the scope of this paper. Nonetheless, it is not clear that bisphosphonate initiation would alter one's willingness to undergo KR or access to KR such that a protective effect would be noted in our study. We included both total and unicompartmental primary knee arthroplasty since either surgery would indicate substantially symptomatic knee osteoarthritis, but did not include revision surgery since the reasons for that procedure are not for severe/end-stage osteoarthritis; this is the same approach used by the other studies we had cited on this matter. As per standard time-to-event analyses, subjects were censored at the first KR, among the other censoring factors outlined in the paper.

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Can we prescribe TMP/SMX prophylaxis without any concerns equally for all patients with rheumatic disease?

We read with great interest the recent article by Park *et al*[1] and appreciate the authors' efforts to assess the benefit and safety of trimethoprim-sulfamethoxazole (TMP/SMX) as primary prophylaxis for pneumocystis pneumonia in patients with rheumatic diseases, exposed to prolonged high-dose glucocorticoids.

However, we would like to point out one concern in the incidence of adverse drug reactions related to TMP/SMX prophylaxis. Despite its efficacy, TMP/SMX could induce adverse events that could cause some patients to discontinue prophylaxis and increase the risk for *Pneumocystis jirovecii* pneumonia. There could be a disease gap for the development of adverse events, and higher risk was indicated in patients with systemic lupus erythematosus (SLE) compared with other rheumatic diseases. In patients with SLE, the reaction rate was estimated to be up to 27.3%–53%[2–5] and anti-Ro/SS-A antibody was especially warned to be a prognostic factor.[2] In addition, a prophylactic regimen is also important to assess safety. This is because adverse events requiring discontinuation of TMP/SMX prophylaxis were higher in patients with usual prophylaxis of a single-strength TMP/SMX tablet daily compared with graded administration.[2 6] For these reasons, detailed description revealing safety profiles based on individual diseases and prophylactic regimens would be required.

In conclusion, we acknowledge the interesting results provided by the authors, confirming the safety and efficacy of TMP/SMX prophylaxis. However, we believe that evaluating safety in patients with SLE would guide the readers in having a better understanding regarding the TMP/SMX prophylaxis in patients with rheumatic diseases.

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Response to: 'Can we prescribe TMP/SMX prophylaxis without any concerns equally for all patients with rheumatic disease?' by Suyama and Okada

We deeply appreciate the comments by Suyama and Okada on our recent report regarding efficacy and safety of primary prophylaxis for pneumocystis pneumonia (PCP) using trimethoprim-sulfamethoxazole (TMP-SMX) in patients with rheumatic disease receiving prolonged, high-dose glucocorticoid treatment.[1 2] They pointed out the possibility that patients with systemic lupus erythematosus (SLE) could have higher risk for adverse events related to TMP-SMX. They also indicated that discontinuation due to adverse events can be lowered by a graded administration strategy. In our cohort, the incidence rate of overall adverse drug reactions (ADR) was numerically higher in patients with SLE as compared with those with other rheumatic diseases, which is in line with the comment by Suyama and Okada (27.8 vs 16.6 per 100 person-years; incidence rate ratio 1.63, 95% CI 0.84 to 3.14). However, all ADRs in our SLE subgroup were mild to moderate in severity, and did not require urgent intervention or immediate discontinuation of TMP-SMX prophylaxis. Various clinical factors such as patient's ethnicity, concomitant medications or underlying rheumatic diseases can affect the frequency and seriousness of adverse events. However, we would like to remind that the previous studies reporting high adverse event rate of sulfa-antibiotics, which Suyama and Okada cited, were case–control studies and that most of the information was obtained by survey.[3–5] In addition, there were no data on the severity of the adverse events. Considering high mortality and morbidity of PCP in rheumatic diseases, the risk benefit of TMP-SMX prophylaxis should be estimated by the incidence of adverse events and by their severity.

There remain many issues that need to be addressed before making a universal recommendation for primary PCP prophylaxis in patients with rheumatic diseases receiving high-dose glucocorticoids. An evidence-based, protocolised approach may be the first step. Establishment of the risk-benefit ratio of PCP prophylaxis for specific rheumatic diseases could then be a logical next step, as Suyama and Okada suggested, and we thank them for their important comment.

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