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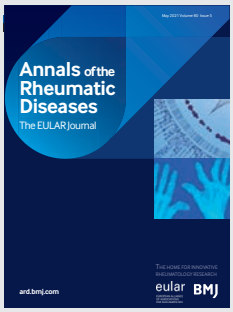

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Vitamin K and osteoarthritis: is there a link?

Richard F Loeser,[1] Francis Berenbaum ,[2] Margreet Kloppenburg[3]

Vitamin K is best known for its role in blood coagulation. The reduced form of vitamin K is a necessary cofactor for the γ-carboxylase enzyme that converts specific glutamic acid residues to γ-carboxyglutamic acid (Gla) in the coagulation factors II, XII, IX, X and protein C and protein S.[1] Proteins containing one or more Gla residues are often referred to as vitamin K-dependent proteins. The presence of calcium-binding Gla residues is critical to the structure and function of the vitamin K-dependent coagulation proteins. The anticoagulant drugs warfarin and acenocoumarol interfere with the reduction of vitamin K to its active form by inhibiting vitamin K epoxide reductase complex 1 (VKORC1) and so are known as vitamin K antagonists (VKAs).[1] These drugs are widely used clinically to treat patients with blood clots or at high risk of blood clots, although newer anticoagulants are available that do not act by inhibiting vitamin K function. These are often referred to as direct oral anticoagulants (DOACs).

The vitamin K-dependent coagulation proteins are produced primarily in the liver and function within the systemic circulation and vasculature. However, there are a number of vitamin K-dependent proteins not involved in blood coagulation, including several found in joint tissues.[2–5] The latter include matrix Gla protein (MGP), Gla-rich protein (GRP) and growth arrest specific gene 6 (Gas6) found in cartilage, the bone proteins bone Gla protein (osteocalcin) and transforming growth factor β (TGFβ)-induced protein, and periostin found in cartilage and the periosteum.[5–10] Periostin and TGFβ-induced protein may not be γ-carboxylated in all tissues,[11] and their γ-carboxylation status has not been determined in the joint. Although the precise function of the vitamin K-dependent proteins in the joint is not known, MGP and GRP have been shown to inhibit mineralisation, Gas6 promotes chondrocyte survival, and osteocalcin regulates bone turnover. Genetic studies have shown that MGP variants that result in reduced MGP expression are associated with hand and knee osteoarthritis (OA).[12–14] MGP knockdown in chondrocytes increased expression of genes related to chondrocyte hypertrophy (type X collagen) and cartilage degradation (MMP-13, ADAMTS4).[15] Importantly, when compared with healthy articular cartilage, uncarboxylated MGP and GRP are more abundant in human osteoarthritic articular cartilage suggesting a deficiency in γ-carboxylase activity or decreased availability of reduced vitamin K in OA cartilage.[7 8] Mice with the γ-glutamyl carboxylase enzyme deleted in osteoblasts have thicker cortical bone width and increased bone formation,[16] a characteristic of the OA joint. In addition, mice aged on a low vitamin K diet were noted to have greater articular cartilage proteoglycan loss than mice aged on a control diet.[17] Together, these studies suggest that vitamin K-dependent proteins have a role in maintaining healthy joints (figure 1).

Additional evidence that vitamin K is important for joint health comes from observational studies examining vitamin K intake and vitamin K blood levels as well as the level of uncarboxylated MGP as a functional measure of vitamin K status. Higher vitamin K intake and/or vitamin K status have been associated with a lower prevalence,[18 19] incidence[20] and progression of OA.[21] Furthermore, low vitamin K status is associated with worse physical performance and more functional decline.[22–24] In the Health, Ageing and Body Composition (Health ABC) knee OA study, those with low plasma vitamin K had slower gait speed and worse physical performance battery scores over 4–5 years of follow-up.[22] Health ABC participants with low plasma vitamin K also had 1.7- and 2.6- fold higher odds of worsening articular cartilage damage and meniscus damage over 3 years.[21] In the Multicenter Osteoarthritis Study (MOST), participants with low plasma vitamin K were 56% more likely to develop radiographic knee OA and had a greater than twofold higher risk of developing MRI-based cartilage lesions over 30 months.[20]

In *Annals of he Rheumatic Diseases*, two new studies provide additional compelling evidence for a role of vitamin K-dependent proteins in joint health. Both studies used observational databases to examine the relationship between VKA use and OA and both found a positive association, one by examining acenocoumarol use and risk of incident and progressive knee and hip OA[25] and the other examining warfarin use and risk of knee or hip replacement.[26] Both studies controlled for age, sex and body mass index, as well as cardiovascular and metabolic factors which might present confounding by indication bias for use of anticoagulants. In addition, the Ballal[26] study examined indication bias by including only patients prescribed anticoagulants for atrial fibrillation and by comparing those prescribed warfarin with patients matched for age and sex prescribed a DOAC. The study by Boer *et al*[25] examined over 4000 participants in the Rotterdam Study and noted that acenocoumarol users had a combined risk of radiographic knee and hip OA incidence and progression of 2.5 (95% CI 1.94 to 3.20) compared with controls not using anticoagulants, while Ballal *et al* used a UK general practitioner database and noted that compared with treatment with DOACs, individuals with atrial fibrillation prescribed warfarin had a 1.59 times higher risk (95% CI 1.31 to 1.92) of knee or hip replacement.

The study by Boer *et al*[25] also examined the potential additive effects of MGP and VKORC1 gene variants that would affect the levels of MGP expression, and the dose of acenocoumarol needed for adequate anticoagulation, respectively, and found over a four times higher risk of knee and hip OA incidence and progression in acenocoumarol users who carried both gene variants. It is noteworthy that neither study examined OA pain as an outcome but only radiographic progression or total joint replacement. Although there is no evidence at this time that vitamin K-dependent proteins play a role in regulating pain pathways, at least one of them, osteocalcin, is expressed in sensory neurons.[27] So the relationship between VKA use and pain remains an unanswered question.

Although both studies are observational, limiting causal inferences, the results presented in the studies by Boer *et al*[25]

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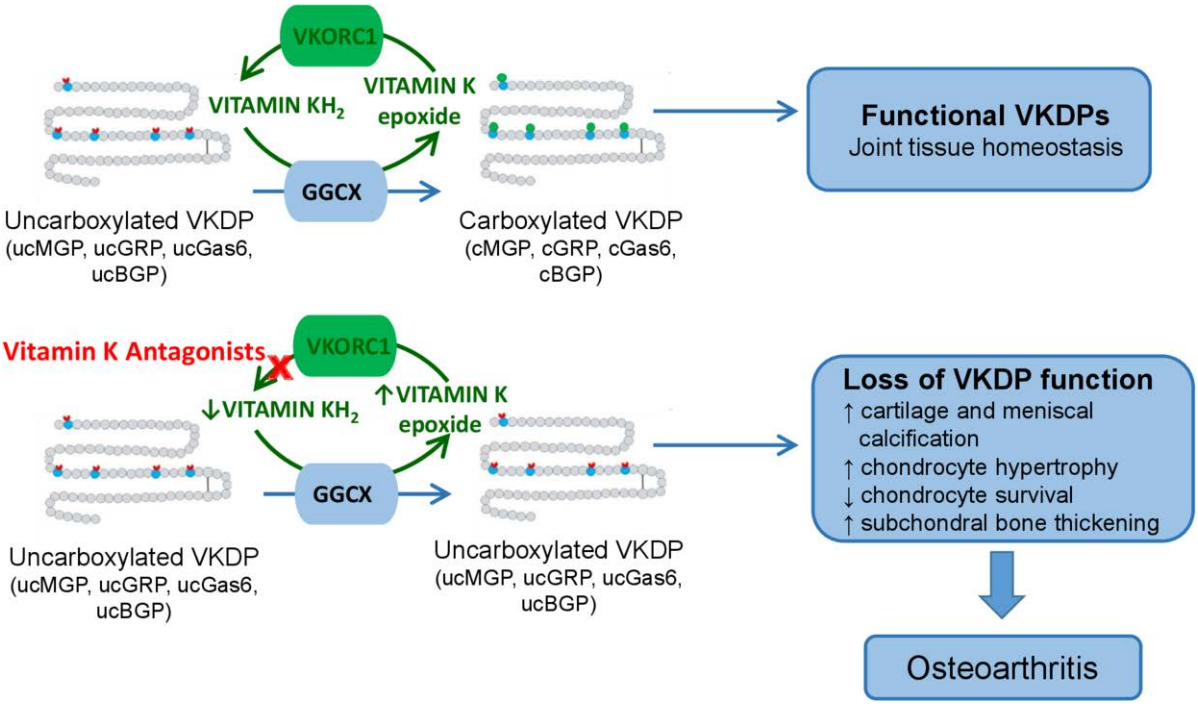

Figure 1 Synthesis and function of vitamin K-dependent proteins (VKDPs) in the joint. The reduced form of vitamin K (KH2) serves as a cofactor for the gamma carboxylase enzyme (GGCX) that post-translationally converts uncarboxylated (uc) glutamic acid residues in VKDPs to form functional γ-carboxylated (c) glutamic acid (Gla) residues. During this process, vitamin KH2 is oxidised to vitamin K epoxide which is reduced by the vitamin K epoxide reductase complex 1 (VKORC1) back to the active vitamin KH2. Vitamin K antagonists function by inhibiting VKORC1 and thus decrease the amount of reduced vitamin K and, in turn, the production of functional Gla-containing VKDPs. These include specific coagulation proteins but also proteins necessary to maintain joint tissue homeostasis including matrix Gla protein (MGP), Gla-rich protein (GRP), growth arrest specific gene 6 (Gas6) and bone Gla protein (BGP, also called osteocalcin).

and Ballal *et al*[26] demonstrating increased OA risk with VKA use, taken together with the prior epidemiologic and biologic studies on vitamin K intake and vitamin K functional status relevant to OA noted above, suggest two important conclusions. First, when prescribing anticoagulant drugs, healthcare providers need to weigh the risk of the potentially harmful effects of the VKA class of anticoagulants on joint tissues that may worsen OA in those already with OA and potentially those at higher risk of OA and decide if, for approved indications, other classes of anticoagulants such as the DOACs would be more appropriate. Second, a randomised clinical trial is needed to determine if individuals with vitamin K insufficiency and OA would benefit from vitamin K supplementation. The only published clinical trial examining the effects of vitamin K supplementation on OA was an ancillary study of hand OA that analysed data from a trial designed to examine vitamin K supplementation for bone loss and vascular calcification.[28] Although in a comparison with placebo, randomization to the vitamin K supplement was not associated with a difference in radiographic hand OA, only radiographs taken at the end of the study were available. In a subgroup analysis of participants with insufficient vitamin K levels at baseline, less joint space narrowing was seen in those given vitamin K supplements. A properly powered randomised controlled trial of vitamin K in people with knee or hip OA who are vitamin K insufficient is needed. Given the large number of individuals with OA across the globe, the present studies and future work have important implications for public health.

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Anti-inflammatory therapy for COVID-19 infection: the case for colchicine

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ABSTRACT

The search for effective COVID-19 management strategies continues to evolve. Current understanding of SARS-CoV-2 mechanisms suggests a central role for exaggerated activation of the innate immune system as an important contributor to COVID-19 adverse outcomes. The actions of colchicine, one of the oldest anti-inflammatory therapeutics, target multiple mechanisms associated with COVID-19 excessive inflammation. While many COVID-19 trials have sought to manipulate SARS-CoV-2 or dampen the inflammatory response once patients are hospitalised, few examine therapeutics to prevent the need for hospitalisation. Colchicine is easily administered, generally well tolerated and inexpensive, and holds particular promise to reduce the risk of hospitalisation and mortality due to COVID-19 in the outpatient setting. Successful outpatient treatment of COVID-19 could greatly reduce morbidity, mortality and the demand for rare or expensive care resources (front-line healthcare workers, hospital beds, ventilators, biological therapies), to the benefit of both resource-replete and resource-poor regions.

INTRODUCTION

As of 27 October 2020, almost 1 year after the first reported cases, the SARS-CoV-2 had resulted in over 43 million people infected and over 1.1 million deaths from COVID-19 worldwide.[1] Clinical experience and data underline the role of excessive inflammation in the pathophysiology of the disease and suggest a potential role for colchicine, a drug with pleiotropic effects.

BIOLOGY OF COVID-19: THE ROLE OF INFLAMMATION

COVID-19 progression can be divided into three distinct phases (figure 1) including: (1) early infection phase, wherein the virus infiltrates host cells in the lung parenchyma; (2) pulmonary phase, in which viral propagation causes lung tissue injury as the host immune response is activated and (3) the inflammatory cascade, which is triggered by pathogen-associated molecular patterns (ie, viral RNA) and damage-associated molecular patterns (DAMPs, ie, cellular debris released during pyroptosis) exposed during active viral replication and release. This third phase of the inflammatory cascade may occur even as viral titers are falling and is comprised of components targeted by colchicine (activation of the inflammasome that drives the cytokine storm, activation of neutrophils and the neutrophil/thrombosis interface)[2] (figure 2).

Activation of the inflammasome

Signals driven by SARS-CoV-2 act on macrophages and other myeloid cells to drive assembly of a proinflammatory protein complex, the nod-like receptor protein 3 (NLRP3) inflammasome,[3] composed of NLRP3, apoptosis-associated speck-like protein adaptor and cysteine-dependent aspartate-directed protease-1 (caspase-1).[4] Activated caspase-1 activity then converts the precursors pro-interleukin (IL)-1β and pro-IL-18 to their active forms. Additionally, caspase-1 activates Gasdermin-D, forming pores in the cell membrane permitting large-scale secretion of IL-1β that, among other actions, induces macrophages to release large quantities of additional pro-inflammatory cytokines.[5 6] IL-1β, tumour necrosis factor (TNF) and ligation of toll-like receptors activate NF-κB[3] and further upregulate the inflammasome. IL-1β and other cytokines additionally recruit large numbers of leukocytes from the marrow, which in turn undergo activation and cytokine production in an accelerating spiral. In the related SARS-CoV-1, a small envelope (E) protein augments this reaction by self-assembling into an ion channel within the host cell membrane, causing calcium dysregulation that promotes further assembly and activation of the NLRP3 inflammasome.[7] More study is needed to determine if the E protein of SARS-CoV-2 has a similar effect on the inflammasome.

The production of IL-1β drives the synthesis of IL-6, a cytokine that induces C reactive protein (CRP) and has been especially implicated as a major proinflammatory agent in the COVID-19 cytokine storm.[8–11]

Activation of neutrophils

Cytokines including IL-1β and IL-6 prime neutrophils for activation by chemoattractants and upregulate intercellular adhesion molecules on endothelial cells. The resulting neutrophil adhesion to the vasculature promotes neutrophil diapedesis and infiltration into the affected tissues—in COVID-19 infection, initially into lung parenchyma, but later into other organs. Once neutrophils migrate to sites of inflamed tissue, they degranulate and release proinflammatory cytokines and chemokines, proteases, antiviral proteins and toxic oxygen radicals. In the myocardium, neutrophils -play a prominent role in the development of myocarditis and cardiogenic shock.[12–14]

Neutrophil/thrombosis interface

Neutrophils trigger a cascade of events in arteries that promote plaque destabilisation/rupture and

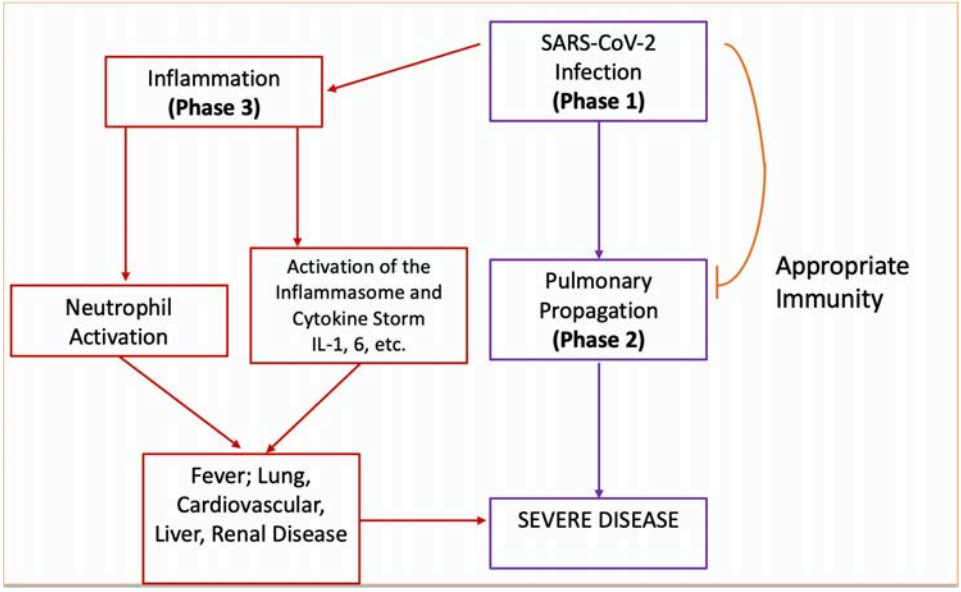

Figure 1 Model of COVID-19 severity. IL, interleukin.

thrombosis.[15–18] Neutrophils release the serine protease neutrophil elastase, which inhibits tissue factor pathway inhibitor and leads to generation of thrombin, the most potent activator of platelets. Neutrophil extracellular traps provide a platform to activate coagulation via active neutrophil elastase adherent to extracellular neutrophil DNA.[19 20] Activated neutrophils and other leukocytes also aggregate with platelets directly to further exacerbate inflammothrombosis.[21–23 24] In the setting of extreme inflammatory states, activated neutrophils adhere directly to each other (leukoaggregation), producing effective but usually transient vascular occlusions.[25] Finally, neutrophils contribute to thrombosis via cytokine-induced release of α-defensin from neutrophil granules.[26 27] Murine studies suggest that α-defensin, at concentrations similar to those observed in inflammatory conditions, results in accelerated, larger and denser thrombus formation.[28 29] Human data suggest that patients with COVID-19

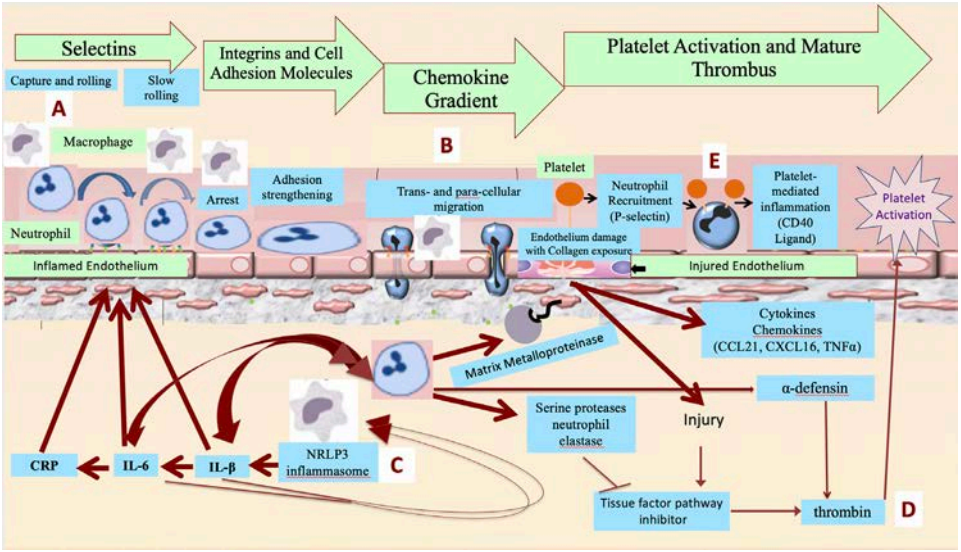

Figure 2 Proposed pathophysiology of acute vascular inflammation in SARS-CoV-2 viral illness and potential therapeutic targets of colchicine. (A) Macrophage-driven inflammation leads to inflammasome activity, cytokine production and endothelial and neutrophil activation, with surface expression of selectins, integrins and intercellular adhesion molecules promoting neutrophil adhesion to the vasculature. Colchicine inhibits E-selectin and L-selectin expression on neutrophil and endothelial surfaces. (B) Neutrophils migrate through the endothelium following chemoattractant gradients. Colchicine impairs the rheologic properties of the neutrophil cytoskeleton, limiting theirability to transmigrate. (C) Inflammasome-generated cytokines, including IL-1β and IL-6, drive additional macrophage activation and cytokine production, in an accelerating pattern known as a cytokine storm. Colchicine inhibits the NLRP3 inflammasome, with the potential to prevent the development of cytokine storm. (D) Neutrophil activation releases neutrophil elastase, which inhibits tissue factor pathway inhibitor. Diminished tissue factor pathway inhibitor activity, along with endothelial injury, promote thrombin generation and platelet activation. In addition, neutrophils release α-defensin, associated with larger and more extensive thrombi. Colchicine inhibits neutrophil elastase and α-defensin release. (E) Neutrophils interact with platelets to form aggregates that are a feature of thrombosis. Colchicine decreases neutrophil-platelet aggregation. CRP, C reactive protein; IL, interleukin; NLRP3, nod-like receptor protein 3; TNF, tumour necrosis factor.

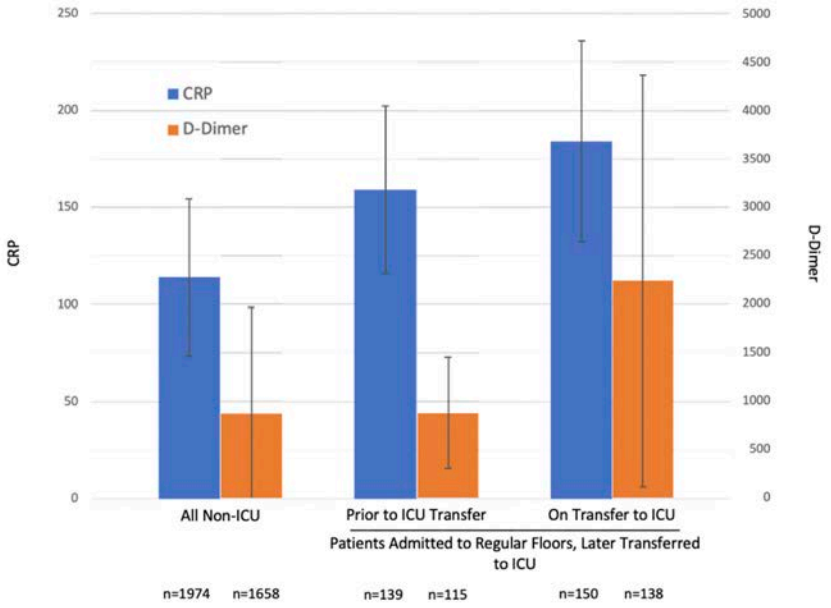

Figure 3 Markers of inflammation and thrombosis in patients admitted to the hospital for COVID-19. Admission inflammatory markers were obtained for all patients admitted to the regular (non-ICU) floors of NYU Langone Hospital for the first weeks (March–April 2020) of the COVID-19 pandemic surge in New York City. Among patients admitted to the regular floors, those who were subsequently transferred to the intensive care unit (ICU) had higher C reactive protein (CRP) levels than the group overall; among those transferred to the ICU, both CRP and D-dimer levels in the ICU were increased compared with prior to transfer, indicating that a worsening inflammatory state is a feature of more severe disease. Not shown in the figure: individuals admitted to the regular floors who were subsequently transferred to directly to the ICU also had higher ferritin levels than the non-ICU group overall (1452 vs 1178 mg/dL), and their mean ferritin level was found to be increased further on transfer to the ICU (1876 mg/dL).

infection have elevated levels of serum α-defensin proportional to COVID-19 disease severity.[30]

Clinical implications

The connections between inflammation, thrombosis and poor COVID-19 outcomes are well established. On admission, patients from our own institution who were admitted to regular floors but subsequently transferred to the intensive care unit (ICU) had higher CRP concentrations (159±86 mg/L) than patients admitted to the regular floors overall (114±81 mg/L). On transfer to the ICU, CRP concentrations (184 mg/L±104) were higher still (unpublished, figure 3). Manifestations of profound inflammation in severe COVID-19 include acute respiratory distress syndrome and distributive shock.[14 15 17] Myocardial injury due to acute coronary syndrome (type 1) and/or supply-demand mismatch in the setting of profound inflammatory response and haemodynamic changes (type 2) is also significantly greater in those with severe COVID-19.[31] Vascular inflammation is associated with a large burden of both venous (deep venous thrombosis, pulmonary embolism) and arterial (myocardial infarction, stroke) thrombus.

Severe COVID-19 has also been characterised by extrapulmonary and extravascular manifestations. Acute kidney injury may be a result of direct inflammatory injury, given evidence of acute tubular necrosis with lymphocyte and macrophage infiltration of the tubulointerstitium on histopathology.[32] The mechanism(s) of COVID-related hepatic injury remains unclear but preliminary studies suggest that the ACE2 receptor is preferentially expressed in cholangiocytes, suggesting that liver involvement may require direct SARS-CoV-2 infection and injury of cholangiocytes.[33 34] Cytokine storm itself can drive multisystem organ injury overall.

Together, these observations suggest that an anti-inflammatory agent with limited immunosuppressive potential could prove useful in preventing severe inflammatory injury and promoting improved patient outcomes.

COLCHICINE

Historical perspective

Although colchicine first received approval from the US Food and Drug Administration in 2009, its modern use dates back two centuries. Indeed, papyri dating from 1500 BC describe the use of colchicine's source plant—*Colchicum autumnale*—for pain and inflammation, making colchicine one of the world's oldest anti-inflammatory therapeutics.[35] Currently, colchicine is approved for treating and preventing acute gout and familial Mediterranean fever, and is used off label in Behçet's disease, pericarditis and other inflammatory conditions.[36]

Colchicine and microtubules: inhibition of neutrophil activity

Microtubules are dynamic proteins that form via polymerisation of α-/β-tubulin dimers. Colchicine irreversibly intercalates into free α/β dimers that incorporate into and block microtubule extension.[37] During inflammation, microtubules facilitate the movement of adhesion molecules onto cell surfaces. Colchicine concentrations are much higher in neutrophils than other leukocytes due to diminished activity of the P-glycoprotein membrane efflux pump that serves as an energy-dependent colchicine efflux

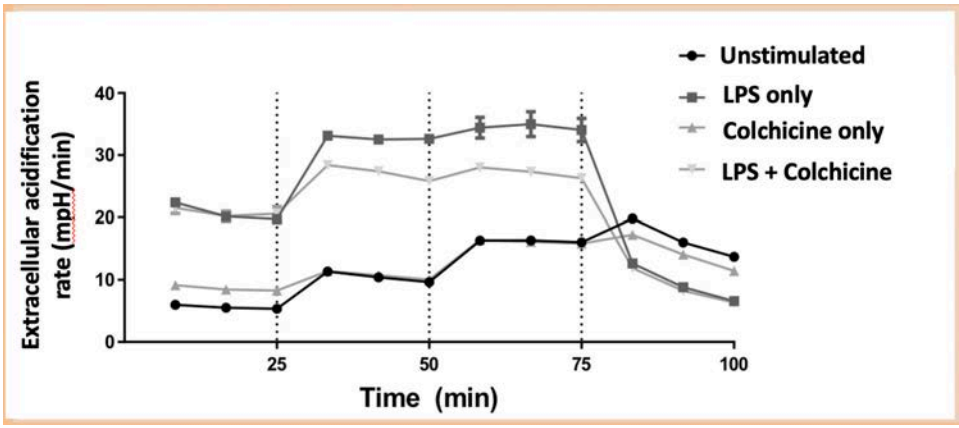

Figure 4 Neutrophil metabolism in the presence of colchicine. Neutrophils were purified from healthy volunteer whole blood using the MACSxpress whole blood neutrophil isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated into four aliquots. Neutrophils were coincubated with and without lipopolysaccharide (LPS) and with and without colchicine. In vitro quantification of neutrophil metabolism, measured as extracellular acidification rate (ECAR) (mpH/min), was evaluated using a glycolysis stress test using a Seahorse XFe24 analyzer (Agilent Technologies, Santa Clara, California, USA). Using a modified assay, cells were first incubated with activators (LPS 10 ng/mL with or without colchicine 15 nM) for 10 min.

transporter.[38] Thus, neutrophils appear to be more sensitive than other cells to lower serum concentrations of colchicine. Cronstein *et al* demonstrated that colchicine causes a quantitative decrease in leucocyte (L)-selectin expression and diminishes qualitative expression of endothelial (E)-selectin, two proteins involved in rolling and adhesion of neutrophils on endothelium.[39] Disruption of microtubules also inhibits neutrophil rheologic capacity, inhibiting their transmigration out of blood vessels.[40]

Additional studies show that colchicine directly inhibits intracellular neutrophil signalling and lysosomal enzyme release during phagocytosis. Colchicine-mediated inhibition of chemoattractant release (eg, leukotriene B_4) suppresses neutrophil adhesion to inflamed endothelium.[41] Colchicine also inhibits calcium influx, which raises intracellular cyclic adenosine monophosphate (cAMP) levels and dampens neutrophil responses.[42] In lipopolysachharide-stimulated neutrophils, we observed that colchicine can dampen stimulated neutrophil metabolism as measured by extracellular acidification (unpublished, figure 4).

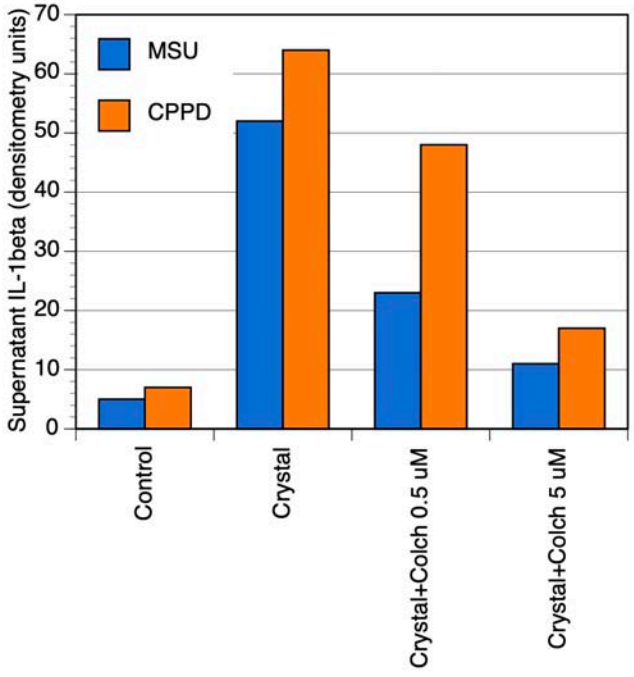

Figure 5 Colchicine inhibits inflammasome action and reduces supernatant levels of IL-1β. THP1 cells (macrophage cell line) were stimulated with monosodium urate (MSU) or calcium pyrophosphate dihydrate (CPPD) crystals in the presence or absence of colchicine. Supernatants were analysed for IL-1β by Western blot. For the purposes of this figure, the original published blot was quantified using Image J. Adapted from Martinon *et al*.[43]

Colchicine and the inflammasome: inhibition of IL-1β and prevention of the cytokine storm

More recently, colchicine has been shown to decrease cytokine production by inhibiting activation of the NLRP3 inflammasome (figure 5). The mechanism(s) of colchicine's action on the inflammasome remain an area of ongoing investigation.[43 44] Colchicine's interruption of inflammasome activation reduces IL-1β production, which in turn prevents the induction of IL-6 and TNF and the recruitment of additional neutrophils and macrophages.[45 46] Whereas the effect of specific anti-IL-6 inhibition for COVID-19 treatment is somewhat controversial (online supplemental text 1), the ability of colchicine to affect multiple cytokines may offer unique advantages.

Colchicine and the Inflammation/thrombosis interface

Murine models show that colchicine inhibits neutrophil release of α-defensin, thereby potentially preventing large thrombus burdens.[29 47] At supratherapeutic concentrations, colchicine, through its microtubule effects, converts normal discoid platelets to rounded, irregular structures and inhibits platelet activation by decreasing calcium entry.[48] These mechanisms diminish in vitro platelet-to-platelet aggregation. In contrast, we demonstrated that standard clinical doses of colchicine do not decrease platelet-to-platelet aggregation but do diminish neutrophil-to-platelet aggregation,[49] suggesting that colchicine at physiological doses may provide an inhibitory role at the inflammation/thrombosis interface without comprising homeostatic platelet-to-platelet function. Indeed, in vivo colchicine has not been shown to inhibit non-inflammatory-related thrombosis.

Adverse effects of colchicine

Colchicine metabolism occurs primarily inside hepatocytes via the cytochrome P450 3A4 (CYP3A4). Medications that strongly

inhibit CYP3A4 metabolism (eg, ritonavir, ketoconazole, clarithromycin, cyclosporine, diltiazem, verapamil) pose a risk of drug-drug interactions. A small number of publications report cases of death after coadministration of clarithromycin and colchicine in patients with severe chronic renal disease.[50 51] Similar cases have been rarely reported in patients receiving atorvastatin, a statin that is also processed by CYP-3A4, but not with statins that are not metabolised through CYP3A4. In a recent placebo-controlled randomised trial of 4745 patient with a recent myocardial infarction, patients receiving daily colchicine experienced no adverse effects related to the coadministration of statins, including atorvastatin.[52] In another recent placebo-controlled randomised trial of 5522 patients with stable coronary artery disease, daily colchicine resulted in numerically higher rates of myalgia (HR 1.15, 95% CI 1.01 to 1.31) and one case of rhabdomyolysis (the patient made a full recovery).[53] However, a non-significant trend towards increased non-cardiovascular death was observed that requires further investigation. Overall, reports of severe colchicine toxicity tend to occur in the setting of errors in colchicine prescribing.

Approximately 10%–20% of colchicine is excreted renally.[36] However, dose reductions may only be necessary in patients with severe renal impairment.[54] As a lipophilic molecule, colchicine is usually protein-bound in plasma, with P-glycoprotein in the intestinal lining serving as the primary protein for gut excretion of colchicine. Cyclosporine and ranolazine compete for the ligand site on P-glycoprotein and can therefore lead to delayed elimination. At higher concentrations for longer durations, particularly in the setting of kidney disease, colchicine has been reported to occasionally induce a reversible neuromyopathy. Acute overdose may cause multiorgan system failure and death. Furthermore, increased adverse events may be noted in the simultaneous presence of moderate renal insufficiency with use of multiple CYP3A4 inhibitors.

A meta-analysis of 35 randomised trials of colchicine versus placebo found that the most common and significant adverse effect was diarrhoea.[55 56] The only other adverse effect that occurred at a greater frequency than placebo was a set of pooled gastrointestinal symptoms including nausea, vomiting, diarrhoea, abdominal pain, loss of appetite, and bloating. A striking finding in this meta-analysis was the absence of increased infection rates in the colchicine compared with the placebo arm. However, in contrast to most available data, one retrospective and one prospective study did report increased pneumonia risk with colchicine (online supplemental table 1).

COLCHICINE AND COVID-19: THE CLINICAL CASE

Several of the biological therapies that have been studied and/or used in the setting of severe SARS-CoV-2 infection target some of the same pathways as colchicine, including IL-1β (ie, anakinra) and IL-6 (ie, tocilizumab and sarilumab).[57] Colchicine differs from these agents in having pleotropic mechanisms of action, being less potent on any single target, and being an oral agent. In contrast to the biological agents used in the midst of cytokine storm, colchicine is not immunosuppressive, is not known to increase risk of infection, and is inexpensive. A review of the mechanisms of SARS-CoV-2 and colchicine in parallel reveals a potential intervention point that may prevent the progression from inflammatory activation (phase 2) to a hyperinflammatory state (phase 3). Taken together with the clinical data described herein, the potential benefits of colchicine are suggested to be maximised when used early in the disease process (ideally prior to phase 2, but certainly prior to phase 3), such as in non-hospitalised patients within a few days of diagnosis regardless of symptoms and/or within a few days of hospitalisation if not already critically ill. However, the optimal timing continues to require further investigation.

Colchicine in non-rheumatological inflammatory conditions

Multiple randomised studies have evaluated the use of colchicine in non-rheumatologic inflammatory conditions. Two randomised trials in acute pericarditis demonstrated lower recurrence rate with colchicine versus conventional or placebo therapy.[58] Colchicine reduced symptom persistence 72 hours after treatment initiation, and colchicine was beneficial even in the setting of recurrent pericarditis.[59] Used after cardiac surgery, colchicine appears to prevent the inflammatory postpericardiotomy syndrome.[60]

Colchicine may reduce risk of acute myocardial infarction (AMI). We demonstrated an association between daily colchicine use and decreased prevalence of AMI in patients with gout, a non-traditional cardiovascular risk factor.[61 62] These findings were subsequently reproduced in an independent gout population.[63] Two open-label prospective studies of daily colchicine use versus no colchicine use in patients with stable coronary artery disease already on aspirin and high-intensity statin therapy demonstrated a decrease in CRP levels with low-dose colchicine, and a significant reduction in cardiovascular events with daily colchicine vs no colchicine.[64 65] The reduction in the primary clinical outcome was driven primarily by a reduction in AMI.[65] The multicentre, double-blind COLchicine Cardiovascular Outcomes Trial (COLCOT) randomised 4745 patients within 30 days of AMI to colchicine or placebo and demonstrated a reduction in the primary composite endpoint of cardiovascular death, resuscitated cardiac arrest, AMI, stroke or urgent revascularisation with colchicine.[52] The multicentre, double-blind Low Dose Colchicine 2 (LoDoCo 2) trial randomised 5522 patients with stable coronary artery disease and also demonstrated a reduction in the primary composite endpoint of cardiovascular death, AMI, stroke or urgent revascularisation.[53] Finally, in cases where the thrombus burden remains refractory to standard antiplatelet and anticoagulant therapies, colchicine has been shown to be associated with thrombus resolution.[66]

Our 400-patient randomised Colchicine in Percutaneous Coronary Intervention (Colchicine-PCI) trial demonstrated that when given as a standard loading dose prior to tissue injury (coronary stent placement), colchicine significantly dampened the upregulation of IL-6 and CRP.[67] These effects were observed 22–24 hours after the acute event, providing a rationale to administer colchicine earlier in the disease process to prevent clinical manifestations of cytokine-induced injury. Consistent with a possible preventive role, colchicine is effective to prevent cytokine-based disease flares in gout and familial Mediterranean fever.[45] Finally, colchicine has also been shown to dampen the inflammatory response and reduce CRP levels among subjects with metabolic syndrome.[68] These data support the general anti-inflammatory effect of colchicine, independent of a specific disease state.

Colchicine trials in COVID-19

The recent open-label, multicentre Randomised Evaluation of COVID-19 Therapy (RECOVERY) trial in the UK demonstrated a reduction in 28-day mortality with dexamethasone (n=2104) vs usual care (n=4321) in patients hospitalised with severe COVID-19.[69] These data support the principle that an anti-inflammatory strategy in COVID-19 may be helpful. However,

glucocorticoids such as dexamethasone have intrinsic immunosuppressive drawbacks that colchicine does not share.

Several early studies have evaluated the benefit of colchicine in COVID-19 patients. A retrospective single-centre study of 87 ICU patients with COVID-19 demonstrated a lower risk of death in patients on colchicine (adjusted HR 0.41, 95% CI 0.17 to 0.98).[70] The Greek Effects of Colchicine in COVID-19 (GRECO-19) trial was the first prospective open-label randomised trial evaluating colchicine versus usual care in early hospitalised patients. This study of 105 patients found a significant reduction in the primary clinical outcome of a two-point deterioration on WHO disease severity scale.[71] The authors additionally noted suppression of D-dimer levels in the colchicine vs control group.[71] An Italian study compared 122 hospitalised patients who received colchicine plus standard-of-care (lopinavir/ritonavir, dexamethasone or hydroxychloroquine) with 140 hospitalised patients receiving standard-of-care alone. Colchicine had a significant mortality benefit (84% vs 64% survival) vs controls.[72] A third prospective study randomised 38 hospitalised COVID-19 patients to colchicine or placebo in a double-blinded manner.[73] Patients receiving colchicine had less need for supplemental oxygen at day 7 (6% vs 39%) and were more likely to be discharged at day 10 (94% vs 83%). Colchicine subjects also had greater reduction of CRP, and no increase in serious adverse events.[73] Additional inpatient studies are ongoing (online supplemental table 2). Although the permitted use of other treatments could have biased the impact of colchicine in these studies, in the GRECO-19 trial no glucocorticoids were administered and other medications did not differ between the two groups; in the Italian study, there was no difference in outcomes among patients given colchicine who did or did not also receive dexamethasone.

Given its ease of use, tolerability and low cost, an argument for studying colchicine in the outpatient setting, to reduce hospitalisation and adverse outcomes, may be even more compelling. Unfortunately, data on the use of colchicine in the setting of outpatient COVID-19 cases are sparse. In a very small case series from Italy, nine outpatients with COVID-19 were administered colchicine, of whom only one subject was ultimately hospitalised. The hospitalised patient received 4 days of oxygen therapy and was discharged.[74] Moreover, all patients experienced defervescence within 72 hours of colchicine initiation, suggesting an antipyretic effect. While these reports are insufficient to recommend colchicine for COVID-19 in clinical practice, they provide support for further study of colchicine in COVID-19, including in the outpatient setting. The ongoing ColCorona Trial (www.colcorona.net) is a large placebo-controlled trial of colchicine use within 2 days of COVID-19 diagnosis, regardless of symptoms, in patients with comorbidities that place patients at a higher risk of developing complications related to COVID-19 that may provide additional information.

CONCLUSIONS

Given the large body of data demonstrating colchicine's inhibitory effects on neutrophil activity, cytokine generation and the inflammation/thrombosis interface, together with an overall lack of evidence for systemic immunosuppression, there is a rationale to study colchicine as a potential treatment for COVID-19. Given that colchicine is generally well tolerated, simple to take and inexpensive, demonstration of colchicine as a useful agent in COVID-19 would potentially spare patients morbidity and mortality, help to conserve valuable clinical resources (hospital floor and ICU beds, ventilators, etc), and dramatically reduce the cost of COVID-19 care. Colchicine might be of particular use in resource-poor rural and developing world settings, both of which have been increasingly affected by COVID-19. However, unless and until evidence is obtained from adequately designed and randomised placebo-controlled trials, this hypothesis must remain speculative.

The optimal dose of colchicine for daily use, even in well-established conditions such as gout, is unknown. Many but not all patients tolerate up to 1.2 mg daily in divided doses; whether lower doses such as 0.5 mg or less daily can be equally effective is unknown. The largest colchicine study for COVID-19 (ColCorona) is testing a dose of 0.5 mg daily based on prior cardiology trials. The duration of colchicine therapy for SARS-COV2 infection would also need to be determined. Most studies to date test a treatment duration of 2–4 weeks, concordant with the acute course of the infection; whether a shorter or longer treatment would be optimal is unknown. Finally, the timing of colchicine initiation is uncertain, with some studies beginning treatment in the outpatient setting, and others in the early inpatient setting. Given the recent track record of failure of treatment of severe COVID-19 treatment with anti-IL-6 biologics such as tocilizumab (a much more potent but also more specific immunosuppressive agent), it is likely that the severe inpatient setting is not the optimal condition under which to assess colchicine efficacy.

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TRANSLATIONAL SCIENCE

Large-scale meta-analysis across East Asian and European populations updated genetic architecture and variant-driven biology of rheumatoid arthritis, identifying 11 novel susceptibility loci

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ABSTRACT

Objectives Nearly 110 susceptibility loci for rheumatoid arthritis (RA) with modest effect sizes have been identified by population-based genetic association studies, suggesting a large number of undiscovered variants behind a highly polygenic genetic architecture of RA. Here, we performed the largest-ever trans-ancestral meta-analysis with the aim to identify new RA loci and to better understand RA biology underlying genetic associations.

Methods Genome-wide RA association summary statistics in three large case–control collections consisting of 311 292 individuals of Korean, Japanese and European populations were used in an inverse-variance-weighted fixed-effects meta-analysis. Several computational analyses using public omics resources were conducted to prioritise causal variants and genes, RA variant-implicating features (tissues, pathways and transcription factors) and potentially repurposable drugs for RA treatment.

Results We identified 11 new RA susceptibility loci that explained 6.9% and 1.8% of the single-nucleotide polymorphism-based heritability in East Asians and Europeans, respectively, and confirmed 71 known non-human leukocyte antigens (HLA) susceptibility loci, identifying 90 independent association signals. The RA variants were preferentially located in binding sites of various transcription factors and in cell type-specific transcription–activation histone marks that simultaneously highlighted the importance of $CD4^+$ T-cell activation and the potential role of non-immune organs in RA pathogenesis. A total of 615 plausible effector genes, based on gene-based associations, expression-associated variants and chromatin interaction, included targets of drugs approved for RA treatments and potentially repurposable drugs approved for other indications.

Conclusion Our findings provide useful insights regarding RA genetic aetiology and variant-driven RA pathogenesis.

Key messages

What is already known about this subject?

- Genetic factors are strongly involved in the aetiology of rheumatoid arthritis (RA).
- Although approximately 110 loci were reported as RA susceptibility loci by genome-wide association studies, a large fraction of genetic heritability estimated in twin studies is still hidden behind many genetic variants with weak effect sizes.

What does this study add?

- We discovered 11 novel loci (*DGUOK-AS1*, *DAP*, *BAD*, *TPCN2*, *LOC107984408*, *LOC105369698*, *IQGAP1*, *PRKCB*, *ZNF689*, *C20orf181* and *SMC1B*) associated with RA surpassing the genome-wide significance level ($p=5\times10^{-8}$) in a large-scale meta-analysis integrating genetic associations in East Asians and Europeans.
- We catalogued possible causal variants and genes, RA-relevant biological features (tissues, pathways and transcription factors) and repurposable drugs for RA treatment from the genetic association results.

How might this impact on clinical practice or future developments?

- The genetic liability explained by these novel variants will improve precision medicine in RA and provide novel druggable targets for RA treatment.

INTRODUCTION

Rheumatoid arthritis (RA) is a common autoimmune disease characterised mainly by pain, swelling and deformity of joints due to prolonged inflammation.[1] A substantial portion of patients with RA are seropositive for rheumatoid factor or anticitrullinated protein antibodies (ACPA).[2] RA is most common in women aged 30–50 years and affects approximately 1% of the general population.[3] Multifactorial causes, including genetic and environmental factors, are involved in the development of RA.[1] Twin concordance studies demonstrated that genetic predisposition plays a major role in the pathogenesis of RA, accounting for approximately 60% of the liability for RA.[4] In the past decade, genome-wide association studies (GWASs) in various populations identified nearly 110 RA susceptibility loci across the human genome.[5] However, the heritability explained by the identified variants was estimated to be ~20%,[5 6] suggesting that a much larger number of disease variants remain unidentified. Consistent with these results, Bayesian inference predicted hundreds of common susceptibility

variants with modest effect sizes and rare variants with large effect sizes.[7]

Here, we carried out a large-scale meta-analysis on RA with summary association data from East Asian and European cohorts comprising 22 628 RA cases and 288 664 controls and identified 11 new RA susceptibility loci. In addition, the integration of accumulated knowledge of RA variants with emerging high-throughput omics data facilitated various so-called post-GWAS approaches that helped unravel the biology of RA based on disease-risk variants in actual human patients, suggesting potentially repurposable drugs for RA treatment.

METHODS

Association summary statistics

RA association summary statistics were retrieved from previous GWASs in Korean, Japanese and European populations.[6 8 9] The sample size of each GWAS was considerable (4068 RA cases and 36 487 controls in the Korean population[8]; 4199 cases and 208 254 controls in the Japanese population[9]; 14 361 cases and 43 923 controls in the European population[6]), which brought the final sample size to more than 310 000. The summary statistics from Japanese and European populations were available at the Japanese Encyclopedia of Genetic associations by RIKEN (http://jenger.riken.jp/en/result). All data included over 13 million imputed variants with reliable imputation quality scores and minor allele frequency (MAF) of ≥0.5%. Each study performed a whole-genome imputation using the imputation reference panel from the 1000 Genomes Projects (1KGP) phase III data with or without ancestry-matched whole-genome sequencing data.

Genome-wide meta-analysis

An inverse-variance-weighted fixed-effects model with genomic control was used in a meta-analysis of the association summary statistics in three studies by meta-analysis of genome-wide association studies (METAL)[10] to calculate the disease effect sizes and SEs of query variants (n=13 810 676). Heterogeneity of effect sizes between East Asian and European populations was assessed using Cochran's Q test.[11] We defined an RA-associated locus by merging the neighbouring significant variants ($p \leq 5 \times 10^{-8}$), between which the minimum distance should not exceed 250 kb. Downstream analyses excluded the extended major histocompatibility complex (MHC) region (24–37 Mb in chromosome 6 in the human genome assembly GRCh37).

Estimation of RA heritability

Single-nucleotide polymorphism (SNP)-based heritability (h^2_{SNP}) of RA in East Asian (Korean+Japanese) or European populations was calculated in a liability scale using LD score regression (LDSC)[12] with precalculated linkage disequilibrium (LD) scores, regression weights and allele frequencies from the 1KGP phase III data in a relevant ancestral population (https://data.broadinstitute.org/alkesgroup/LDSCORE/), setting the disease prevalence parameter to 1%.

Correlation analysis of genome-wide SNP effects between ancestries

We employed Popcorn[13] to calculate the trans-ethnic genetic correlation of effect sizes of the genome-wide variants based on genetic effect scores via LD optimisation similar to LDSC.

Conditional association analysis in RA loci

To identify independent association signals in 82 non-MHC loci, a stepwise approximate conditional association analysis was performed for each population by genome-wide complex trait analysis (GCTA)[14] using ancestry-matched LD matrices. Genotype data of 5288 Korean and 2502 European individuals were used to estimate LD matrices. The Korean genotype/imputation data were previously described in our recent study,[8] and the European genotype data were obtained from European Genome-phenome Archive (EGAD00010000290).[15] We newly imputed untyped variants in the European individuals from quality-controlled variants (with missing rate≤0.05, MAF≥0.5%, and p value in Hardy-Weinberg equilibrium exact test$\leq 1 \times 10^{-7}$) in each RA locus using SHAPEIT2 and Minimac3 with the 1KGP imputation reference panel. The polymorphic variants with imputation quality score of ≥0.3 and MAF of ≥0.5% were used to calculate variant–variant correlations. A conditional analysis with adjustment of a primary association signal (the lead variant) in each RA locus was performed for each ancestral group and followed by a cross-ancestry meta-analysis to identify a secondary association signal with conditional p_{meta} of $\leq 5 \times 10^{-8}$. This procedure conditioning on all independent association signals was repeated until no more signals were significant. When a lead variant was not available in one ancestry, we performed a stepwise conditional analysis in the other ancestry. All annotation of lead variants and their proxies ($r^2 \geq 0.9$) were retrieved from HaploReg[16] and RegulomeDB.[17]

Heritability-partitioning analysis using transcription factor-binding sites (TFBSs)

We calculated RA heritability attributed to SNPs within binding sites of 161 transcription factors (TFs) using stratified LDSC[18] to estimate relative heritability enrichment. All tested TFBSs were provided by an annotation library from PAINTOR (https://github.com/gkichaev/PAINTOR_V3.0/wiki/2b.-Overlapping-annotations). For each TF, heritability enrichment value is equal to the proportion of heritability ($= h^2_{TFBS\_SNP} / h^2_{SNP}$) divided by the proportion of variants (= *number of SNPs in TFBS* / *number of total SNPs*). The enrichment scores and significance levels were estimated in East Asian and European populations separately using ancestry-matched LD scores, and the cross-ancestry significance level for each TF was calculated by Fisher's combined probability test.[19] A statistically significant threshold was set at a false discovery rate (FDR) of <5%.

Enrichment analysis of RA variants using histone modification marks

We assessed the statistical significance for the colocalisation of RA-associated lead variants (plus their proxies in $r^2 \geq 0.8$ with the lead variants in a relevant ancestry) with tissue-specific epigenomic regulatory elements in East Asians and Europeans separately using GREGOR[20] by comparing 1000 feature-matched control sets. The genomic locations of six histone marks (H3K4me1, H3K4me3, H3K9me3, H3K27me3, H3K36me3 and H3K27ac) of various cells or tissues were retrieved from the Roadmap Epigenomics Project data.[21] The number of lead variants in RA loci outside of the MHC regions was 38 in East Asians and 43 in Europeans. The feature-matched control sets were randomly selected based on the 1KGP phase III data to be matched for three properties of RA-associated lead variants (the number of LD proxies, MAF and distance to the most proximal gene). The enrichment p value was defined as a probability of detecting more than the observed number of overlaps between the query variant sets and a selected histone mark, based on the control distribution approximated using feature-matched control

sets by the saddle point method.[20] Fisher's combined probability test was used to calculate the combined p value.

Nomination of potential effector genes in RA loci

We catalogued potential effector genes (=disease-driving genes) from the meta-analysis association summary statistics using FUMA[22] and MAGMA[23] with LD information in all 1KGP individuals. First, FUMA analysis mapped candidate genes using the SNP2GENE module with default parameters when RA-risk variants were located within 10 kb of the genes, known to have expression quantitative trait loci (eQTLs) of the genes or interacted with the genes via chromatin interaction. Briefly, we retrieved reported eQTLs in selected tissues (blood cells, spleen, small intestine and lung) from the EBI eQTL catalogue (https://www.ebi.ac.uk/eqtl), single-cell RNA eQTLs,[24] DICE,[25] GTEx[26] and other blood eQTL sets.[27–30] Chromatin interaction mapping was performed using publicly available Hi-C data[31] in the same four tissues and FANTOM5 enhancers and promoters.[32] To retain more likely candidate effector genes, physically mapped genes were narrowed down at the Bonferroni-corrected gene-based p value cut-off of 0.05 in MAGMA.

Prediction of repurposable drugs for RA

We investigated potential drug candidates for RA treatment using Genome for REPositioning drugs (GREP).[33] Proteins directly interacting with the products of 615 potential RA effector genes were obtained from HumanNet V.2 resources.[34] The genes of these interacting proteins were used as query genes, along with the 615 RA genes in the GREP analysis to search for potential repurposable drugs that target effector proteins or their network members. Anatomical therapeutic chemical (ATC) classes[35] (n=85) were tested for the enrichment of the identified drugs. The most likely repurposable drugs were defined as belonging to any ATC groups with FDR-corrected enrichment p value of ≤0.05.

RESULTS

Identification of 11 novel susceptibility loci for RA

A genome-wide meta-analysis of RA associations in 22 628 patients with RA and 288 664 healthy controls in East Asian (8267 cases and 244 741 controls) and European (14 361 cases and 43 923 controls) populations detected 11 novel loci associated with RA risk at the genome-wide significance level ($4.6\times10^{-8}\leq p_{meta}\leq1.5\times10^{-10}$), replicating the known RA associations in 71 non-MHC loci[8 36 37] (figure 1 and online supplemental table S1). The meta-analysis association summary statistics in this study are available online (https://doi.org/10.5061/dryad.ns1rn8pr0). Excluding MHC variants, we found that the meta-analysis results displayed an inflation factor λ of 1.01, indicating little systemic bias in our study (online supplemental figure S1). The most significant variants in the newly identified 11 loci (with the following nearest genes: *DGUOK-AS1*, *DAP*, *BAD*, *TPCN2*, *LOC107984408*, *LOC105369698*, *IQGAP1*, *PRKCB*, *ZNF689*, *C20orf181* and *SMC1B*) showed modest effect sizes on RA development (OR for a risk allele ≤1.13, figure 2, table 1). The observed effect sizes were highly consistent between Korean and Japanese populations (heterogeneity p value≥0.38, online supplemental table S2) and between East Asians and Europeans (heterogeneity p value≥0.67, table 1) for all the tested lead variants, although four lead variants were not tested in Europeans. Of the 11 lead variants, four were insertion/deletion variants. The lead variant in *DGUOK-AS1* was not common in Europeans (MAF=0.7%), yielding an insignificant association p value but a consistent effect size (table 1).

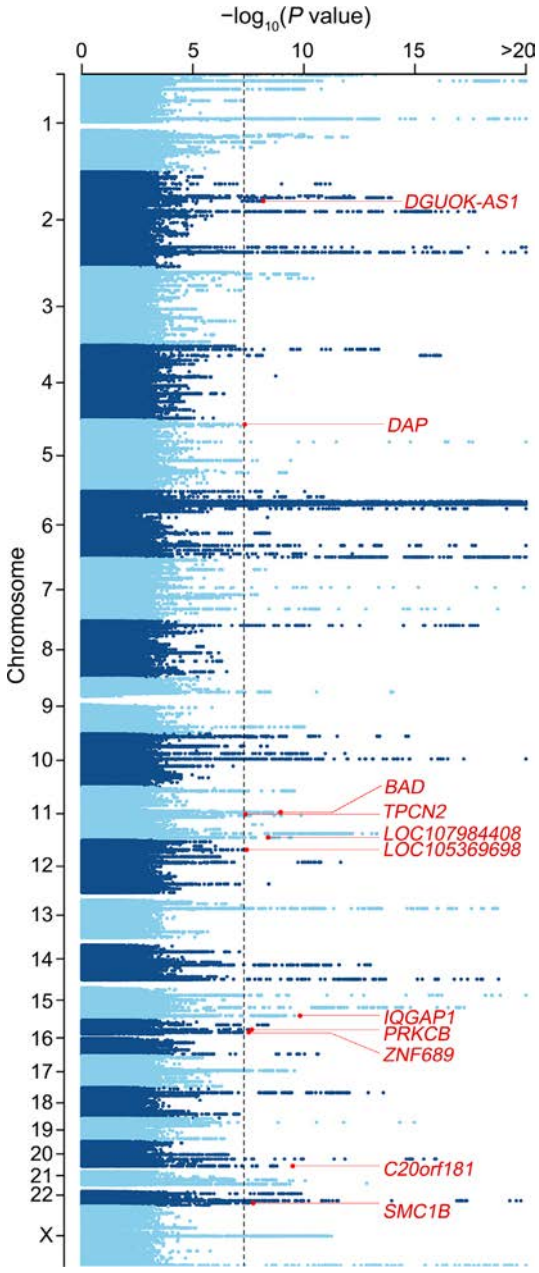

Figure 1 Manhattan plot of trans-ancestral genome-wide association meta-analysis results for RA. The y-axis represents negative natural logarithms of the p value for each variant in a meta-analysis using an inverse-variance-weighted fixed-effects model for the genomic-control associations in Korean, Japanese and European populations. The x-axis indicates chromosomal positions. The dashed line represents the genome-wide significance threshold ($p=5\times10^{-8}$). Eleven novel loci are marked in red with the nearest genes. RA, rheumatoid arthritis.

Genetic liability explained by the tested genome-wide variants was estimated as 0.176 in East Asians and 0.275 in Europeans in an LDSC heritability analysis[12] (table 2). The variants in 11 novel loci accounted for 6.9% and 1.8% of the non-MHC SNP-based heritability in East Asians and Europeans, respectively. Although the estimated SNP-based heritability was higher in Europeans than in East Asians, we observed a highly strong association correlation between the two ancestries in whole-genome

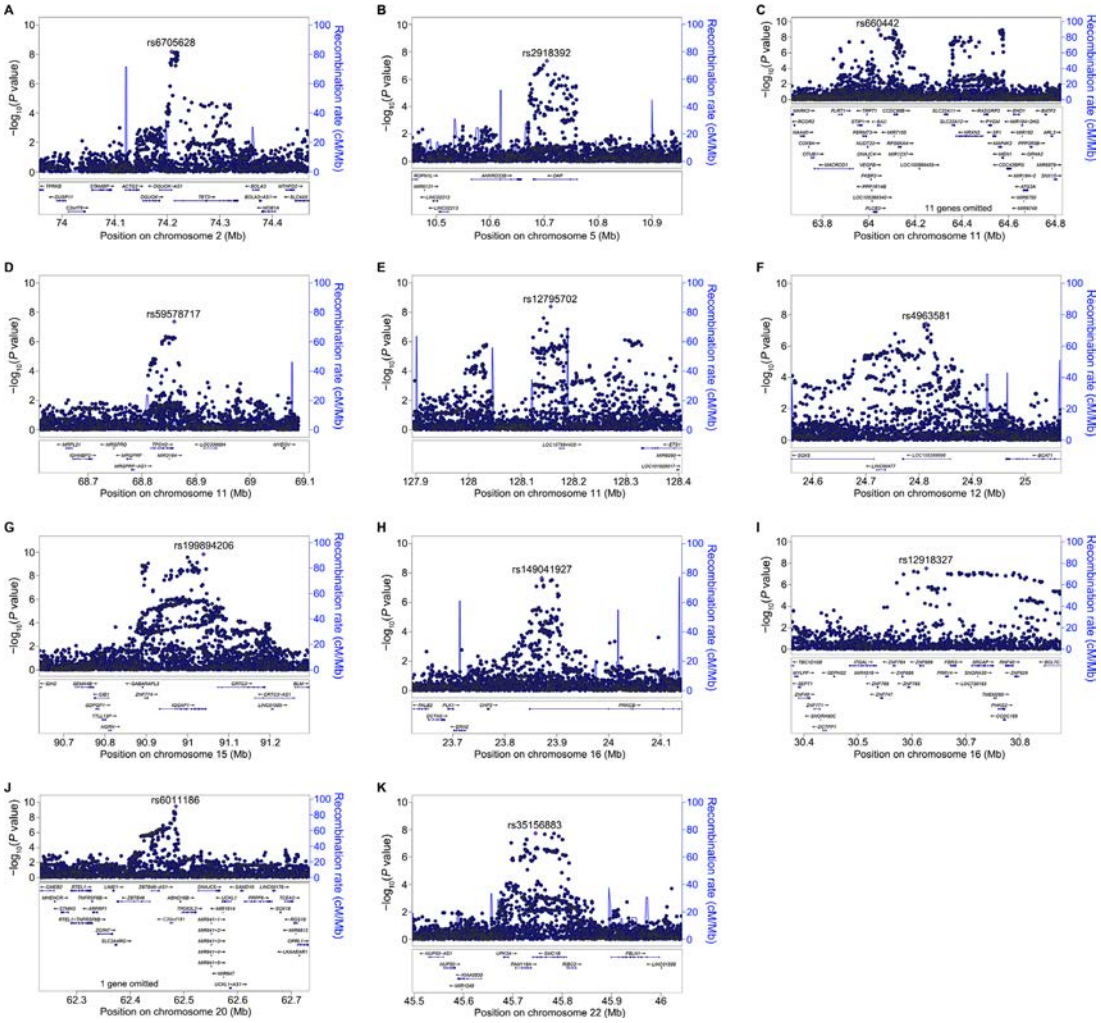

Figure 2 Regional association plots for the newly identified RA loci. The association significance levels in the loci of interest were plotted in negative logarithm scale according the chromosomal position of variants. The most significant variants are denoted as purple diamonds. (A) *DGUOK-AS1*, (B) *DAP*, (C) BAD, (D) *TPCN2*, (E) *LOC107984408,* (F) *LOC105369698*, (G) *IQGAP1*, (H) *PRKCB*, (I) *ZNF689*, (J) *C20orf181* and (K) *SMC1B*. RA, rheumatoid arthritis.

scale ($r=0.69\pm0.09$, p value$=2.12\times10^{-14}$), which supports a high degree of risk allele sharing between the two populations.

To narrow down the potentially functional variants, we found 130 proxy variants in high LD ($r^2\geq0.9$) with lead variants in both East Asian and European populations (for eight loci tested in both populations) or in East Asians (for four loci tested in only East Asians). Among the 141 potentially causal variants, two (rs11556482 and rs6007594) were missense variants in *FAM118A* (near *SMC1B*). In addition, 29 variants in six loci were likely to affect TF binding or linked to gene expression in an allele-specific manner, being located with highly functional annotations (RegulomeDB[17] category=1 or 2, online supplemental table S3).

Dissecting association signals

To determine the number and sources of association signals in 82 non-MHC loci, we performed a stepwise approximate conditional association analysis for each ancestral group followed by a meta-analysis of conditional association results in two groups. There were at least two independent association signals in each of seven loci (*PADI4*, *CTLA4*, *TNFAIP3*, *IL2RA*, *PRKCQ*, *ARID5B* and *LOC145837*) with a conditional p value$\leq5\times10^{-8}$ (online supplemental table S4).

Enrichment of RA variants on TFBSs and tissue-specific epigenetic features

The degree of enrichment of RA heritability on binding sites of 161 TFs was assessed using the population-specific LDSC followed by Fisher's combined probability tests. We observed that RA heritability was significantly enriched in variants within binding sites of 29 TFs (p value for heritability enrichment ≤0.05 in both populations and FDR-corrected $p_{meta}\leq0.05$, figure 3A and online supplemental table S5). Among the identified TFs, 12 displayed extremely large heritability enrichment in their TFBSs (enrichment>40 in both populations), and these TFs have been significantly associated with T-cell receptor (TCR) signalling transduction mediated by mitogen-activated protein kinases, nuclear factor-kappa B and nuclear factor of activated T-cells[38] (online supplemental table S6). These results reinforce the importance of $CD4^+$ T-cell activation in RA pathogenesis,[5] suggesting that heritability-explaining RA variants may play an allele-specific transcription-regulatory role in $CD4^+$ T cells, especially on activation.

The regulatory effects of RA-risk variants even in relevant TFBSs highly depend on chromatin accessibility associated with highly cell type-specific histone modification marks. Given this knowledge, we searched for RA-relevant tissues, in which histone

Table 1 RA associations of the newly identified loci in a trans-ancestral genome-wide association meta-analysis

Variant	Chr:position*	Nearest gene	EA/NEA	East Asian					European			Meta-analysis		
				$Freq_{ASN}$	OR_{KOR}	OR_{JAP}	OR_{ASN} (95% CI)	P_{ASN}	$Freq_{EUR}$	OR_{EUR} (95% CI)	P_{EUR}	OR_{META} (95% CI)	P_{META}	P_{HET}
rs6705628	2:74208362	*DGUOK-AS1*	T/C	0.18	0.87	0.89	0.88 (0.84 to 0.92)	2.1×10^{-8}	0.007	0.90 (0.79 to 1.03)	0.12	0.88 (0.85 to 0.92)	6.7×10^{-9}	0.95
rs2918392	5:10704797	*DAP*	T/C	0.29	0.95	0.95	0.95 (0.91 to 0.98)	3.9×10^{-3}	0.32	0.93 (0.90 to 0.96)	1.6×10^{-6}	0.94 (0.91 to 0.96)	4.6×10^{-8}	0.89
rs660442	11:64042997	*BAD*	A/G	0.08	0.82	0.86	0.84 (0.79 to 0.89)	3.9×10^{-8}	0.10	0.93 (0.89 to 0.96)	2.0×10^{-4}	0.90 (0.87 to 0.93)	1.1×10^{-9}	0.67
rs59578717	11:68859848	*TPCN2*	Ins1†/C	0.51	0.90	0.92	0.91 (0.88 to 0.94)	4.3×10^{-8}	0.86‡	NA	NA	0.91 (0.88 to 0.94)	4.3×10^{-8}	NA
rs12795702	11:128156314	*LOC107984408*	A/G	0.77	1.14	1.07	1.11 (1.06 to 1.15)	5.8×10^{-7}	0.18	1.07 (1.03 to 1.11)	8.5×10^{-4}	1.09 (1.06 to 1.12)	4.1×10^{-9}	0.89
rs4963581	12:24813281	*LOC105369698*	A/G	0.75	1.09	1.09	1.09 (1.05 to 1.13)	6.6×10^{-6}	0.09	1.09 (1.03 to 1.14)	1.4×10^{-3}	1.09 (1.06 to 1.12)	3.8×10^{-8}	0.98
rs199894206	15:91038883	*IQGAP1*	CT/C	0.36	1.12	1.12	1.12 (1.08 to 1.16)	1.5×10^{-10}	0.19‡	NA	NA	1.12 (1.08 to 1.16)	1.5×10^{-10}	NA
rs1490419278§	16:23871206	*PRKCB*	A/Ins2¶	0.38	0.90	0.91	0.91 (0.88 to 0.94)	2.2×10^{-8}	0.44‡	NA	NA	0.91 (0.88 to 0.94)	2.2×10^{-8}	NA
rs12918327	16:30626616	*ZNF689*	T/C	0.10	1.06	1.12	1.09 (1.03 to 1.16)	1.6×10^{-3}	0.12	1.09 (1.05 to 1.13)	3.9×10^{-6}	1.09 (1.06 to 1.12)	3.0×10^{-8}	0.98
rs6011186	20:62484008	*C20orf181*	T/C	0.38	0.87	0.92	0.90 (0.87 to 0.93)	3.7×10^{-9}	0.035	0.90 (0.82 to 0.99)	2.6×10^{-2}	0.90 (0.87 to 0.93)	3.2×10^{-10}	0.99
rs35156883	22:45746152	*SMC1B*	A/AT	0.52	1.12	1.08	1.10 (1.06 to 1.13)	1.9×10^{-8}	0.63	NA	NA	1.10 (1.06 to 1.13)	1.9×10^{-8}	NA

*Based on hg19.
†CGGCTAAGGGGAGAGAGCCGGAAGCCACGCACACCGGCGCAAGGGGCGGATA.
‡Frequency data were retrieved from European statistics in the 1000 Genomes Project phase III.
§or rs59279623.
¶AAGGGAG.
Chr, chromosome; EA, effect allele; EUR, European; Freq, frequency of EA; JAP, Japanese; KOR, Korean; NEA, non-effect allele; P_{HET}, p value from a Cochrane's Q test for heterogeneity in effect sizes; RA, rheumatoid arthritis.

Table 2 Liability-scale RA heritability h^2 estimated from the tested genome-wide variants in each ancestry

	All tested variants		Non-MHC variants*			
					Proportion of h^2 explained by	
Ancestry	h^2	SE of h^2	h^2	SE of h^2	Known RA loci	Novel RA loci
East Asian	0.176	0.053	0. 123	0. 012	43.0%	6.9%
European	0. 275	0.096	0. 185	0. 021	38.1%	1.8%

*The MHC region was defined as spanning as the 24–37 Mb region of chromosome 6 in hg19.
MHC, major histocompatibility complex; RA, rheumatoid arthritis.

marks colocalise with significantly more RA-risk variants. We employed the GERGOR algorithm[20] to test the enrichment on four transcription-activating histone modifications (H3K4me1, H3K27ac, H3K4me3 and H3K36me3) and two repressing histone modifications (H3K27me3 and H3K9me3) in diverse human cell types. Transcription-activating histone marks were strongly associated with RA-risk variants in various immune cells, especially in $CD4^+$ T-cell subtypes (figure 3B). Among the $CD4^+$ T cells, memory $CD4^+$ T cells (E037 and E040) rather than naïve $CD4^+$ T cells (E038 and E039) presented relatively strong significance levels for the RA-variant enrichment. Furthermore, chromatin changes on T-cell activation and T_{reg} differentiation were strongly associated with RA variants (figure 3B). In addition, this analysis replicated our recent findings on the involvement of two non-immune organs,[8] lung and small intestine, in disease pathogenesis (figure 3B and online supplemental table S7).

Candidates for repurposable drugs targeting RA genes

We narrowed down the potential effector genes to 615 genes based on three categories: gene-level association significance levels (estimated from genome-wide variant associations, gene-level p value≤0.05/19 644), known eQTL effects, and chromatin interactions between RA-variants and neighbouring genes (online supplemental table S8). A total of 132 genes belonged to more than two categories. For example, *DAP* in a novel locus encodes a member of mTOR signal transduction[39] and was identified as a new plausible effector for RA, as the gene-based association of *DAP* with RA was significant ($p_{MAGMA}=1.62\times10^{-6}$) and a lead variant (rs2918392) was a known eQTL for *DAP* in blood cells.[25 26 29]

We further investigated potentially repurposable drugs for RA that target the 615 effector gene products and their 1543 direct interactors (=2158 RA-relevant genes). We found that the tested genes were significantly enriched in the targets of immunosuppressants, immunostimulants and antineoplastic agents in Fisher's exact tests (FDR-corrected p values≤3.34×10^{-4}). For example, 18 RA-relevant genes are targeted by 21 immunosuppressants, including known RA drugs (eg, abatacept, tocilizumab, tofacitinib, etanercept, sarilumab, baricitinib, infliximab, adalimumab, certolizumab pegol, golimumab and azathioprine) and potential repurposable drugs previously approved for other indications, such as systemic lupus erythematosus and multiple sclerosis (eg, eculizumab, alefacept, belatacept, daclizumab, siltuximab, mycophenolic acid, lenalidomide, basiliximab and pomalidomide; figure 4 and online supplemental table S9).

DISCUSSION

This study had the advantage of analysing two distinct ancestral populations. As two ancestral populations have highly different

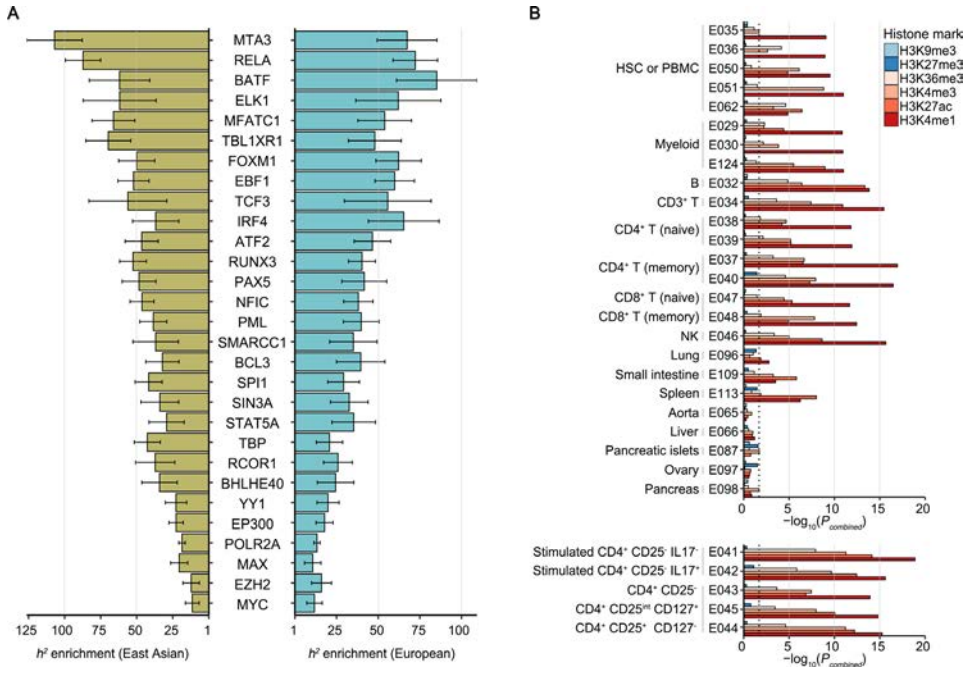

Figure 3 Transcription factors and cell types implicated by genetic associations in RA. (A) RA heritability was significantly enriched in binding sites of 29 TFs with p<0.05 in both populations and FDR-corrected p_{meta} <0.05. Erichment estimates in East Asians (left) and Europeans (right) were plotted for each TF (y-axis). (B) Enrichment of RA-risk variants on roadmap histone modification marks in diverse immune and non-immune cell types are assessed in each population. The combined p values for the enrichment were plotted in negative logarithm scale according to 25 selected cell types and 5 additional $CD4^+$ subtypes, with roadmap epigenome codes and cell-type names. FDR, false discovery rate; RA, rheumatoid arthritis; TF, transcription factor.

LD architectures, most analyses in this study (eg, association meta-analysis, conditional association analysis, TFBS heritability analysis and cell-type histone mark enrichment analysis) were performed for each population, separately, and the independent results were merged. Results in each population were mutually validated by the results in the other population, which provided population-shared, reliable insights into RA aetiology and pathogenesis.

This study increased the explained proportion of genetic liability for RA, especially more in East Asians. We detected 82 non-MHC RA-risk loci, including 11 novel loci, and identified 90 distinct signals by combining conditional results for both ancestry groups. The gene-level approaches nominated 615 plausible effector genes from the 82 RA loci based on genic variant association, eQTL and chromatin interaction data. In addition, we provided complete lists of the most likely causal variants in novel loci based on LD in both populations and regulatory annotation/statistics. The catalogues of the most likely RA-risk variants and genes may be useful in choosing targets for experimental validation to deepen understanding of variant-driven

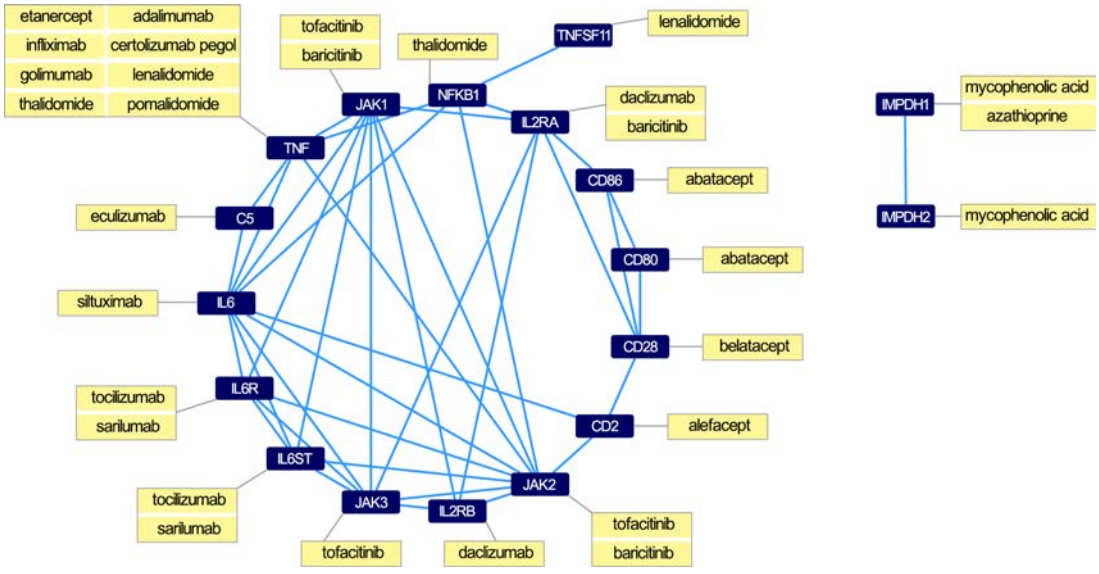

Figure 4 Approved or potentially repurposable immunosuppressants for RA treatment. Existing immunosuppressants (shown in yellow boxes) are connected with drug targets (shown in dark blue boxes) that were extracted from potential RA effector gene products and their interaction partners. A network of the drug targets was retrieved from HumanNet v2-XN and visualised by Cytoscape. RA, rheumatoid arthritis.

pathological processes and to identify druggable targets. Indeed, we showed that the RA effector genes and their interaction partners were actual targets of known RA drugs and suggested other drugs that may be repurposable for RA treatment.

$CD4^+$ T-cell biology was emphasised in RA pathogenesis by large-heritability variants that preferentially spanned binding sites of various TFs related with TCR-mediated signalling and that were preferentially located with transcription-activating histone marks in $CD4^+$ T cells, including stimulated, memory and/or regulatory T cells.

In the novel loci, 88 genes were detected as potential effector genes (8 genes per locus). The number of nominated genes are quite large but likely include true RA genes. It is possible to further narrow them down based on the number of categories (gene-based p value, eQTL and chromatin interaction) to which they are assigned (online supplemental table S8). For example, the number of genes with more than two categorical hits is decreased to 16 in 11 novel loci (*DAP*, *CCDC88B*, *RPS6KA4*, *NRXN2*, *MEN1*, *ZNF774*, *IQGAP1*, *CRTC3*, *PRKCB*, *AC002310.12*, *PRR14*, *FBRS*, *SRCAP*, *BCL7C*, *FAM118A* and *SMC1B*). Some have been documented for their functional relationship with immune cells or immune disorders (*DAP*,[40] *CCDC88B*,[41] *RPS6KA4*,[42] *IQGAP1*,[43] *CRTC3*[44] and *PRKCB*[45]). For example, *IQGAP1*, encoding a controller of tumour nectosis factor costimulatory receptor CD134,[43] modulates immune responses (eg, T-cell cosignalling pathway) possibly by an allele-specific regulatory effect of an RA-risk eQTL mediated by chromatin interaction in relevant tissues (online supplemental table S8).

CCDC88B is known to be essential for T-cell maturation and activation.[41] Variants in *CCDC88B* have been associated with the risk of inflammatory bowel diseases,[46] possibly leading to $CD4^+$ T-cell-induced colitis.[47] The lead variant rs660442 in our study was demonstrated to regulate expression of *CCDC88B* in immune cells (online supplemental table S3).

Another variant rs3826259 in *PRKCB*, an LD proxy (r^2=0.98) of a lead variant, is located in a highly conserved genomic element and is likely to influence binding affinity of several TFs according to the RegulomeDB.[17] The regulatory effect of the variant on *PRKCB* expression in RA-relevant cells is supported by eQTL catalogues (online supplemental table S3). *PRKCB* was a hub gene of the gene network constructed by differentially expressed genes in $CD4^+$ T cells in RA, involved in diverse signalling pathways.[45]

In summary, we performed the largest genome-wide meta-analysis using RA associations in three large cohorts comprising >300 000 East Asian and European individuals. Our computational analyses provided new insights and enhanced evidence regarding the genetic architecture/liability, disease-driving variants/genes/TFs/pathways/tissues and potential therapeutic targets.

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Contributors KK and S-CB designed the study. EH and KK performed all data analyses. All authors interpreted the results. All authors wrote, reviewed and approved the manuscript.

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EPIDEMIOLOGICAL SCIENCE

Disease activity, cytokines, chemokines and the risk of incident diabetes in rheumatoid arthritis

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ABSTRACT

Purpose Rheumatoid arthritis (RA) is associated with a higher risk of diabetes mellitus (DM). Our aim was to determine associations between inflammatory disease activity (including evaluation of specific cytokines and chemokines) and incident DM.

Methods Participants were adults with physician-confirmed RA from Veteran's Affairs Rheumatoid Arthritis Registry. Disease activity and clinical assessments occur longitudinally as part of clinical care. Thirty cytokines and chemokines were measured in banked serum obtained at the time of enrolment. Cytokine/chemokine values were log-adjusted and standardised (per SD). Incident DM was defined based on validated algorithms using diagnostic codes and medications. Multivariable Cox proportional hazard models evaluated associations between clinical factors and incident DM. Independent associations between cytokines/chemokines and incident DM were assessed adjusting for age, sex, race, smoking, body mass index (BMI) and medication use at baseline.

Results Among 1866 patients with RA without prevalent DM at enrolment, there were 130 incident cases over 9223 person-years of follow-up. High Disease Activity Score (DAS28)-C reactive protein (CRP), obese BMI, older age and male sex were associated with greater risk for incident DM while current smoking and methotrexate use were protective. Patients using methotrexate were at lower risk. Several cytokines/chemokines evaluated were independently associated (per 1 SD) with DM incidence including interleukin(IL)-1, IL-6 and select macrophage-derived cytokines/chemokines (HR range 1.11–1.26). These associations were independent of the DAS28-CRP.

Conclusions Higher disease activity and elevated levels of cytokines/chemokines are associated with a higher risk of incident DM in patients with RA. Future study may help to determine if targeted treatments in at-risk individuals could prevent the development of DM.

Key messages

What is already known about this subject?
- Some studies suggested a greater risk of diabetes in patients with rheumatoid arthritis and some suggest that certain therapies may reduce that risk.

What does this study add?
- In this study, elevated disease activity was associated with a greater risk of diabetes.
- Elevations in inflammatory cytokines and chemokines were also associated with a greater risk of diabetes.

How might this impact on clinical practice or future developments?
- These data support closer attention to the risk of diabetes among patients with elevated disease activity and may support more aggressive treatment to reduce the risk.

INTRODUCTION

Patients with rheumatoid arthritis (RA) may be at greater risk of developing diabetes mellitus (DM), though studies are conflicted.[1–5] This increase in risk is important since it might contribute to the observed higher risk of cardiovascular disease and premature mortality in this population.[6] Some evidence suggests that systemic inflammation might directly lead to insulin resistance and poor insulin production by interfering with cellular functions in the pancreas, liver and skeletal muscle.[7 8] Systemic inflammation has been considered to be a potential risk factor and has been associated with incident diabetes in the general population.[9]

Despite an emerging awareness of a potential link between inflammation and insulin resistance, few studies have evaluated the relationship between disease activity in patients with inflammatory diseases such as RA and the subsequent risk of DM. Some studies have demonstrated that certain treatments are associated with a lower risk of DM in patients with RA and psoriatic arthritis, perhaps suggesting a benefit of superior disease control, but these studies did not directly assess the effect of disease activity itself.[10 11]

Furthermore, a few studies have evaluated whether there are specific circulating inflammatory mediators that correlate more closely with the risk of diabetes in this population. In the general population, several cytokines and chemokines have been implicated in the development of DM including interleukin(IL)−1, IL-6, tumour necrosis factor (TNF)-α, IL-8, IL-18 and macrophage chemoattractant protein-1 (MCP-1).[12–14] The identification of specific circulating cytokines or chemokines that are implicated in the risk of DM would have potential implications for predicting long-term risk and possibly guiding the choice of targeted therapies in patients with RA who are at risk for diabetes.[15]

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Our specific aims were to evaluate associations between clinical disease activity and inflammatory markers, including individual cytokines/chemokines, with incident diabetes in a population of patients with RA. We hypothesised that inflammation related to RA disease activity would be associated with a higher risk of incident DM independent of other clinical factors. We also hypothesised that proinflammatory cytokines and chemokines might contribute to this increase in risk beyond traditional RA disease activity assessment.

METHODS

Study setting

The Veterans' Affairs Rheumatoid Arthritis (VARA) study is an ongoing national repository and multicentre RA registry that has been active for more than 17 years (initiated 2003).[16–23] At the time this study was conducted, 13 VA sites had contributed data. Veterans with RA are identified during routine care by the treating rheumatologist at individual sites and consented for enrolment. All Veterans who fulfill the 1987 American College of Rheumatology classification criteria for RA and are over 18 years of age are eligible.[24] Physician investigators at each site record clinical data at enrolment and at routine follow-up visits as part of normal clinical care. All study patients provided informed written consent. Patients and the public were not involved in the design of the VARA registry or the current study.

RA disease activity

The results of clinical testing of C reactive protein (CRP, mg/dL), clinical joint counts (0–28) and patient/physician global scores were extracted from the registry and from the VA electronic medical record by querying data in the Corporate Data Warehouse (CDW). Our primary disease activity measure was the Disease Activity Score in 28 Joints with the CRP (DAS28(CRP)) and was categorised as remission (<2.6); low disease activity (2.61–3.2); moderate activity (3.21–5.09) and high activity (≥5.1).[25] Missing components for the DAS28(CRP) were imputed by carrying forward from the prior visit.

Serologies, inflammatory markers, cytokine and chemokine assays

Cytokine and chemokines levels were determined by the V-PLEX multiplex panel from Meso Scale Discovery (Rockville, Massachusetts USA). These analytes were measured from serum obtained at the time of registry enrolment, the only time point for which samples are routinely banked for these study participants. Following sample collection, specimens were processed and stored at −70°C until time of measurement. Thirty analytes were examined at enrolment: IL-1α, IL-1β, IL-1 receptor antagonist (RA), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-16, IL-17α, IL-23, IL-27, interferon (IFN)-γ, granulocyte-macrophage colony-stimulating factor, macrophage-derived chemokine (MDC), MCP-1, MCP-4, macrophage inflammatory protein (MIP)−1α, MIP-1β, MIP-3α, Vascular Endothelial Growth Factor, eotaxin and TNF-α. Assays were performed as per manufacture protocols and analysed on the MESO QuickPlex SQ 120 imager (Meso Scale Discovery). From banked serum, a second-generation commercial anti-cyclic citrullinated peptide antibody and high sensitivity CRP (hsCRP) were also measured from banked enrolment serum as previously reported.[26]

DM outcome definition

We identified incident DM by querying inpatient and outpatient diagnoses as well as antidiabetic medications within the VA CDW. Incident diabetes was defined as fulfilling one of the following: (1) two or more outpatient diagnosis codes (International Classification of Diseases (ICD)−9 clinical modification (CM) or ICD-10-CM (online supplemental table 1), (2) one or more discharge diagnosis codes or (3) one outpatient diagnosis code and one or more antidiabetic medication (VA or non-VA medications). Similar approaches have demonstrated excellent sensitivity and specificity (83.2%/99.2%) as well as high positive and negative predictive value in administrative data (92.5%/98.1%).[27] Any patient with a medication or diagnosis code occurring within 180 days after enrolment in the registry was considered a prevalent case.

Covariables

Demographics and disease-specific characteristics at baseline and during follow-up were obtained from the VARA registry database. Current smoking was considered time-invariant (presence or absence of the exposure reported at baseline). Body mass index (BMI) was extracted from the vital sign packages available in the CDW and the closest BMI value (within 30 days) to the visit date was used. Observations with missing BMI data were imputed by carrying forward from the prior observation. BMI categories were defined as (underweight, <20 kg/m^2; normal weight, ≥20–25 kg/m^2; overweight, ≥25–30 kg/m^2; obese, ≥30–35 kg/m^2 and severely obese (≥35 kg/m^2).

RA treatments were extracted from VA pharmacy databases. Each prescription fill of a drug was defined as a dispensing episode.[19] For each episode, the amount of the drug dispensed and the expected duration of the treatment episode were determined. The expected days of supply were determined based on the dosing instructions. A drug course was defined as a period of continuous treatment consisting of one or more dispensing episodes without a gap of ≥90 days between the expected end of the days of supply for that episode and the start of the subsequent dispensing episode. Participants were considered exposed to the therapy if the current visit occurred during a defined medication course. Active glucocorticoid use was physician-reported and extracted from the registry database.

Statistical analysis

Characteristics of the study population were described among participants with prevalent and non-prevalent DM at enrolment. The primary analyses used Cox proportional hazards models to assess associations between baseline characteristics and the time to the development of incident DM among participants without prevalent DM at baseline, clustering on study site. Secondary analyses also incorporated time-varying assessments of disease activity, BMI and RA treatments (including glucocorticoids). Time-varying models provide an opportunity to assess the association with the most recently collected measure of the exposure rather than focusing on the baseline assessment. We focused on methotrexate, hydroxychloroquine, TNF-inhibitors and abatacept as potential confounders given prior data demonstrating potential reductions in risk with these therapies.[11] Other hypothesised confounders included demographics, smoking, BMI, disease duration, anti-citrullinated peptide antibody (ACPA) status (positive vs negative) and calendar year.

Cytokines and chemokines were log-adjusted to approximate a normal distribution and standardised so that a 1-unit difference in the value represented a 1 SD difference for all individual

analytes. Separate multivariable Cox proportional hazards models evaluated associations between each individual cytokine/chemokine and the time to the development of DM. Each of these models was adjusted for the factors defined above. Primary models did not adjust for clinical disease activity, however, we also explored models adjusting for disease activity. We performed Simes-Benjamini-Hochberg adjustment for multiple comparisons and noted cytokines and chemokines that remained significant after adjustment ($p<0.008$). We also assessed the improvement in model fit with the inclusion of key cytokines that remained significant after adjustment for multiple comparisons.

RESULTS

A total of 2541 registry participants were evaluated at baseline. Of these, 2341 had cytokines and chemokine data available and 667 (26%) had prevalent DM. The enrolment characteristics of participants with and without prevalent DM are shown in table 1. Participants with diabetes were older, were less likely to be female, had higher BMI and had higher DAS28(CRP). Diabetics had lower levels of IL-4, IL-12, TNF-α and MCP-1 and higher levels of IL-1RA and IL-17α. There were no other significant differences in circulating cytokines or chemokines between diabetics and non-diabetics at enrollment.

There were 1866 participants without DM at baseline that were included in longitudinal analyses. There were 130 RA patients who developed incident diabetes over 9223 person-years of follow-up, a rate of 1.41 cases per 100 person-years. Among those that developed diabetes, the median time to DM diagnosis was 4.7 (3.3) years. Higher disease activity was independently associated with a greater risk of DM in a dose-dependent manner, with high disease activity at baseline being associated with a significant increase in risk compared with those in remission (HR: 2.07 (95% CI 1.34 to 2.85) $p<0.001$) after adjustment for confounders (figure 1, table 2). A test for trend was significant ($p<0.001$).

In models including time-varying measures of disease activity and covariates (including glucocorticoids), higher disease activity was again observed to be independently associated with a higher risk of DM (test for trend: $p<0.001$). Specifically, in time-varying models, moderate disease activity was associated with a 58% higher risk (HR 1.58 (95% CI 1.26 to 1.90) $p<0.001$). High disease activity was not significantly associated, though with low precision due to a low number of observations (HR 1.52 (95% CI 0.77 to 2.99) $p=0.23$). The average DAS28(CRP) over all prior observations was also associated with incident DM (HR (per one unit): 1.16 (95% CI 1.03 to 1.30) $p=0.02$). Low disease activity on average over all prior observations was not associated with a higher risk compared with remission (HR 1.00 (95% CI 0.75 to 1.34) $p=0.99$). In models incorporating all components of time-varying disease activity separately (swollen joint count, tender joint count, patient global score and CRP), only CRP was independently associated with the risk of DM (HR (per 1 mg/dL): 1.05 (95% CI 1.00 to 1.09) $p=0.03$ (full model not shown).

Prior to imputation for longitudinal measures, BMI was missing in 12% of observations and DAS28(CRP) was missing in 23%. In regression models that used only unimputed data, results were highly similar to the primary analysis, though high disease activity was significantly associated with the risk of DM (HR 1.97 (95% CI 1.01 to 3.87) $p=0.04$) (full model not shown).

Methotrexate use at baseline and as a time-varying covariate was associated with a lower risk of DM in these models. Baseline and time-varying prednisone use was not significantly associated with a higher risk of incident DM independent of measures of disease activity, BMI and other factors (table 2). Hydroxychloroquine and TNFi were not associated with diabetes independent

Table 1 Enrolment characteristics of those with prevalent diabetes compared with those without prevalent diabetes at baseline in the VARA registry

	Diabetes	No diabetes	P value
N (%)	667 (26)	1874 (74%)	
Age (years)	73.0 (8.6)	69.8 (11.1)	<0.001
Female, N (%)	51 (8)	227 (12%)	0.002
White, N (%)	486 (73)	1428 (76%)	0.09
Current Smoking, N (%)	124 (19)	505 (27%)	<0.001
BMI (kg/m^2) (n=1787)	30.5 (6.1)	28.2 (5.3)	<0.001
DAS28 (n=1611)	3.81 (1.53)	3.53 (1.43)	<0.001
ACPA positive, N (%)	500 (75)	1424 (76%)	0.25
Disease duration (years)	10.4 (11.8)	10.4 (11.0)	0.96
hsCRP, mg/dL	0.63 (0.22 to 1.47)	0.53 (0.2 to 1.3)	0.10
Methotrexate, N (%)	345 (52)	892 (48%)	0.07
Glucocorticoids, N (%)	238 (36)	664 (35%)	0.91
TNFi Therapy, N (%)	137 (21)	452 (24%)	0.06
Enrolled <2010, N (%)	344 (59)	981 (52%)	0.73
Cytokines and chemokines (n=613/1728) (by SD score)			
IL-1ß	−0.028 (1.02)	0.001 (1.00)	0.55
IL-1 RA	0.071 (1.02)	−0.059 (0.96)	0.005
IL-1α	−0.020 (0.99)	0.010 (1.02)	0.52
IL-2	−0.004 (0.98)	0.027 (0.99)	0.50
IL-3	−0.023 (0.99)	−0.007 (1.01)	0.73
IL-4	−0.050 (1.07)	0.049 (0.97)	0.03
IL-5	0.028 (1.01)	0.028 (0.98)	1.00
IL-6	0.024 (1.00)	−0.004 (1.01)	0.55
IL-7	−0.051 (1.03)	0.012 (1.00)	0.18
IL-8	−0.013 (1.01)	−0.021 (1.00)	0.86
IL-9	−0.048 (1.11)	0.027 (0.97)	0.11
IL-10	0.022 (1.00)	0.015 (1.01)	0.18
IL-12 (p70)	−0.048 (1.01)	0.015 (0.99)	0.04
IL-13	−0.015 (1.00)	−0.003 (1.01)	0.80
IL-15	0.045 (0.99)	−0.037 (0.99)	0.08
IL-16	−0.098 (1.11)	0.023 (0.95)	0.01
IL-17α	0.066 (0.98)	−0.059 (1.00)	0.008
IL-23	0.017 (1.00)	0.008 (1.02)	0.84
IL-27	−0.058 (1.08)	0.024 (0.95)	0.08
TNF-α	−0.11 (1.05)	0.016 (0.99)	0.009
MDC	−0.055 (1.04)	0.006 (1.00)	0.19
MCP-1	−0.021 (1.01)	0.005 (1.00)	0.58
MCP-4	−0.054 (1.05)	0.017 (0.98)	0.13
MIP-1α	−0.031 (1.03)	0.022 (1.02)	0.27
MIP-1ß	−0.048 (1.07)	−0.001 (0.99)	0.32
MIP-3α	0.061 (1.10)	−0.032 (0.98)	0.051
Eotaxin	0.021 (1.00)	−0.004 (0.99)	0.61
GMCSF	−0.036 (1.02)	0.026 (1.00)	0.20
IFN-γ	−0.022 (1.00)	0.025 (0.99)	0.33
VEGF	−0.037 (1.04)	0.010 (0.99)	0.31

Data are presented as mean (SD) or median (IQR) for skewed data.
ACPA, anti-citrullinated peptide antibody; BMI, body mass index; DAS, Disease Activity Score; GMCSF, granulocyte-macrophage colony-stimulating factor; hsCRP, high sensitivity C reactive protein; IL, interleukin; MCP, macrophage chemoattractant protein; MIP, macrophage inflammatory protein; RA, rheumatoid arthritis; TNF, tumour necrosis factor; VARA, Veterans' Affairs Rheumatoid Arthritis; VEGF, vascular endothelial growth factor.

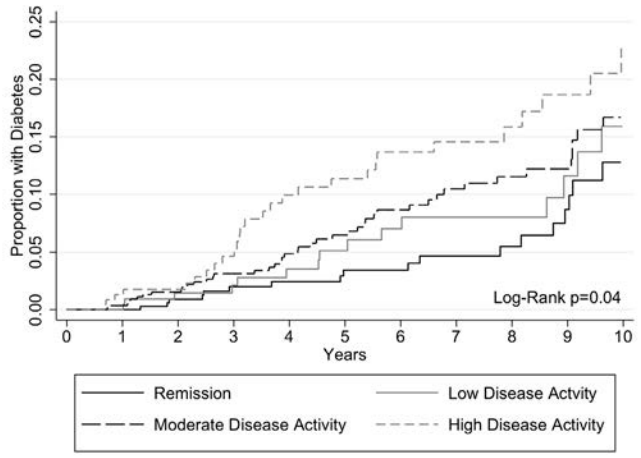

Figure 1 Proportion of remaining subjects with diabetes by baseline disease activity category (unadjusted).

of other factors. Female sex and current smoking were also associated with a substantially lower risk of DM. Higher BMI was the strongest risk factor for DM in these models, with severely obese participants at baseline having a sevenfold higher risk compared with those in the normal weight category (HR 6.80 (95% CI 3.27 to 14.10) p<0.001) (table 2).

There was substantial intercorrelation between cytokines and chemokines studied (online supplemental table 2). Levels of a number of specific individual cytokines and chemokines were associated with a higher risk of DM (figure 2) after adjustment for multiple potential confounders. Of the cytokines and chemokines analysed in this study, IL-1 RA, IL-1α, IL-1β, IL-4, IL-6, IL-12p70, IL-15, MDC, MCP-1, MCP-4, MIP-1β, MIP-3α and eotaxin were each significantly associated with the risk of DM (HR per SD range: 1.11–1.26; p<0.05) (figure 2). These associations were independent of clinical disease activity (online supplemental figure 1). Associations with IL-6 were not attenuated after excluding two participants receiving anti-IL-6 therapies at baseline. TNF-α was associated with the development of DM only after adjusting for DAS28(CRP) (HR (per 1 SD): 1.07 (95% CI 1.01 to 1.15) p=0.03). The effect was numerically similar but not statistically significant when excluding TNF-inhibitor treated patients at baseline ((N=1348) HR 1.06 (0.90, 1.24) p=0.50). hsCRP measured at enrolment was not significantly associated with the risk of DM.

After inclusion of all cytokines/chemokines separately associated with DM (after accounting for multiple comparisons), only IL-6 and IL-1α remained independently associated with DM (p<0.05; full model not shown). Inclusion of IL-6 and IL-1α in the model improved model fit compared with model 1, table 2 (p=0.03).

DISCUSSION

This cohort study demonstrates strong associations between clinical disease activity and the risk of incident DM in patients

Table 2 Multivariable associations between disease activity and other clinical factors and the risk of incident diabetes among participants without diabetes at enrolment (all variables included in a single model)

	Model 1: Baseline predictors N=1866 P-Y=9223; events: 130		Model 2: Time-varying exposures N=1866 P-Y=9223; events: 130	
	HR (95% CI)	P value	HR (95% CI)	P value
Age (per 1 year)	1.01 (1.00 to 1.02)	0.056	1.01 (1.00 to 1.03)	0.07
Female	0.25 (0.12 to 0.52)	<0.001	0.25 (0.12 to 0.52)	<0.001
White	1.01 (0.64 to 1.63)	0.83	1.06 (0.79 to 1.44)	0.66
Current smoking	0.64 (0.50 to 0.82)	<0.001	0.63 (0.45 to 0.87)	0.006
Former smoking	0.70 (0.49 to 1.00)	0.05	0.69 (0.41 to 1.41)	0.15
BMI category				
<20 kg/m^2	1.00 (0.12 to 8.19)	1.00	0.96 (0.39 to 3.27)	0.93
20–25 kg/m^2	(reference)	—	(reference)	—
25–30 kg/m^2	3.60 (1.84 to 7.03)	<0.001	2.64 (1.72 to 4.06)	<0.001
30–35 kg/m^2	4.39 (2.69 to 7.29)	<0.001	4.33 (2.54 to 7.39)	<0.001
>35 kg/m^2	6.82 (3.26 to 14.28)	<0.001	6.55 (4.40 to 9.76)	<0.001
DAS28(CRP)				
Remission	(reference)	—	(reference)	—
Low	1.10 (0.53 to 2.48)	0.81	1.29 (0.49 to 3.40)	0.61
Moderate	1.50 (0.89 to 2.34)	0.14	1.58 (1.26 to 1.90)	<0.001
High	2.12 (1.45 to 3.10)	<0.001	1.52 (0.77 to 3.01)	0.23
Methotrexate	0.68 (0.50 to 0.81)	0.006	0.68 (0.50 to 0.93)	0.02
Hydroxychloroquine	1.07 (0.57 to 2.02)	0.84	0.89 (0.65 to 1.21)	0.45
TNFi	1.47 (0.83 to 2.60)	0.17	1.01 (0.75 to 1.36)	0.94
Abatacept	—	—	0.81 (0.17 to 3.82)	0.79
Prednisone	1.05 (0.87 to 1.26)	0.58	1.25 (0.88 to 1.80)	0.22

Disease activity, BMI and therapies evaluated as time-varying covariates in model 2. Abatacept was not included in model 1 due to insufficient numbers of patients on this medication at enrolment.
Model 2: Time-varying exposures include BMI, DAS28(CRP), methotrexate, TNFi, prednisone and hydroxychloroquine.
Considered but not included in the final models: disease duration, ACPA serostatus, geographic region, calendar year, number of prior biologics.
. BMI, body mass index; CRP, C reactive protein; DAS, Disease Activity Score; P-Y, person years; TNFi, tumour necrosis factor inhibitor.

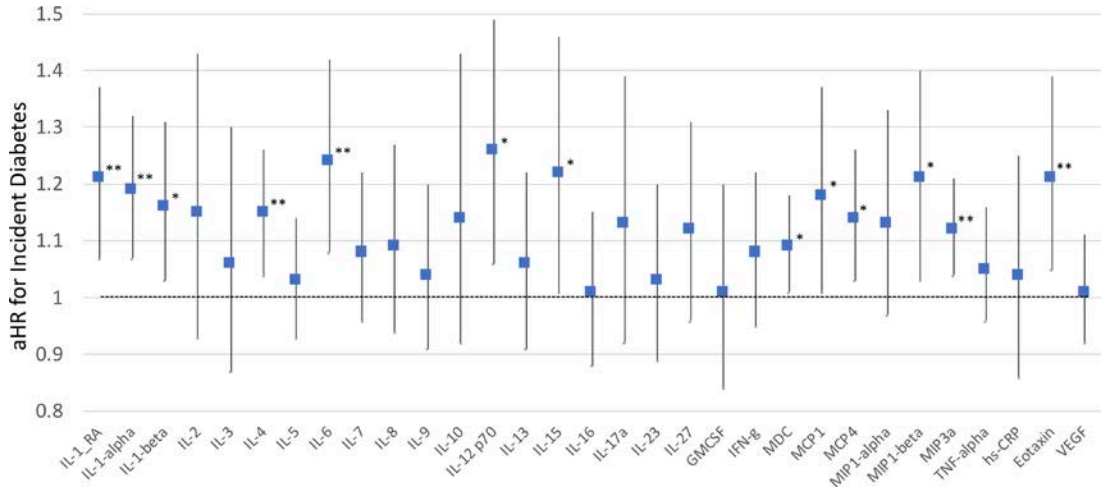

Figure 2 Adjusted HRs (aHR) for the risk of incident diabetes among patients with rheumatoid arthritis and no history of diabetes (n=1635, events=119) by individual cytokine concentrations (per 1 SD) measured on banked serum from enrolment. Each point estimate and CI shown represents results of the individual analyte examined in a separate multivariable model adjusting for age, sex, race, current smoking, baseline body mass index and baseline use of methotrexate, TNF-biologics and prednisone. *P<0.05, **p<0.008 (Simes-Benjamini-Hochberg adjustment for multiple comparisons). IL, interleukin; IFN, interferon; GMCSF, granulocyte macrophage colony stimulating factor; hs-CRP, high-sensitivity C reactive protein; MCP, macrophage chemoattractant protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

with RA. This association does not appear to be explained by differences in age, BMI or the use of RA treatments, including glucocorticoids. Overall, the findings support the hypothesis that systemic inflammation promotes insulin resistance in patients with RA and supports efforts to prevent diabetes through optimal control of disease activity. Further evidence of a relationship between inflammation and DM is the associations observed between several circulating proinflammatory cytokines and chemokines and the development of DM in this population. These associations might suggest that preventative approaches targeting these pathways could be of value or that measurement of inflammatory mediators may help predict populations at risk for this condition.

While evidence has suggested that RA is associated with a higher risk of DM[3–5] and that therapies for the disease appear to reduce that risk,[10] this study is among the first to demonstrate an important impact of the clinical disease activity itself. The immediate clinical implication of this work is the support of efforts to optimally control disease activity in order to reduce a patient's risk of DM. The observations from this study also provide insight into understanding the relationship between inflammation and the development of DM in RA and in other populations. While moderate and high disease activity were associated with an increased risk, we found no clear evidence that patients with low disease activity were at greater risk compared with those that were in remission. Thus, these data do not support a more aggressive approach to treatment beyond achievement of low disease activity in order to lower the risk of DM, though a small benefit of a more aggressive treatment target is not ruled out.

This study identified several cytokines and chemokines associated with DM risk of potential interest including IL-1α, IL-1β and IL-1β RA. IL-1β has been shown to be associated with DM in the general population and has been considered a potential target of therapy in DM.[13] However, a recent large randomised trial performed in the general population, did not demonstrate a reduced risk of DM among participants receiving canakinumab, an anti-IL-1β therapy.[28] Thus, while the current study supports upregulation of IL-1 and its RA as an important marker of DM risk in this population, it is not sufficient to support the use of anti-IL-1β therapy with the goal of preventing this outcome.

IL-6 was also demonstrated to be independently associated with incident diabetes as has been shown in other populations.[13] It has been proposed that obesity-related inflammation may result in high levels of IL-6.[29] In our study, elevated IL-6 levels were associated with DM independent of BMI, but we were not able to directly assess fat in the visceral compartment. Whether obesity-related inflammation is a primary driver of the metabolic complications of obesity or simply a marker of the severity of visceral adiposity is not fully elucidated. Further study with longitudinal IL-6 measurement is needed in order to determine if IL-6 is in the causal pathway leading from obesity to diabetes. It therefore remains unknown whether blocking IL-6 might prevent the onset of DM in the general population and in patients with RA.

Several MDCs were identified in this study that were associated with the risk of DM. MCP-1 is a chemokine that recruits monocytes and other inflammatory cells to the site of inflammation in response to inflammatory stimuli. MCP-1 may be produced in visceral fat and higher levels have been linked to a greater risk of DM in other settings.[14 30] Gene polymorphisms resulting in lower levels appear protective, supporting MCP-1 as a potential target of intervention.[31 32] Prior studies in the general population have similarly demonstrated expression of MIP-1β in visceral fat of diabetic patients.[33 34] It remains unclear if elevations in MDCs are a consequence of RA-related inflammation or reflect inflammation related to obesity and visceral adiposity.

In this study, prednisone use was not significantly associated with a higher risk of DM independent of the effect of disease activity. The effect of prednisone on the risk of DM is well known. It is possible that the tendency for providers to use low-dose prednisone in patients with RA and to avoid its use in obese patients and in patients with pre-diabetes explains why there appears to be no significant increase in risk. However, the current study is limited in the accurate assessment of dose to further delineate 'safe' doses of prednisone. Furthermore, the tendency for providers to use of prednisone in patients with the

most active disease and those with contraindications to other therapies suggests that confounding may limit our ability to make causal inferences in this study that was not aimed at this question. Non-differential misclassification of prednisone use is also possible, given the reliance on physician and this may have biased results to the null.

Methotrexate use was associated with a lower risk of DM. It is tempting to attribute this association to causal reduction in risk that is perhaps independent of the effects of the drug on measures of disease activity. Few clinical studies have addressed this question and have yielded conflicting results.[35 36] Despite a desire to attribute causal benefit, there may be residual confounding at play given that patients with hepatic steatosis may simultaneously be both less likely to use methotrexate and also are at higher risk of developing DM. Further study is needed to determine if there are drug-specific benefits of methotrexate or whether this noted association is the result of channelling. We did not identify significant associations with other RA therapies such as hydroxychloroquine (HCQ), TNFi or abatacept independent of other factors such as disease activity, though the study was not designed with the primary aim of characterising DM risk with these therapies.

Further study with alternative designs is needed to better determine if particular therapies might have a greater impact on reducing the risk of DM. The nature of the registry does not provide an opportunity to study regional changes in adiposity, such as visceral deposition, nor behavioural risk factors such as diet and physical activity, which may play a role in understanding the risk of DM in RA. The size of this study limits the ability to identify weaker relationships between specific cytokines and chemokines with a relatively uncommon outcome. In addition, the observation of strong interanalyte correlation limits our ability to identify key cytokines that may play a pathogenic role. Furthermore, cytokines and chemokines were only available at enrolment and the impact of changes in these measures over time was not possible to assess. We also present results of multiple comparisons and some significant findings may have occurred by chance. While we found no significant differences between men and women in this study, the VARA registry, which is predominantly male, may not be entirely generalisable to other populations with RA. Finally, misclassification between type 1 and type 2 DM, though in this age group the incidence of type 1 DM is expected to be quite rare. Notable strengths of the study include the long-term follow-up of patients with rheumatologist-diagnosed RA, the availability of longitudinal clinical assessments, and the direct assessment of inflammatory cytokines and chemokines.

In conclusion, greater disease activity is associated with a greater risk of DM among patients with RA. Circulating levels of cytokines and chemokines are independently associated with the development of DM supporting the hypothesis that inflammation is an important factor in risk. These data may also support the targeting of specific inflammatory pathways for intervention, though further study is needed. Better control of disease activity and related systemic inflammation may help to reduce the long-term risks of metabolic complications of RA such as the observed increase in risk of DM.

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TRANSLATIONAL SCIENCE

Streptococcus species enriched in the oral cavity of patients with RA are a source of peptidoglycan-polysaccharide polymers that can induce arthritis in mice

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ABSTRACT

Objectives Analysis of oral dysbiosis in individuals sharing genetic and environmental risk factors with rheumatoid arthritis (RA) patients may illuminate how microbiota contribute to disease susceptibility. We studied the oral microbiota in a prospective cohort of patients with RA, first-degree relatives (FDR) and healthy controls (HC), then genomically and functionally characterised streptococcal species from each group to understand their potential contribution to RA development.

Methods After DNA extraction from tongue swabs, targeted 16S rRNA gene sequencing and statistical analysis, we defined a microbial dysbiosis score based on an operational taxonomic unit signature of disease. After selective culture from swabs, we identified streptococci by sequencing. We examined the ability of streptococcal cell walls (SCW) from isolates to induce cytokines from splenocytes and arthritis in ZAP-70-mutant SKG mice.

Results RA and FDR were more likely to have periodontitis symptoms. An oral microbial dysbiosis score discriminated RA and HC subjects and predicted similarity of FDR to RA. *Streptococcaceae* were major contributors to the score. We identified 10 out of 15 streptococcal isolates as *S. parasalivarius* sp. nov., a distinct sister species to *S. salivarius*. Tumour necrosis factor and interleukin 6 production in vitro differed in response to individual *S. parasalivarius* isolates, suggesting strain specific effects on innate immunity. Cytokine secretion was associated with the presence of proteins potentially involved in *S. parasalivarius* SCW synthesis. Systemic administration of SCW from RA and HC-associated *S. parasalivarius* strains induced similar chronic arthritis.

Conclusions Dysbiosis-associated periodontal inflammation and barrier dysfunction may permit arthritogenic insoluble pro-inflammatory pathogen-associated molecules, like SCW, to reach synovial tissue.

Key messages

What is already known about this subject?

- The prevalence of periodontitis is significantly increased in patients with rheumatoid arthritis (RA), and its severity is associated with RA severity.
- Periodontitis is driven by pathobiont periodontal bacteria derived from the oral microbiota. Susceptibility to periodontal infections with pathobiont strains, such as *Porphyromonas gingivalis*, is supported by streptococcal-rich plaques.
- Inflammatory arthritis in mice and rats can be induced by non-viable fragments of streptococcal cell walls (SCW), which is poorly degraded, pro-inflammatory and persistent.
- Microbial remnants have been identified in synovia of patients with RA and in rodent models of arthritis, induced after microbial infection.

What does this study add?

- RA and first-degree relatives were more likely to have periodontitis symptoms, and were discriminated from healthy controls (HC) by an oral microbial dysbiosis score.
- Members of the bacterial family *Streptococcaceae* were major contributors to the score.
- We propose a novel streptococcal species *S. parasalivarius* sp. nov., with strain-level genetic variation.
- SCW from RA and HC-associated *S. parasalivarius* strains induced similar mild chronic arthritis in mice.

How might this impact on clinical practice or future developments?

- Barrier dysfunction—associated with poor dental health, smoking and poor nutrition—may facilitate spread of insoluble microbial remnants to the synovium. Controlling mucosal inflammation and barrier dysfunction in genetically predisposed individuals may reduce dysbiosis and propensity for arthritis development.

INTRODUCTION

The aetiopathogenesis of rheumatoid arthritis (RA) interlinks female sex, age, genetic predisposition and environmental factors. Contributory environmental risks including smoking, particulate exposure, periodontitis and bronchiectasis implicate mucosal microbe/host immune interactions in disease development.[1 2] The gut and oral resident

microbial communities, or microbiota, maintain homoeostasis by promoting immune cell function, tolerance and barrier functions. These include production of mucus, IgA and antimicrobial peptides.[3] Barrier protective mechanisms likely dictate the microbiota composition by supporting the environment of commensals in health or disrupting barrier function with pathobiont outgrowth in disease. Shifts in microbiota, termed dysbiosis, are associated with disease susceptibility. Dysbiosis of faecal and oral microbiota, and increased abundance of oral-resident bacteria in the faecal microbiome in patients with RA suggest that differential abundance of particular taxonomic groups may contribute to RA development or control.[4–8] Pathogen-associated molecular patterns (PAMPs) associated with pathobionts, which thrive or outgrow in dysbiotic mucosal environments, could activate the innate immune system in genetically-susceptible individuals, such as patients with or at risk of RA.[3] Microbial DNA remnants identifiable in the synovial fluid, membranes and serum of patients with RA may contribute PAMPs, as demonstrated in experimental models of arthritis induced after microbial infection.[9–11] However, the contribution of such potentially inflammatory microbial products to disease development or perpetuation is not fully understood.

The prevalence of periodontitis is significantly increased in patients with RA, and its severity is associated with RA severity.[12] Periodontitis is driven by pathobiont periodontal bacteria derived from the oral microbiota, including *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*.[13] *P. gingivalis* may function as a 'keystone pathogen', central to the transition to a dysbiotic community by promoting growth of and communicating with accessory bacteria, such as *Streptococcus gordonii*.[14] *A. actinomycetemcomitans* may also function as a keystone pathogen, where its combination with *Filifactor alocis* and *Streptococcus parasanguinis* is associated with localised aggressive periodontitis.[15] While *P. gingivalis* may contribute to the aetiology of RA,[16 17] the evidence is still associative. For example, *P. gingivalis* antibody titres correlated with RA disease activity,[18] treatment of periodontitis reduced RA severity and disease-modifying anti-rheumatic drugs (DMARDs) simultaneously reduced RA disease activity, *P. gingivalis* antibody titres and periodontitis.[18 19] However, a prospective cohort failed to show that subjects with periodontal disease had an increased risk of later RA.[20] *P. gingivalis* cell-associated DNA was more likely to be detected in synovial fluid of patients with RA than in healthy controls.[21] These data suggest that while not causative, periodontitis-derived bacteria or debris reaching the blood may enhance inflammatory responses and autoantibody generation by spreading to synovial inflammatory tissue.[22]

Streptococci are a major group of early colonisers of dental plaque, making up about 80% of early biofilm constituents.[23 24] *S. sanguinis* is a pioneer organism that adheres to the tooth enamel through specific surface adhesins, allowing later more virulent colonisers, such as *S. mutans* to build a stratified, complex biofilm.[23] Such biofilms, coupled with poor oral hygiene, permit expansion of opportunistic pathogenic bacteria, thus increasing susceptibility to periodontal infections like *P. gingivalis*, supported by streptococcal-rich plaques.[25] Increased and decreased abundance of *Streptococcus* spp have been reported within the oral microbiome of patients with RA.[26–28] *Streptococcus* was observed at increased abundance in the gut of patients with RA and genetically at-risk individuals prior to disease onset.[29–31] Non-viable fragments of streptococcal cell wall (SCW) peptidoglycan-polysaccharide (PG-PS) induce inflammatory arthritis in certain strains of mice and rats after systemic administration. Notably, PG-PS is poorly degraded, pro-inflammatory and persistent in tissues.[32–34] Therefore, further exploration of the abundance of streptococci in RA is of clinical interest both for their potential role in the development of periodontitis and as a source of inflammation.

The tongue has the most stable, diverse and dense bacterial biofilm of the oral cavity.[35] To evaluate oral dysbiosis of the tongue biofilm and potential contributors supporting periodontal disease in patients with RA and genetically and environmentally at-risk individuals, we studied oral microbiota derived from tongue swabs in patients with RA, first-degree relatives (FDR) of RA probands and individuals without RA matched for age and sex (healthy controls, HC). We then employed a susceptible mouse strain to discern how systemic spread of pathobiont-derived PAMPs might contribute to inflammatory arthritis.

MATERIALS AND METHODS

Cohort and oral swab collection

In a study approved by the University of Queensland, and Gallipoli Human Research Ethics Committees, 116 patients with RA, 63 FDR and 43 HC, predominantly from an Australian urban environment, provided informed consent and clinical, lifestyle and sociodemographic data. Patients with RA and FDR were recruited in the waiting rooms of metropolitan rheumatologists, through community advertising and word of mouth (friends and neighbours). Each individual came to a clinical appointment involving history, questionnaire (online supplemental table 1) and a 68 tender and swollen joint count. Adult patients with RA were included if they either met American College of Rheumatology (ACR) 2010 classification criteria or had a confirmed diagnosis by a rheumatologist. FDR included parents, full siblings or offspring of patients with RA and were clinically ascertained as non-RA. HC were community volunteers without a family history of RA, but sometimes had other diseases. Two oral swabs taken from the centre of the tongue of each participant were stored at −80°C within 3 hours.

SCW arthritis induction in SKG mice

Female ZAP70^{W163C} BALB/c (SKG) mice aged 7 to 16 weeks were maintained under specific pathogen free conditions at Translational Research Institute. The University of Queensland animal ethics committee approved all experiments. Female SKG mice (n=6 per group) were injected intraperitoneally two times (days 0 and 22) with either 15 µg/gr body weight 10S PG-PS or SCW from axenic cultures, no treatment control or once with 3 mg curdlan. Joints were scored visually by an independent technician.[36] Decalcified ankle joints were stained with H&E for histological scoring.[37]

Additional methods for DNA extraction, amplicon sequencing, sequence processing and statistical analysis, axenic cultures, *Streptococcus* genus verification and characterisation, SCW isolation and responses in vitro, are provided in the online supplemental material.

RESULTS

The oral (tongue) microbiome differs in patients with RA and healthy controls

Two hundred and twenty-two individuals, comprising 116 treated, established patients with RA, 63 FDR and 43 HC subjects (table 1) were included. FDR all had a family history of RA. Of the control subjects, 24% had a relative with RA but none was a FDR. While the age of patients with RA and control subjects did not differ, FDR were significantly younger. Sixty-five per cent of patients with RA and 4% of FDR were anti-citrullinated peptide

Table 1 Demographic features of the cohort

Feature	RA (n=116)	FDR (n=63)	HC (n=43)	P value
Age (mean, SD)	53.5 (14)	45.7 (16)	51.2 (15)	0.003
RA duration (median, range)	12 (1 to 46)	0	0	
Female sex (%)	92 (79.3)	42 (66.7)	31 (72.1)	0.169
Family history RA (%)	60 (51.7)	63 (100)	11 (25.5)	**0.015**
Alcohol consumer (%)	75 (64.7)	53 (84.1)	36 (83.7)	**0.003**
Smoking: never (%)	61 (52.6)	43 (68.3)	27 (62.8)	**0.018**
Ex	46 (39.7)	11 (17.5)	15 (34.9)	
Current	8 (6.9)	8 (12.7)	1 (2.3)	
Treatment, cs/b DMARDs (%)	96 (82.8)	0 (0)	2 (4.7)	**<0.0001**
Treatment, prednisone (%)	31 (26.7)	0 (0)	0 (0)	**<0.0001**
Bleeding gums last 5 years (%)	28 (24.1)	18 (28.6)	4 (9.3)	**0.055**
Tooth or gum infection in last 2 years	15 (12.9)	6 (9.5)	2 (4.7)	0.304
Streptococcal or throat infection last 2 years (%)	20 (17.2)	8 (12.7)	4 (9.3)	0.404
Chest infection requiring antibiotics last 2 years (%)	27 (23.3)	16 (25.4)	6 (14.0)	0.341
RF^+ (%)	70 (61.9)	6 (9.5)	2 (4.7)	**<0.0001**
$ACPA^+$ (%)	61 (54)	2 (3)	0 (0)	**<0.0001**
Tender joint count, of 68 (median, range)	0 (0 to 46)	0 (0 to 2)	0 (0)	**<0.0001**
Swollen joint count, of 68 (median, range)	0 (0 to 34)	0 (0 to 1)	0 (0)	**<0.0001**

$ACPA^+$, anti-citrullinated peptide autoantibody; cs/b, conventional synthetic/biological; DMARDs, disease-modifying anti-rheumatic drugs; FDR, first-degree relatives; HC, healthy controls; RA, rheumatoid arthritis; RF^+, rheumatoid factor.

autoantibody $(ACPA)^+$, respectively, while 56% of patients with RA and 12% of FDR were rheumatoid factor $(RF)^+$. Patients with RA were significantly less likely to drink alcohol or to have never smoked. Patients with RA and FDR tended to be more likely to have a history of bleeding gums—a symptom of periodontitis—than HC (p=0.055). Other infections were not more frequent in patients with RA.

After targeted 16S rRNA gene amplicon sequencing of one oral (tongue) swab from each individual, in two batches, preliminary principal component analysis (PCA) and one-way analysis of variance based on the first three components from PCA confirmed the absence of batch effect. We identified a microbial signature discriminating between patients with RA and HC using sPLS-DA (figure 1A) which included representatives of several bacterial families including *Enterobacteriaceae*, *Peptostreptococcaceae*, *Veilonellaceae* and *Streptococcaceae*. The importance and contribution of each operational taxonomic unit (OTU) defining the sPLS-DA component is represented in figure 1B.

The OTUs constituting this signature are combined in a sPLS-DA component and represent a 'microbial dysbiosis score' for each subject. A receiver operating characteristic curve constructed based on the dysbiosis score (AUC (area under

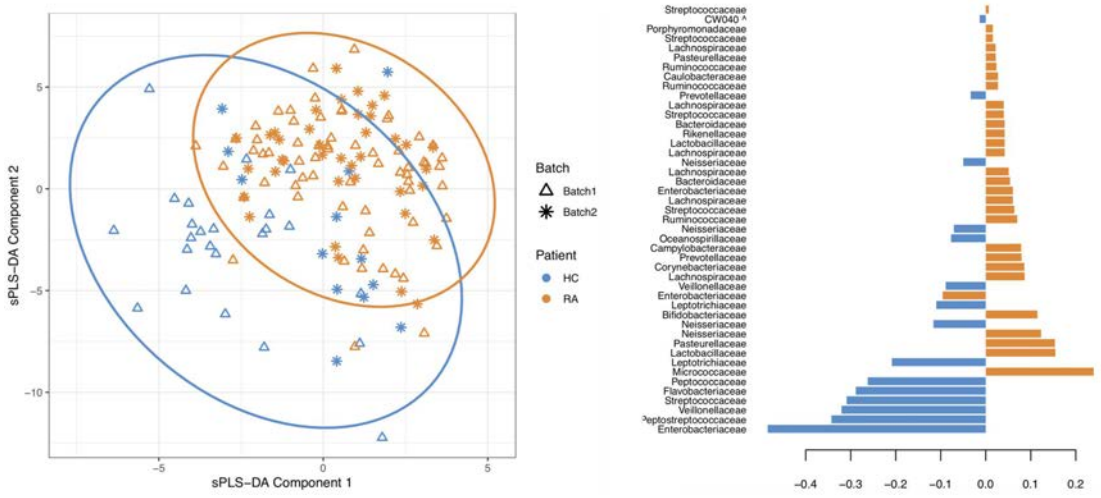

Figure 1 sPLS-DA sample plot of oral microbiome and OTU selection and contribution comparing patients with RA and healthy controls. (A) sPLS-DA sample plot of oral microbiome. The overall abundance of oral microbiota based on 16S rRNA gene amplicon sequencing is compared in HC subjects (HC, blue) and patients with RA (RA, orange). Samples were run in two sequencing batches, represented by open triangle and star symbols. The sPLS-DA model identified 45 OTUs on each component, which in combination help to discriminate the sample groups. 95% confidence ellipse plots are represented within each sample group. (B) Selected OTUs and their contribution in the sPLS-DA model when comparing patients with RA and HC. To reflect the importance of each OTU selected in sPLS-DA component 1, their loading weight coefficient is represented on the x-axis. A high (absolute) value indicate a highly discriminant OTU (bottom of the plot). Colours indicate the sample group (RA orange, HC blue) for which a given OTU is most abundant when considering their median value. HC, healthy controls; OTU, operational taxonomicunit; RA, rheumatoid arthritis.

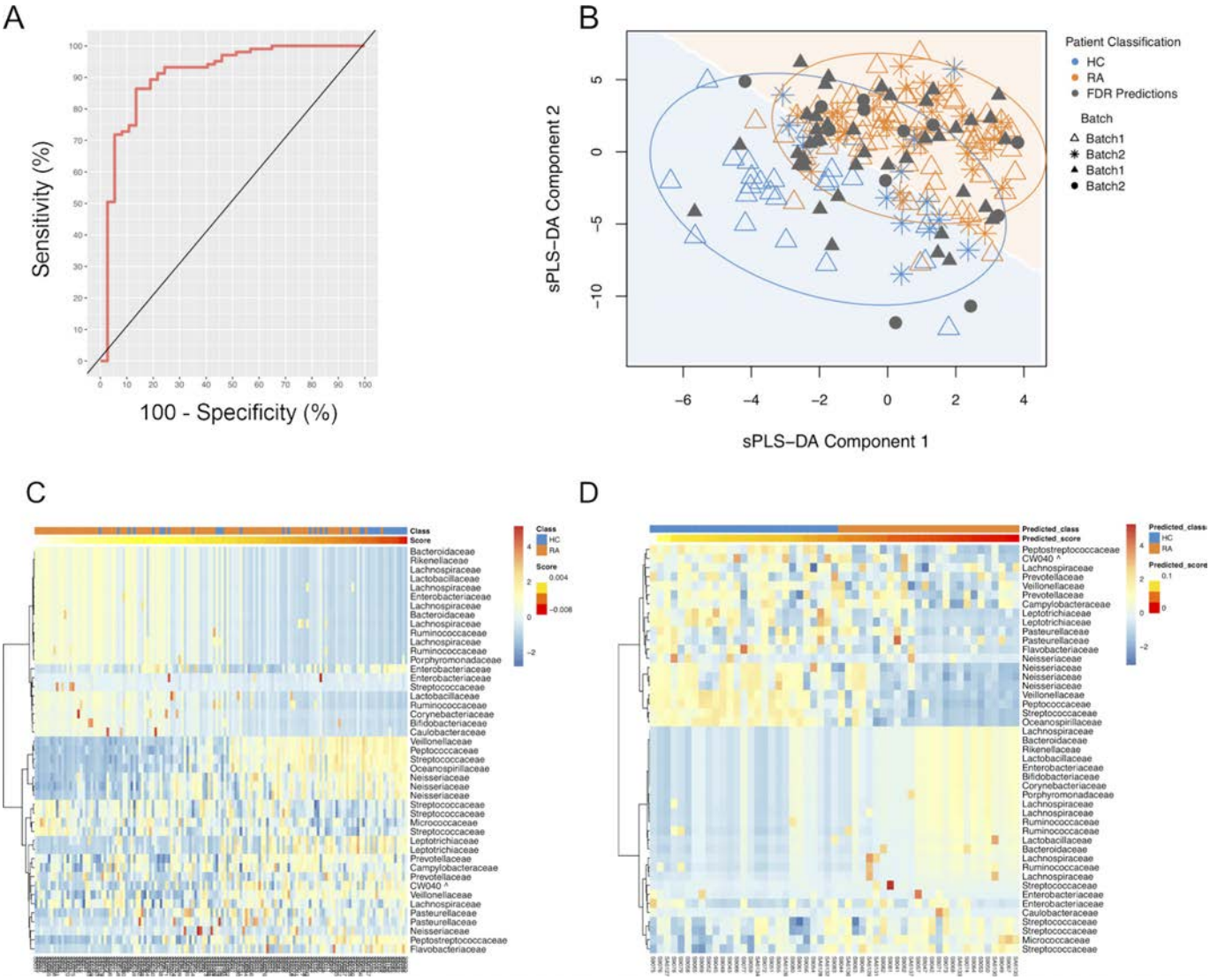

Figure 2 Oral microbiome signature to predict RA and classification of FDR according to the signature (A) Receiver operating characteristic curve (red) illustrates the ability of sPLS-DA to discriminate HC versus RA based on the identified microbial signature, with an AUC=0.9079. (B) The FDR samples are overlaid onto the sample plot from figure 1A for group prediction based on the sPLS-DA model fitted on the HC versus RA samples and the identified microbial signature (figure 1B). The background colour indicates the prediction of the two batches of FDR sample sequencing are denoted by grey circle and triangle symbols. (C) Heatmap representation of the oral microbiome signature from RA and HC samples. Abundance ranges from low (blue) to high (red) for each OTU (rows, Family or Order taxonomy) and each sample (columns). In rows, OTU are clustered according to Euclidean distance and Ward linkage method, while in columns the samples are ordered according to their microbial score (as indicated at the top of the graphic, second row) to highlight patterns of microbial abundance from the sPLS-DA. To illustrate the abundance patterns of the microbial signature identified by sPLS-DA and to summarise as a continuous disease score, the first row indicates the known group status, and the second row the proposed dysbiosis score. (D) Heatmap representation of the OTU signature for the FDR samples calculated from the sPLS-DA model and signature identified from the RA versus HC. In rows, OTU are clustered according to Euclidean distance and Ward linkage method while in columns the samples are ordered according to their predicted microbial score. The first row indicates the predicted status (as disease class is unknown), and the second row the proposed dysbiosis score showing a continuum ranging from RA-like (left columns) to HC-like score (right columns). AUC, area under the curve; FDR, first-degree relatives; HC, healthy controls; OTU,operational taxonomic unit; RA, rheumatoid arthritis.

the curve)=0.9079) discriminated non-RA from RA samples (figure 2A). The dysbiosis score was then used to predict which FDR were most or least 'RA-like' (figure 2B) based on the model fitted on the HC versus RA samples (figure 1). The oral microbiome signature (figure 2C and D) displays a heat map of abundance for each OTU, ranked according to the dysbiosis score, along with group status (for RA and HC) or predicted RA or non-RA status (for FDR) (online supplemental table 4). These data indicate that oral community profiles can be distinguished in patients with RA, which we suggest represents a dysbiotic state. Using sPLS-DA as a prediction tool, the oral microbiome of FDRs can be classified, or assigned to a spectrum, based on similarity to the RA oral dysbiosis score. However, we found no association between measured clinical parameters of the RA and HC samples and the dysbiosis score (online supplemental table 5).

Since Streptococcaceae were major contributors to oral dysbiosis in patients with RA and RA-like FDR, and their cell walls promote immune activation and arthritis in susceptible rodent strains, we isolated axenic streptococcal cultures from the

remaining stored tongue swab of three patients with RA, three FDRs and three HC, to recover 15 isolates. Each isolate was identified based on morphology (online supplemental figure 1), 16S rRNA gene sequence similarity to existing strains and subsequent whole genome sequencing (table 2). Clinical features and dysbiosis scores of the subjects are also indicated (table 2). The majority (n=10) of the isolated strains, including all RA-associated isolates, were *S. sp001556435*, defined by the Genome Taxonomy Database as a distinct sister species to *S. salivarius*[38] and for which we propose the name *S. parasalivarius* sp nov. Other recovered isolates were either *S. salivarius* (n=1) or *S. parasanguinis_B* (n=4). Comparison of the recovered genomes to the *Streptococcus* OTUs within the discriminatory OTU profile (online supplemental table 4) suggested that two of the five OTUs likely belong to either *S. parasalivarius* or *S. salivarius* (100% identity to both species) and one OTU to *S. parasanguinis_B* (online supplemental table 6). The remaining two discriminatory streptococcal OTUs had highest matches to two unrecovered streptococci, *S. sp000187445* and *S. pneumoniae*, with the latter being a low-confidence match (94.4% identity).

To determine whether streptococcal strains of the same species isolated from patients with RA and HC differed at the genomic level—potentially translating to strain functional differences and inflammatory properties—we compared the genomes of the *S. parasalivarius* isolates RA: 21.1, 22.1 and 23.2, and HC: 2.1 (table 2, green shading). Phylogenetic comparison of these, and *S. parasalivarius* and *S. salivarius* isolates recovered from FDRs, clearly distinguished each person's strain (figure 3). Where multiple isolates were obtained from the same individual, all were closely related, for example, 2.1 and 3.1 (figure 3). SNP and indel analysis in comparison to the *S. parasalivarius* reference isolate CCH5-D3 identified over 250 RA-associated mutations (conserved among isolates from patients with RA) classified as moderate or high risk to protein structure, for example, missense, non-sense and frameshift mutations (online supplemental table 7). At the protein level, comparison of homologous proteins identified the three RA-associated isolates encode the galactose/lactose operon described in *S. salivarius*,[39] and a multidrug resistance efflux transporter (EmrE superfamily) absent from the *S. parasalivarius* isolate obtained from a HC (online supplemental table 8).

Since the genomic diversity observed between the RA-associated strains might impact functional differentiation, we compared the inflammatory potential of *S. parasalivarius* isolates 21.1, 22.1 and 23.2. We incubated heat-killed cells of these RA-associated isolates with splenocytes from naïve SKG mice, which are genetically susceptible hosts for Th17-dependent autoimmune arthritis in response to microbial triggers.[40] Two additional *S. parasalivarius* isolates, (HC isolate 2.1 and FDR isolate 16.1) and one *S. salivarius* isolate (FDR isolate 11.1) were assayed for reference. While in vitro production of tumour necrosis factor (TNF) and interleukin (IL)-6 differed between the RA-associated *S. parasalivarius* strains, with 21.1 and 22.1 inducing significantly lower levels of both cytokines than 23.2, TNF was the dominant cytokine induced by all isolates. TNF was produced at 10-fold higher levels than IL-6 and 100-fold higher levels than IL-10 (figure 4A–C). We compared SNPs and indels of each strain to ascertain genetic differences that might account for fine differences in TNF production between strains. Over 340 SNPs and indels classified as moderate or high risk were shared between strains 21.1 and 22.1 while absent from other assayed *S. parasalivarius* isolates (online supplemental table 9). Conversely, 171 SNPs and indels were identified as absent from 21.1 and 22.1 while present in the other strains (online

Table 2 Streptococcal isolate genome characteristics and clinical features of subjects

NCBI assembly accession	Isolate	16S rRNA gene-based identification	Genome-based classification	Genome completeness	Genome contamination	Genome contigs	Genome length	Source	Age	Sex	BMI	Infection history	RA drugs	ACPA titre	RF (IU)	Dysbiosis score x10^{-3} (rank)
JACLQX000000000	1.1	*parasanguinis*	*parasanguinis_B*	100	0.07	50	2 103 580	HC				Tooth	–	0	0	−1.43 (96)
JACLQW000000000	2.1	*salivarius*	*parasalivarius*	99.38	0.66	62	2 397 206	HC	35	F	26					
JACLQV000000000	3.1	*salivarius*	*parasalivarius*	99.38	0.66	53	2 395 186	HC								
JACLQU000000000	6.1	*parasanguinis*	*parasanguinis_B*	100	0.35	28	2 135 306	HC	54	M	31	Tooth	–	0	0	−2.26 (104)
JACLQT000000000	7.1	*parasanguinis*	*parasanguinis_B*	100	0.35	30	2 138 533	HC				Throat, sinus				
JACLQS000000000	9.1	*salivarius*	*parasalivarius*	99.76	0.15	39	2 127 501	FDR	22	F	24	Tooth	–	0	0	28.72 (19)
JACLQR000000000	10.1	*salivarius*	*parasalivarius*	99.76	0.15	42	2 128 725	FDR								
JACLQQ000000000	11.1	*salivarius*	*salivarius*	99.84	1.12	35	2 198 364	FDR	67	F	24	Tooth	–	0	0	7.94 (53)
JACLQP000000000	12.1	*parasanguinis*	*parasanguinis_B*	100	0	50	2 088 454	FDR				UTI				
JACLQO000000000	15.1	*salivarius*	*parasalivarius*	99.4	1.46	92	2 228 781	FDR	42	F	34	Tooth	–	11	0	27.02 (20)
JACLQN000000000	16.1	*salivarius*	*parasalivarius*	99.4	1.46	79	2 216 454	FDR				Throat				
JACLQM000000000	17.1	*salivarius*	*parasalivarius*	99.4	1.46	60	2 222 428	FDR								
JACLQL000000000	21.1	*salivarius*	*parasalivarius*	99.68	0.22	34	2 114 746	RA	62	F	25	Tooth, GI	MTX	49	976	1.62 (47)
JACLQK000000000	22.1	*salivarius*	*parasalivarius*	99.82	0.88	60	2 394 449	RA	53	F	32	Throat, UTI	MTX	36	23	4.3 (10)
JACLQJ000000000	23.2	*salivarius*	*parasalivarius*	99.69	0.58	44	2 160 453	RA	32	F	18	–	SSZ	110	83	1.68 (46)

Genome sequences were compared for isolates shaded green.
ACPA, anti-citrullinated peptide autoantibody; BMI, body mass index; F, female; FDR, first-degree relatives; GI, gastrointestinal; HC, healthy controls; M, male; MTX, methotrexate; RA, rheumatoid arthritis; RF, rheumatoid factor; SSZ, sulfasalazine; UTI, urinary tract infection.

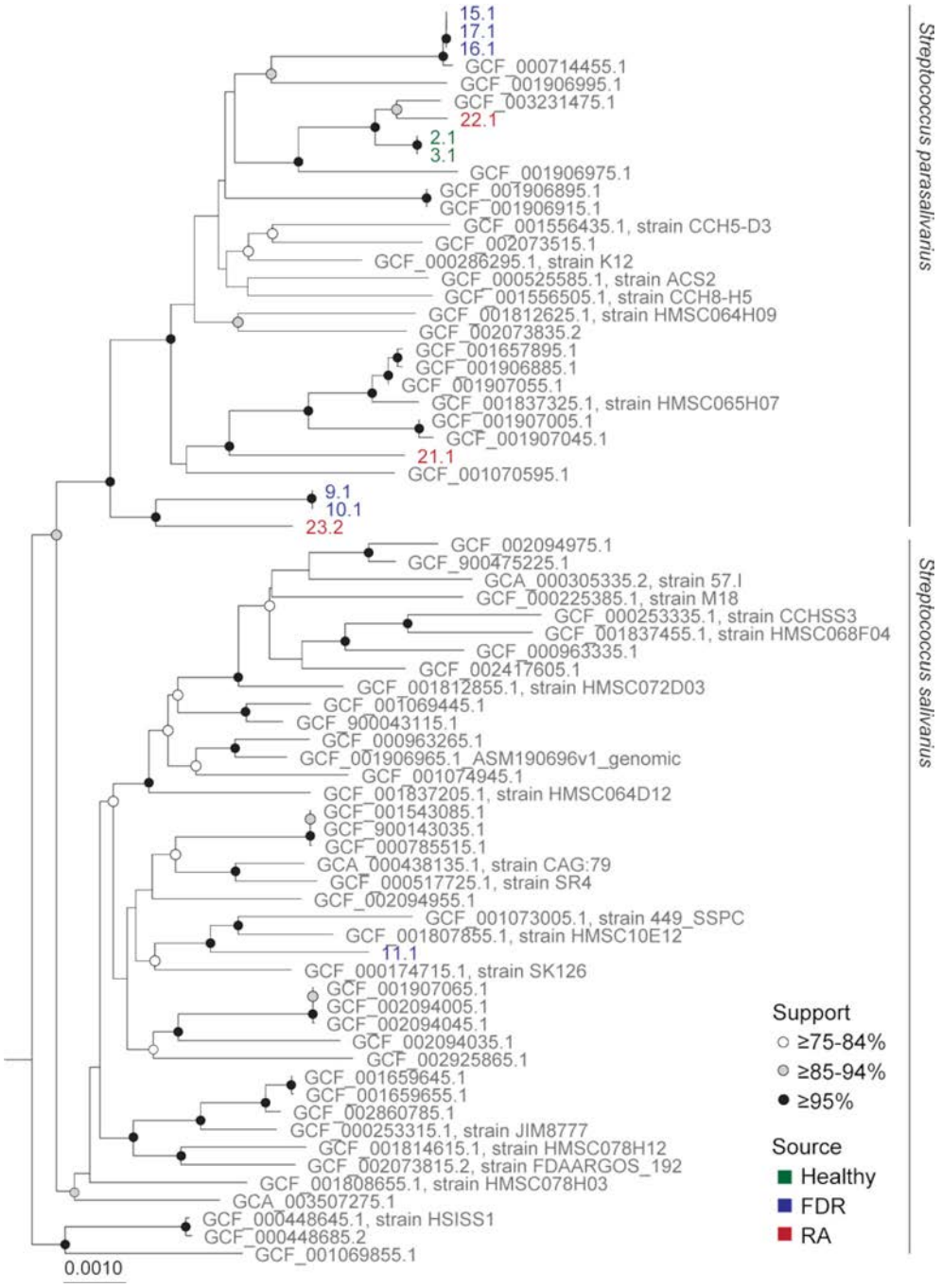

Figure 3 Phylogenetic analysis of *S. parasalivarius* and *S. salivarius* isolates. Maximum-likelihood tree generated using IQ-TREE from alignment of 120 marker genes identified using GTDB-Tk. Alignment filtered for sites conserved in a minimum of 10% of genomes yielding 39 247 residues. IQ-TREE built-in model testing assigned model JTT+F+R5 to data set. Bootstrap support values from IQ-TREE Ultrafast Bootstrapping with 1000 replicates. Isolate genomes marked in red (RA), blue (FDR) and green (healthy). Instances of multiple isolates from the same individual are clustered: 2.1/3.1, 9.1/10.1 and 15.1/16.1/17.1. Public genomes from NCBI representing *S. salivarius* and *S. parasalivarius* included as defined by GTDB R04-RS89 (grey). *S. vestibularis* genome GCF_000188295.1 used as outgroup. FDR, first-degree relatives; RA, rheumatoid arthritis.

supplemental table 10). Multiple encoded proteins displayed a pattern of presence/absence matching the TNF profile, including several potentially involved in cell wall synthesis, such as the FemA/B homologue, which contributes to peptidoglycan bridge formation and several glycosyltransferase proteins—of interest given the known immunogenicity of SCW (online supplemental tables 11 and 12).

Untreated SKG mice are healthy, and 100% of female SKG mice develop arthritis after one intraperitoneal injection of microbial beta-glucan (curdlan).[41] To assess potential for SCW of the different isolates to induce disease, we generated SCW from RA 21.1 and 23.2, and HC 2.1. We compared their arthritogenicity after two intraperitoneal injections, to standard *S. pyogenes* SCW, 10S PS-PG in female SKG mice. While the time course of response differed somewhat between experiments, all mice developed arthritis after 10S PS-PG or study SCW (figure 4D and E). Joint swelling scores were not significantly different between mice receiving the study SCW, and disease was generally more severe after 10S PS-PG. Curdlan-induced arthritis developed more rapidly and was more severe than SCW arthritis

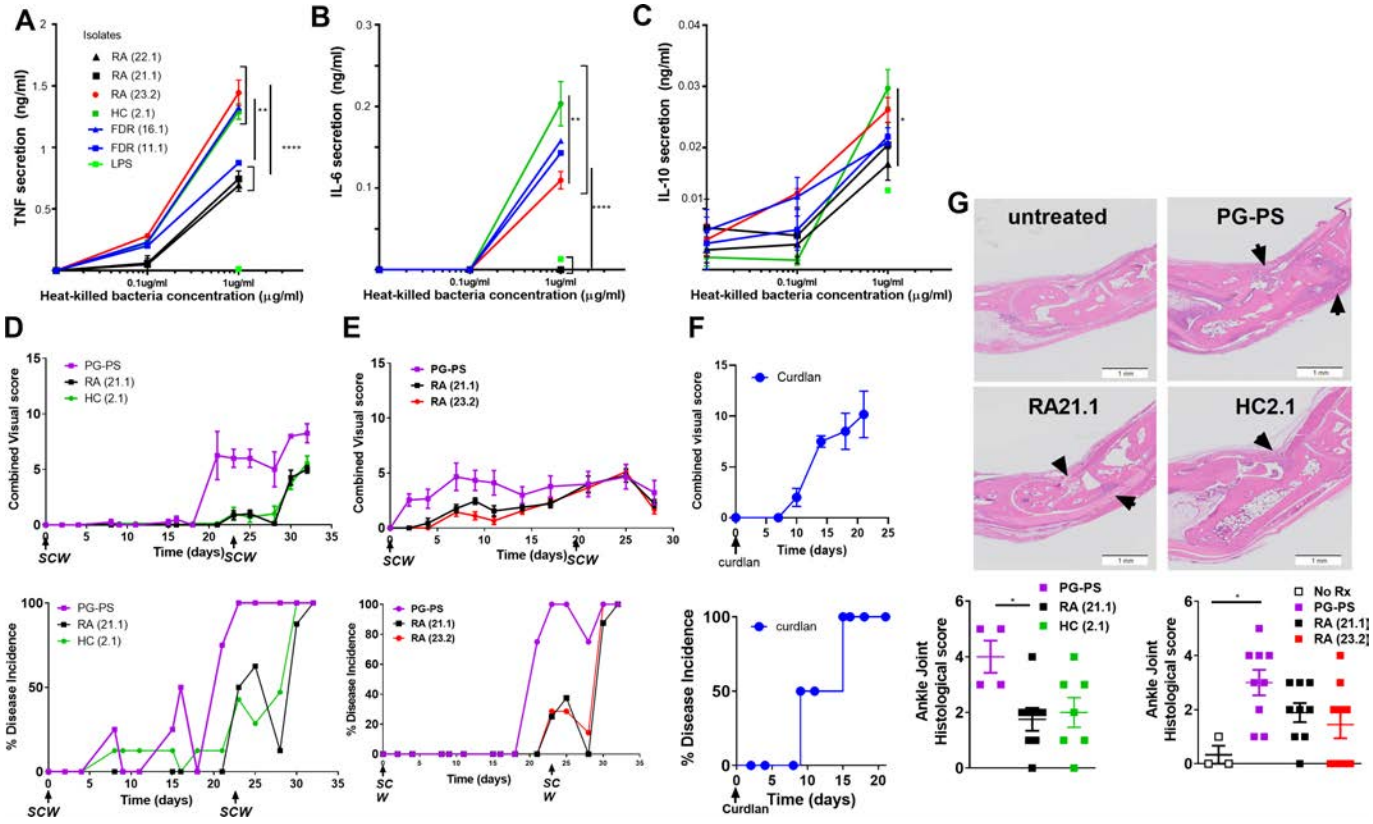

Figure 4 Functional characterisation of streptococcal strains in vitro and in vivo varying concentrations of heat-killed *S. parasalivarius* (2.1, 21.1, 22.1, 23.2, 16.1) and *S. salivarius* (11.1) isolates were incubated with SKG splenocytes for 24 hours then TNF (A), IL-6 (B) and IL-10 (C) were measured in cell free supernatant by cytokine bead array. 10S PG-PS or SCW from Streptococcal isolates 21.1, 2.1 () or 21.1 and 23.2 (E) were administered intraperitoneally to female SKG mice then visual score of ankle and wrists, and arthritis incidence, were monitored three times weekly for 32 () or 28 (E) days. Curdlan was administered intraperitoneally and visual score and arthritis incidence were monitored for 21 days (F). Joint histology was scored at the endpoint of each experiment (). Arrowheads: mild arthritis in the ankle and small joints of foot, arrows: moderate ankle and foot arthritis and soft tissue inflammation. FDR, first-degree relatives; HC, healthy controls; IL, interleukin; LPS, lipopolysaccharide; PG-PS, peptidoglycan-polysaccharide; RA, rheumatoid arthritis; SCW, streptococcal cell wall;TNF, tumour necrosis factor.

(figure 4F). Joint swelling scores were not significantly different between mice receiving the study SCW and disease was generally more severe after 10S PS-PG. Histologically, whereas mice receiving study SCW developed mild arthritis in the small joints of the feet and ankle, 10S PS-PG induced more florid ankle, foot and soft tissue inflammation (figure 4G). Thus, similar to TNF-dependent Chlamydia-induced arthritis in SKG mice,[42] repeated in vivo exposure to SCW is sufficient to trigger mild-to-moderate chronic inflammatory arthritis in this autoimmune-prone host, regardless of the disease-association status of the oral community from which the streptococci derived.

DISCUSSION

Using 16S rRNA gene amplicon sequencing, we show that patients with RA and some FDR, who were more likely to report bleeding gums than age and sex-matched HC, had dysbiotic oral microbiome profiles. Oral dysbiosis in FDR—with unknown disease class—could be classified according to similarity to the RA oral dysbiosis score. The dysbiosis score was not associated with clinical features surveyed among participants—including HLA-DR type, autoantibody titre, history of infection, medications, disease duration, joint count or erythrocyte sedimentation rate/C-reactive protein inflammatory markers in patients with RA or FDR. However, we did not formally measure disability or damage, and no longitudinal data were obtained to assess impact of disease activity on dysbiosis. Our study examined tongue swabs, as the tongue has the most stable, diverse and dense bacterial biofilm of the oral cavity.[35] Tongue microbiota are comparable to that of saliva, of clinical relevance due to the association of oral species with the gut environment in RA.[8] Since this compartment differs from the gingival or supragingival plaque biofilm, it is not surprising that we did not identify differential abundance of periodontitis keystone bacteria. Most of the patients with RA were treated with conventional or biological DMARDs, which may also have influenced the results. Furthermore, in absence of data from a second RA cohort, we could not fully validate the oral dysbiosis score. Nevertheless, our data support previous observations of dysbiosis of gut and oral microbiomes in RA.[5]

The 16S rRNA gene amplicon sequencing revealed five *Streptococcus* OTUs that were differentially abundant between RA and HC samples. With culture-based isolation and whole genome sequencing, we recovered 15 streptococcal isolates across three different species, *S. parasalivarius* (n=10), *S. salivarius* (n=1) and *S. parasanguinis_B* (n=4), with strains of *S. parasalivarius* isolated from RA, FDR and HC subjects. While only *S. parasalivarius* was isolated from patients with RA, culturing efforts were not exhaustive, based on comparison of the genomic data with the amplicon sequencing data (online supplemental table 6). Phylogenetic comparison of the *S. parasalivarius* isolates revealed significant genomic diversity across the RA-associated strains. Exposure of splenocytes to heat-killed *S. parasalivarius*

isolates in vitro differentially promoted the production of innate immune cytokines IL-6 and TNF, potentially reflecting the observed genomic diversity. Importantly though, all strains dominantly induced TNF secretion, and TNF is likely to contribute to their in vivo pathogenicity in SKG mice.[42]

PAMPs associated with multiple pathobionts expanding in the oral dysbiotic environments could influence innate immune control of the mucosal barrier, such as the gingiva, predisposing to or exacerbating periodontitis. In hypervascular inflammatory tissue, permeability to resident mucosal microbes increases, enhancing systemic haematogenous spread of microbes, debris or PAMPs. Ectopic intestinal colonisation by oral bacteria, including streptococcal species, may also elicit intestinal inflammation.[43] Multiple encoded proteins within the *S. parasalivarius* isolates could potentially contribute to their immune-stimulatory function, including several involved in SCW synthesis. SCW consist of peptidoglycan, which induces inflammation, and polysaccharide, which protects against enzyme degradation in vivo.[32] The barrier that polysaccharides present to streptococcal phagocytosis by macrophages necessitates antibody and complement opsonisation for clearance. After intraperitoneal injection of SCW, female Lewis rats were shown to develop a chronic inflammatory arthritis with synovial localisation of the degradation-resistant SCW. Here they were phagocytised, but with persistence of PAMPs within the inflammatory site.[11] Depletion of macrophages with clodronate liposomes suppressed arthritis development.[44] By contrast, in BALB/c mice, SCW acute arthritis resolves spontaneously, and continued joint injections induce chronic arthritis.[33] IL-6, IL-17 and IL-23 are the predominant cytokines released in SCW-induced inflammatory arthritis.[45] SKG mice (BALB/c background) are genetically prone to Th17 or TNF-mediated chronic inflammatory arthritis after exposure to microbial products or chlamydial infection, due to reduced bacterial control by the host immune system.[46] In the present study, two intraperitoneal administrations of SCW derived from *S. parasalivarius* isolates induced mild chronic inflammatory arthritis in SKG mice, demonstrating that the ZAP-70 mutation prevents resolution of SCW arthritis. Furthermore, systemic administration was sufficient for development of arthritis, suggesting focal haematogenous spread of SCW debris.

Together, our data suggest that local inflammation and barrier dysfunction in the gingiva, permitting pro-inflammatory SCW to penetrate and reach the joint, are more important than the source of *Streptococcus* species from which SCW are derived. Controlling mucosal inflammation and barrier dysfunction in genetically predisposed individuals with oral dysbiosis and local periodontal inflammation—associated with poor dental health, smoking and poor nutrition—should reduce their propensity for arthritis development.

Description of *Streptococcus parasalivarius* sp nov

Streptococcus parasalivarius (pa.ra.sal'i.var'i.us. Gr. prep. *para* resembling; N.L. part. adj. *salivarius* specific epithet of *Streptococcus salivarius*; N.L. part. adj. *parasalivarius* (*Streptococcus*) *salivarius*-like). Represented by isolate genome 21.1 (acc. no. JACLQL000000000) obtained from a tongue swab of a patient with RA.

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Patient and public involvement statement At what stage in the research process were patients/the public first involved in the research and how? Patients and the public were involved at the recruitment stage. How were the research question(s) and outcome measures developed and informed by their priorities, experience and preferences? No direct patient involvement. How were patients/the public involved in the design of this study? Not involved. How were they involved in the recruitment to and conduct of the study? Not involved. Were they asked to assess the burden of the intervention and time required to participate in the research? No. How were (or will) they be involved in your plans to disseminate the study results to participants and relevant wider patient communities (eg, by choosing what information/results to share, when and in what format)? Preliminary results were shared via a newsletter to the involved participants to inform them of study progress.

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CLINICAL SCIENCE

Secukinumab in patients with psoriatic arthritis and axial manifestations: results from the double-blind, randomised, phase 3 MAXIMISE trial

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ABSTRACT

Objectives MAXIMISE (Managing AXIal Manifestations in psorIatic arthritis with SEcukinumab) trial was designed to evaluate the efficacy of secukinumab in the management of axial manifestations of psoriatic arthritis (PsA).

Methods This phase 3b, double-blind, placebo-controlled, multi-centre 52-week trial included patients (≥18 years) diagnosed with PsA and classified by ClASsification criteria for Psoriatic Arthritis (CASPAR) criteria, with spinal pain Visual Analogue Score ≥40/100 and Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score ≥4 despite use of at least two non-steroidal anti-inflammatory drugs (NSAIDs). Patients were randomised (1:1:1) to secukinumab 300 mg, secukinumab 150 mg or placebo weekly for 4 weeks and every 4 weeks thereafter. At week 12, placebo patients were re-randomised to secukinumab 300/150 mg. Primary endpoint was ASAS20 (Assessment of SpondyloArthritis international Society) response with secukinumab 300 mg at week 12.

Results Patients were randomly assigned; 167 to secukinumab 300 mg, 165 to secukinumab 150 mg and 166 to placebo. Secukinumab 300 mg and 150 mg significantly improved ASAS20 response versus placebo at week 12 (63% and 66% vs 31% placebo). The OR (95% CI) comparing secukinumab 300 mg and 150 mg versus placebo, using a logistic regression model after multiple imputation, was 3.8 (2.4 and 6.1) and 4.4 (2.7 and 7.0; p<0.0001).

Conclusions Secukinumab 300 mg and 150 mg provided significant improvement in signs and symptoms of axial disease compared with placebo in patients with PsA and axial manifestations with inadequate response to NSAIDs.

Trial registration number NCT02721966.

Key messages

What is already known about this subject?

► Secukinumab, a fully human monoclonal antibody that directly inhibits interleukin (IL)-17A, has demonstrated significant and sustained efficacy across distinct clinical domains in active psoriatic arthritis (PsA). However, the efficacy of secukinumab or any other biological disease modifying anti-rheumatic drugs (bDMARDs) treatment specifically on axial manifestations in patients with PsA has never been investigated in a randomised controlled trial (RCT) setting.

What does this study add?

► MAXIMISE (Managing AXIal Manifestations in psorIatic arthritis with SEcukinumab) is the first RCT to evaluate the efficacy of a bDMARD specifically in the management of the axial manifestations of PsA. Secukinumab 300 mg and 150 mg demonstrated significant improvements across the primary, key secondary and secondary clinical and imaging endpoints at week 12, which were sustained through week 52.

How might this impact on clinical practice or future developments?

► The study provides evidence for the efficacy of IL-17A inhibition with secukinumab for the treatment of axial disease in patients with PsA. The results provide valuable data that will help inform treatment decision-making and deepen the clinical understanding of axial PsA, one of the disease manifestations lacking universally acceptable definition criteria.

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INTRODUCTION

Spondyloarthritis (SpA) refers to a group of inter-related inflammatory musculoskeletal disorders that include either peripheral or axial SpA (axSpA). Psoriatic arthritis (PsA), the main type of peripheral involvement of SpA, is a heterogeneous, chronic, progressive, inflammatory condition, associated with enthesitis, dactylitis, skin and nail psoriasis that can affect peripheral joints but also the axial skeleton, with diverse patterns of involvement that can mimic different inflammatory arthritides.[1 2] AxSpA is an inflammatory condition that can occur with (ankylosing spondylitis (AS) or radiographic axSpA) or without (non-radiographic axSpA) radiographic sacroiliitis. Although PsA and AS have a number of clinical features in common, AS accompanied with psoriasis and PsA with predominant axial involvement (axial PsA) are considered two separate disease entities with overlapping features.[3 4] Axial PsA is not clearly defined, universally accepted criteria for axial PsA are currently

lacking and the available outcome measures do not distinguish improvement of axial or peripheral symptoms.[5 6] The development of classification criteria for axial PsA is currently being undertaken by a common effort of Assessment of SpondyloArthritis international Society (ASAS) and Group for Research and Assessment of Psoriasis and Psoriatic Arthritis (GRAPPA). The prevalence of axial disease in patients with PsA varies with disease duration and the definition used, occurring in 5% to 28% of patients with early-stage disease and in 25% to 70% of patients with long-standing PsA.[3] Psoriatic nail dystrophy, the number of radiographically damaged joints, the number of swollen joints, the presence of periostitis and human leukocyte antigen (HLA)-B27 positivity have been identified as predictive factors associated with early or late axial involvement in previous reports.[7–11] The burden of disease is underestimated in axial PsA because patients under-report axial symptoms as peripheral pain is more prominent and long-standing.[11 12] In 2015, both the GRAPPA[13] and the European League Against Rheumatism (EULAR) presented updated recommendations on the management of PsA.[14] GRAPPA recommendations for the management of axial PsA are developed in accordance with the ASAS guidelines and suggest that biologics approved for axSpA may be used to inform treatment decisions for patients with axial PsA. The recently updated PsA EULAR recommendations are based on current practice and recommend therapy with a biological disease modifying anti-rheumatic drug (bDMARD), namely a tumour necrosis factor (TNF) inhibitor in patients with predominantly axial disease and an interleukin (IL)-17 inhibitor when there is relevant skin involvement.[14] The recommendations from both groups note that the development of optimal recommendations for axial PsA remains a challenge.[15]

To the best of our knowledge, none of the randomised clinical trials performed to date that assessed the effect of biologics in PsA included a targeted assessment of axial disease. The only existing evidence comes from two observational studies based on clinical practice settings.[10 11] Therefore, data from randomised controlled trials are lacking on the efficacy of biological treatment for the management of axial manifestations in patients with PsA.

Secukinumab, a fully human monoclonal antibody that directly inhibits IL-17A, has provided significant and sustained improvement in the signs and symptoms of active PsA and axSpA.[16–18] The objective of the MAXIMISE (Managing AXIal Manifestations in psorIatic arthritis with SEcukinumab; NCT02721966) trial was to specifically evaluate the efficacy and safety of secukinumab 300 mg and 150 mg in managing axial manifestations in patients with PsA with an inadequate response to non-steroidal anti-inflammatory drugs (NSAIDs).

METHODS

Study design

MAXIMISE was a phase 3b, double-blind, placebo-controlled, multi-centre 52-week trial that included 498 patients enrolled in 97 centres in Europe, Russia and Israel, between 3 October 2016 and 12 June 2018. The trial consisted of two treatment periods; a placebo-controlled period from baseline to week 12 followed by an active treatment period from week 12 to 52. After a screening period of up to 8 weeks, eligible patients were randomised (1:1:1) to subcutaneous (s.c.) secukinumab 300 mg, 150 mg or placebo weekly for 4 weeks and every 4 weeks thereafter. At week 12, placebo patients were re-randomised (1:1) to s.c. secukinumab 300 mg or 150 mg (online supplemental figure 1).

Patients

Patients aged ≥18 years diagnosed with PsA and classified by ClASsification criteria for Psoriatic Arthritis (CASPAR) criteria, active spinal disease with a Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score ≥4, spinal pain score ≥40 by Visual Analogue Score (VAS) (0 to 100 mm scale) and inadequate response to at least two NSAIDs over a 4-week period were included in the trial. Patients were excluded if they had a history of prior use of bDMARDs (such as TNF inhibitors, ustekinumab, IL-17, IL-23 inhibitors), active ongoing inflammatory conditions other than PsA, current treatment with conventional synthetic DMARDs other than methotrexate (MTX), and patients taking high potency opioid analgesics. Patients were allowed to continue prior use of NSAIDs, MTX and corticosteroids at enrolment through to the end of trial if on a stable dose from baseline to week 12.

Randomisation and masking

All eligible patients were randomised using Interactive Response Technology in a 1:1:1 ratio to secukinumab 300 mg, secukinumab 150 mg or placebo. At week 12, patients randomised to placebo at baseline were re-randomised in a 1:1 ratio to active treatment with secukinumab 300 mg or secukinumab 150 mg. Patients, investigators, site personnel and persons performing the assessments were blinded to the trial assignment (online supplemental material). To maintain blinding, all treatment groups received a consistent number of injections at each visit. The identity of the treatments were concealed by the use of study treatments in the form of pre-filled syringes for s.c. injection filled with secukinumab or placebo that were identical in appearance. Study treatments were administered by the patient or a caregiver after being instructed by site personnel.

Outcome measures

Key efficacy, safety and tolerability assessments were done at screening, baseline, week 12 (primary endpoint), week 52 and time points in between. Protocol amendments are described in the online supplemental material. The primary endpoint was the proportion of patients achieving an ASAS20 response with secukinumab 300 mg at week 12. The ASAS Response Criteria (ASAS20) is defined as an improvement of ≥20% and ≥1 unit on a scale of 10 in at least three of the four main ASAS domains (namely patients global assessment (PtGA) of disease activity, PtGA of inflammatory back pain, BASFI (Bath Ankylosing Spondylitis Functional Index) and average of the last two questions on the six-question BASDAI) and no worsening of ≥20% and ≥1 unit on a scale of 10 in the remaining domain.[19] The key secondary endpoint was an ASAS20 response with secukinumab 150 mg at week 12 after superiority of 300 mg was established. Other secondary endpoints were ASAS40, BASDAI50 and ACR20 (American College of Rheumatology) responses, mean change from baseline in spinal pain measured by VAS, Health Assessment Questionnaire Disability Index (HAQ-DI) score, Functional Assessment of Chronic Illness Therapy (FACIT)-fatigue scale and ASAS Health Index at week 12.

MRI of the spine and sacroiliac joints (SIJ) was performed at baseline and weeks 12 and 52 for all patients to assess sacroiliac and spinal inflammation as an exploratory endpoint to investigate whether these changes are affected by treatment with secukinumab. For patients who discontinued before or at week 12, an MRI was performed at the time of discontinuation. MRI scans were acquired using scanning techniques appropriate for the measurement of inflammation, bone marrow oedema and

erosion[20] and analysed centrally using the Berlin modification of the ASspiMRI scoring system (Berlin MRI score).[20] MRI imaging of the spine and SIJ was implemented using a standardised scanning procedure monitored by a central imaging service agency to minimise differences among MRI scanners at different imaging centres. Spine images were acquired in two or three overlapping segments to achieve complete sagittal coverage of the spine (from C1 to S1). For SIJ, 3-plane localisers were acquired to have a true mid-sagittal slice showing the entire sacrum, based on which the centre of the joint space between S1 and S2 vertebral bodies was identified and 18 slices were prescribed in oblique coronal orientation. Details of the MRI image acquisition procedure are described in the online supplemental material.

The improvement in AS disease activity score (ASDAS) and Berlin MRI score for the spine and SIJ at week 12 to assess bone marrow oedema were exploratory outcome measures. ASAS20 response rates at week 12 were assessed in the subgroup of patients with positive MRI for spine and/or SIJ at baseline, as well as in the subgroup with or without concomitant MTX. Assessments at week 52 were ASAS20 and ASAS40, BASDAI50, spinal pain (VAS), ACR20, HAQ-DI, ASAS-Health Index, FACIT-fatigue and ASDAS. Safety analyses included all safety data reported up to and including the week 52 visit for each patient who received at least one dose of study drug.

Statistical analyses

Sample size was calculated based on a Fisher's exact test assuming an overall type I error (two-sided) of 5%. To achieve 92% power and conservatively assuming a response rate of 40% in the placebo group, at least 150 patients per group were needed to be recruited under equal allocation to show a response rate of 60% in the secukinumab 300 mg group. Using the same number of patients per group, the second test had at least 80% power to detect a difference, if the true response rates are 57% in the secukinumab 150 mg group and 40% in placebo. To compensate for drop-outs and protocol violations, 165 patients per group (=495 in total) were required to be recruited into this trial. The full analysis set followed the intent-to-treat principle and comprised all patients from the randomised set to whom study treatment was assigned, fulfilling the clinical criteria for active axial disease, that is, spinal pain ≥40 and BASDAI ≥4. Patients were evaluated according to the treatment assigned at randomisation. The safety set included all patients who took at least one dose of study treatment during the entire treatment period. Summary statistics are presented for continuous demographic and baseline characteristic variables for each treatment group and for all patients in the randomised set, which included all patients originally randomised to secukinumab 300 mg or 150 mg and patients originally randomised to placebo who switched to secukinumab 300 mg or 150 mg at week 12 (placebo-secukinumab 300 mg or placebo-secukinumab 150 mg). Missing data up to week 12 for binary efficacy variables were handled using multiple imputation (MI) which imputes missing data based on patients' actual data and observed data from similar patients in similar conditions. Analysis of covariance model was used to analyse continuous variables up to week 12. Data after week 12 through week 52 are reported as observed. Pre-defined exploratory analysis of the ASAS20/40 and BASDAI50 response at week 12 by Baseline Berlin MRI used the last observation carried forward (LOCF) method for imputation of missing data. LOCF technique was also undertaken as post-hoc analyses for ASAS20 and ASAS40 outcome measures.

Patient and public involvement

Patient or public were not involved in the design and conduct of the trial. The trial was conducted in accordance with the Declaration of Helsinki (General Assembly of the World Medical Association 2014) and was approved by institutional review boards or independent ethics committees at each participating centre. Written informed consent was obtained from all enrolled patients. Data were collected in accordance with Good Clinical Practice guidelines by the trial investigators and analysed by the sponsor.

RESULTS

Patients

A total of 498 patients (167 to secukinumab 300 mg, 165 to secukinumab 150 mg and 166 to placebo group) were randomised; of these 425 (85%) patients completed the trial through week 52.

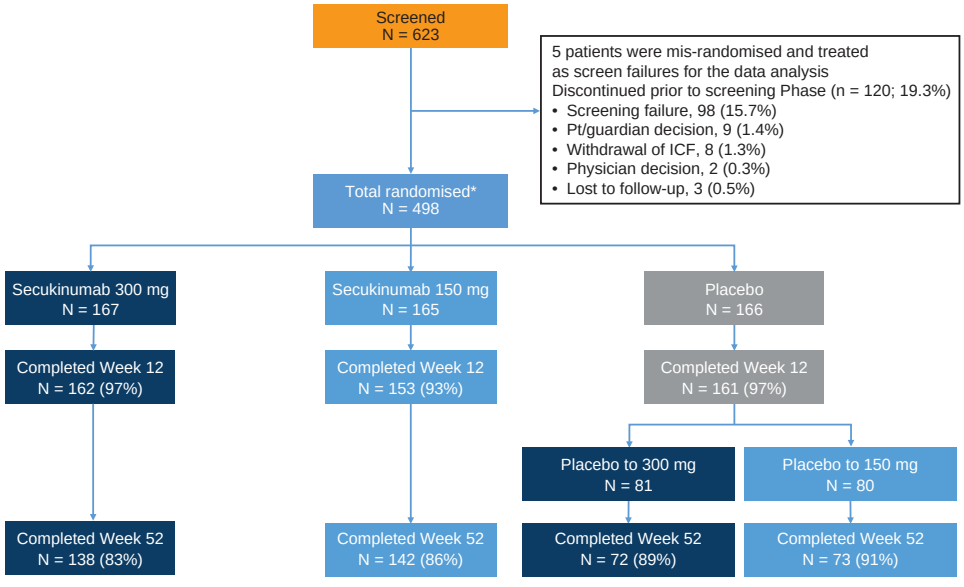

Figure 1 Patient disposition. *5 patients were mis-randomised and were treated like screen failures for the data analysis. AE, adverse event; ICF, informed consent; Pt, patient; N, total number of randomised patients.

Table 1 Demographics and baseline disease characteristics

Characteristics mean (SD) unless specified	Secukinumab 300 mg s.c. (N=167)	Secukinumab 150 mg s.c. (N=165)	Placebo (N=166)
Age (years)	46.2 (12.3)	46.9 (11.5)	46.6 (11.5)
Male, n (%)	77 (46.1)	81 (49.1)	88 (53.0)
Body mass index (kg/m^2)	27.3 (4.8)	29.0 (6.4)	28.3 (5.5)
Smoking status (tobacco), n (%)			
Current	47 (28.1)	39 (23.6)	39 (23.5)
Former	20 (12.0)	34 (20.6)	25 (15.1)
Total spinal pain score, VAS	**72.5 (13.8)**	**73.6 (15.4)**	**74.0 (13.7)**
Inflammatory back pain parameters, n (%)			
Onset of back pain is insidious	150 (89.8)	147 (89.1)	152 (91.6)
Back pain improving with exercise	148 (88.6)	139 (84.2)	146 (88.0)
Back pain worsening with rest	152 (91.0)	151 (91.5)	157 (94.6)
Night pain with improvement on getting up	147 (88.0)	147 (89.1)	143 (86.1)
Awakening due to back pain in second half of night	143 (85.6)	145 (87.9)	137 (82.5)
Alternating buttock pain	102 (61.1)	98 (59.4)	101 (60.8)
Efficacy variables at baseline			
PtGA of disease activity	71.7 (14.4)	74.5 (14.2)	72.4 (15.6)
PGA of disease activity	62.6 (15.7)	62.2 (19.5)	64.0 (17.6)
BASDAI score	7.3 (1.2)	7.2 (1.4)	7.3 (1.2)
TJC	15.3 (15.3)	14.9 (14.5)	15.6 (15.0)
SJC	6.1 (8.7)	5.9 (7.7)	6.2 (9.0)
SPARCC score	4.5 (4.2)	4.7 (4.3)	4.7 (4.4)
HAQ-DI score	1.4 (0.5)	1.4 (0.6)	1.5 (0.5)
FACIT-Fatigue	22.0 (9.4)	21.6 (10.1)	21.0 (9.5)
BASFI, score	6.3 (1.8)	6.5 (1.9)	6.4 (2.0)
Evidence of current PsO, n (%)	152 (91.0)	147 (89.1)	153 (92.2)
hsCRP (mg/L)	11.7 (23.3)	11.5 (21.2)	8.7 (15.4)
Axial PsA history			
Presence of peripheral arthritis, n (%)	**133 (79.6)**	**136 (82.4)**	**137 (82.5)**
Time since first signs and symptoms of arthritis (years)	7.0 (7.1)	7.8 (8.4)	7.9 (8.4)
Time since first diagnosis of peripheral arthritis (years)	5.3 (6.6)	4.7 (5.1)	5.1 (7.0)
Time since first axial signs and symptoms (years)	6.9 (7.7)	7.9 (7.9)	7.7 (9.5)
Time since diagnosis of axial PsA prior to baseline (years)	2.8 (4.4)	3.3 (4.7)	2.9 (5.0)
Patient with diagnosis of AS, n (%)	35 (21.0)	36 (21.8)	42 (25.3)
MRI parameters at baseline*			
Berlin MRI score for the entire spine, Mean (SD)	n=150 2.0 (3.95)	n=144 1.0 (1.68)	n=148 1.5 (2.45)
Berlin MRI score for SIJ, Mean (SD)	n=151 1.7 (2.94)	n=142 1.6 (2.77)	n=146 1.8 (3.32)
HLA-B27 status, n (%)†			
Positive	32 (35.2)	25 (28.4)	28 (34.1)
Negative	59 (64.8)	63 (71.6)	54 (65.9)

*n represents number of patients with evaluable MRI data at baseline and post-baseline.
†Based on available HLA-B27 status data (secukinumab 300 mg (n=91), 150 mg (n=88) and placebo (n=82)).
AS, ankylosing spondylitis; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; FACIT-Fatigue, Functional Assessment of Chronic Illness Therapy Fatigue Scale; HAQ-DI, Health Assessment Questionnaire Disability Index; HLA, human leukocyte antigen; hsCRP, high sensitivity C-reactive protein; MTX, methotrexate; N, total number of randomised patients; PGA, physician global assessment; PsA, psoriatic arthritis; PsO, psoriasis; PtGA, patients global assessment; s.c., subcutaneous; SIJ, sacroiliac joints; SJC, swollen joint count; SPARCC, Spondyloarthritis Research Consortium of Canada enthesitis index; TJC, tender joint count; VAS, Visual Analogue Scale.

The retention rates at week 52 were 83% (138/167) for secukinumab 300 mg, 86% (142/165) for secukinumab 150 mg, 89% (72/81) for placebo-secukinumab 300 mg and 91% (73/80) for placebo-secukinumab 150 mg; (figure 1). A total of 73 patients (15%) discontinued during the entire study period; with the most frequent reason being patient/guardian decision (33% (24/73)) followed by adverse events (AEs; 21% (15/73)) and lack of efficacy (15% (11/73)). Demographic and baseline disease characteristics and efficacy variables were comparable across groups (table 1). Patients had an established diagnosis of PsA with symptoms for around 7 years on average and around 50% were men. One or more of the parameters of inflammatory back pain were reported for the vast majority of the patients. Around 60% of patients had a positive MRI with inflammation in the spine and/

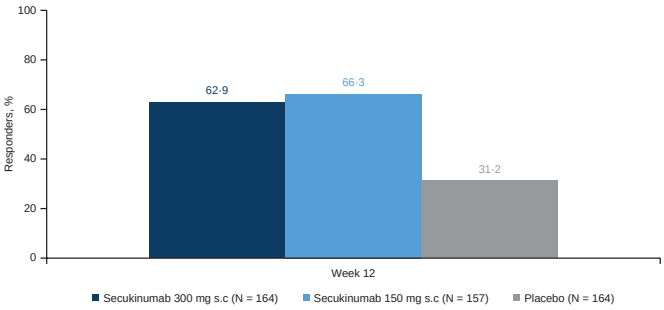

Figure 2 ASAS20 response rates at week 12 (MI). OR secukinumab 300 mg versus placebo: 3.8, **p<0.0001** secukinumab 150 mg versus placebo: 4.4, **p<0.0001**. ASAS, Assessment of SpondyloArthritis International Society; MI, multiple imputation; N, total number of randomised patients (full analysis set); s.c., subcutaneous.

or SIJ. HLA-B27 status, as reported by the investigator, was positive for 33% of the 261 patients for whom this data was available. Investigator reported X-ray data at baseline is summarised in online supplemental table S1. The mean time since last X-ray of SIJ ranged from 1.3 to 1.9 months across treatment arms and approximately two-third of the patient population had Grade 1 to Grade 4 sacroiliitis on either side.

Clinical efficacy

The primary and key secondary endpoints of the study were met; secukinumab 300 mg and 150 mg significantly improved ASAS20 response versus placebo at week 12 (63% and 66% vs 31% placebo). The OR (95% CI) for reaching ASAS20 response in the comparison of secukinumab 300 and 150 mg versus placebo, using a logistic regression model (with MI), was 3.8 (2.4 to 6.1) and 4.4 (2.7 to 7.0; p<0.0001; figure 2). ASAS40 response rates were greater with secukinumab 300 mg and 150 mg versus placebo at week 12 (44% (71/161) and 40% (60/151) vs 12% (20/161) placebo). The OR (95% CI) for reaching ASAS40 response in the comparison of secukinumab 300 mg and 150 mg versus placebo was 5.6 (3.2 to 9.8) and 4.7 (2.7 to 8.3), respectively (p<0.0001). Secukinumab improved other secondary endpoints at week 12 (table 2).

The least square means (LSM) of treatment difference versus placebo in change from baseline in total Berlin MRI score for the entire spine at week 12 was −0.4 (0.1; secukinumab 300 mg; p<0.01) and −0.4 (0.1; secukinumab 150 mg; p<0.05). The LSM of treatment difference versus placebo in change from baseline in total Berlin MRI score for the SIJ at week 12 was −0.5 (0.2; secukinumab 300 mg; p<0.01) and −0.5 (0.2; secukinumab 150 mg; p<0.01). ASAS20 response rates at week 12 in the subgroup of patients with positive MRI for spine and/or SIJ at baseline were similar to the overall population, with 66% (58/88) for secukinumab 300 mg, 70% (51/73) for secukinumab 150 mg versus 27% (26/95) for placebo. ASAS20 response rates at week 12 in patients using concomitant MTX were 67% (secukinumab 300 mg), 67% (secukinumab 150 mg) versus 40% (placebo) and corresponding rates in the group without MTX use were 61%, 67% vs 25%. Pre-defined exploratory analysis of the ASAS20/40 and BASDAI50 response at week 12 by Baseline Berlin MRI score did not indicate a notable difference in the odds of achieving response between patients with either positive or negative Baseline Berlin MRIs in the secukinumab groups (online supplemental table S2).

ASAS20 responses observed with secukinumab were sustained through week 52 and were 81% (113/139), 80% (113/141), 75% (54/72) and 80% (59/74) in the secukinumab 300 mg, 150 mg, placebo to secukinumab 300 mg and 150 mg groups, respectively. Reductions observed at week 12 in mean Berlin MRI score for the entire spine and SIJ were sustained at week 52. Notable reductions were also observed in placebo patients who switched to active treatment at week 12 (online supplemental figure S2). Other efficacy endpoints were sustained with secukinumab treatment through week 52 (table 2). ASAS20 response observed in the post-hoc analysis using LOCF was reported in 76% (123/163), 77% (119/154), 74% (60/81) and 75% (59/79) patients in the secukinumab 300 mg, 150 mg, placebo to secukinumab 300 mg and 150 mg groups, respectively. The corresponding rates for ASAS40 response using LOCF at week 52 respectively were 63% (102/163), 60% (93/154), 63% (51/81) and 51% (40/79) (figure 3). ASAS20 responses at week 52 in patients using concomitant MTX were 84% (secukinumab 300 mg), 82% (secukinumab 150 mg), 85% (placebo-secukinumab 300 mg) and 83% (placebo-secukinumab 150 mg); corresponding values in patients without concomitant MTX use were 80%, 79%, 66% and 77%, respectively.

Safety

The overall frequencies of non-serious AEs up to week 12 were reported in 65/167 (39%) and 60/165 (36%) patients in the secukinumab 300 mg and 150 mg groups, respectively, compared with 78/166 (47%) in the placebo group (table 3). The rate of serious AEs (SAEs) across secukinumab treatment groups over the entire treatment period was 28/493 (6%); none of the SAEs by preferred term were reported more than once in either of the secukinumab treatment groups (300 mg and 150 mg) over the entire treatment period. A total of seven serious infections (system organ class—infections and infestations) were reported over the entire treatment period. Three of these cases caused temporary dose interruption while the others did not warrant study treatment interruption. A total of eight cases of *Candida* infection (high level term) was reported. The cases of *Candida* infection were non-serious skin and mucosal infections of moderate severity and did not warrant study treatment interruption. One case of Crohn's disease was reported through the entire treatment period with secukinumab (150 mg group), which led to study treatment discontinuation. Major adverse cardiovascular event was reported in three patients: one case each of ischaemic cardiomyopathy and cardiogenic shock, myocardial infarction (both in secukinumab 300 mg arm) and ischaemic stroke (secukinumab 150 mg arm). The event of ischaemic cardiomyopathy in a patient with a known history of hypercholesterolaemia and hypertension was fatal. Three cases of 'malignant or unspecified tumour' were reported through the entire treatment period. One was a case of small cell lung cancer (placebo-secukinumab 300 mg group), the second was a case of metastases to the spine (secukinumab 300 mg group) and the third was a case of adrenal neoplasm (secukinumab 150 mg) that was reported as benign by the investigator. One death (secukinumab 300 mg group) was reported in the trial which was a case of ischaemic cardiomyopathy in a 70-year-old male Caucasian patient with a known history of hypercholesterolaemia and hypertension that happened on day 204 and was not considered related to the study drug by the investigator (table 3).

DISCUSSION

MAXIMISE is the first randomised controlled trial (RCT) to demonstrate the efficacy of a bDMARD in the management of the axial manifestations of PsA. Overall, significant improvements

Table 2 Other efficacy endpoints at weeks 12 and 52

Treatment period 1 (week 12)			
Criteria	**Secukinumab 300 mg s.c. n=164**	**Secukinumab 150 mg s.c. n=157**	**Placebo n=164**
ASAS20, % responders	**63%**	**66%**	**31%**
OR vs placebo (95% CI)	3.8 (2.4 to 6.1)*	4.4 (2.7 to 7.0)*	–
ASAS40, % responders	**44%**	**40%**	**12%**
OR vs placebo (95% CI)	5.6 (3.2 to 9.8)*	4.7 (2.7 to 8.3)*	–
BASDAI50, % responders	**37%**	**33%**	**10%**
OR vs placebo (95% CI)	5.6 (3.0 to 10.2)*	4.5 (2.4 to 8.3)*	–
Spinal pain VAS, LSM change (SE)	**−26.5 (1.8)**	**−28.5 (1.9)**	**−13.6 (1.8)**
LSM difference vs placebo (SE)	−12.9 (2.6)*	−14.9 (2.6)*	–
SPARCC score, LSM change (SE)	**−2.4 (0.2)**	**−2.2 (0.2)**	**−1.7 (0.2)**
LSM difference vs placebo (SE)	−0.7 (0.3)	−0.5 (0.3)	–
HAQ-DI score, LSM change (SE)	**−0.4 (0.04)**	**−0.3 (0.04)**	**−0.2 (0.04)**
LSM difference vs placebo (SE)	−0.2 (0.05)*	−0.2 (0.05)†	–
FACIT-Fatigue, LSM change (SE)	**7.6 (0.7)**	**8.0 (0.7)**	**4.2 (0.7)**
LSM difference vs placebo (SE)	3.4 (1.0)†	3.8 (1.0)†	–
ASAS health index, LSM change (SE)	**−2.8 (0.3)**	**−2.9 (0.3)**	**−1.2 (0.3)**
LSM difference vs placebo (SE)	−1.7 (0.4)*	−1.7 (0.4)*	–
ACR20, % responders	**52%**	**57%**	**19%**
OR vs placebo (95% CI)	4.8 (2.8 to 8.2)*	5.7 (3.3,10.0)*	–
ASDAS-CRP, LSM change (SE)	**−1.3 (0.1)**	**−1.3 (0.1)**	**−0.4 (0.01)**
LSM difference vs placebo (SE)	−0.9 (0.1)*	−0.8 (0.1)*	–

Treatment period 2 (week 52)				
	Secukinumab 300 mg s.c. n=164	**Secukinumab 150 mg s.c. n=157**	**Placebo-secukinumab 300 mg s.c. n=81**	**Placebo-secukinumab 150 mg s.c. n=80**
ASAS20, n/M % responders	113/139 (81%)	113/141 (80%)	54/72 (75%)	59/74 (80%)
ASAS40, n/M % responders	96/139 (69%)	91/141 (65%)	45/72 (63%)	40/74 (54%)
BASDAI50, n/M % responders	95/139 (68%)	83/142 (59%)	40/72 (56%)	40/74 (54%)
Spinal pain VAS, Mean change (SD)	n=140 −42.4 (27.0)	n=142 −43.8 (26.2)	n=72 −43.1 (25.0)	n=74 −36.4 (25.2)
SPARCC score, Mean change (SD)	n=139 −3.1 (3.6)	n=141 −3.0 (4.0)	n=72 −3.4 (4.1)	n=73 −3.2 (4.2)
HAQ-DI score, Mean change (SD)	n=140 −0.5 (0.5)	n=142 −0.5 (0.6)	n=72 −0.5 (0.5)	n=74 −0.4 (0.5)
FACIT-fatigue, Mean change (SD)	n=141 11.7 (9.3)	n=146 11.2 (12.4)	n=72 13.3 (11.8)	n=75 10.0 (10.3)
ASAS health index, Mean change (SD)	n=141 −3.9 (4.1)	n=144 −4.2 (5.0)	n=73 −4.0 (4.6)	n=74 −3.0 (4.3)
ACR20, n/M % responders	81/112 (72%)	84/107 (79%)	45/61 (74%)	40/61 (66%)
ASDAS-CRP, Mean change (SD)	n=136 −1.9 (1.1)	n=139 −1.8 (1.0)	n=71 −1.8 (1.1)	n=72 −1.4 (1.0)

*P<0.0001.

†P<0.001 versus placebo. OR and p values versus placebo using logistic regression with treatment and concomitant MTX intake status as factors. LSM treatment difference and p values versus placebo using an analysis of covariance model with treatment group, visit and concomitant MTX intake status, as factors and baseline score as continuous covariate.

ACR, American College of Rheumatology; ASAS, Assessment of Spondyloarthritis international Society; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; FACIT-Fatigue, Functional Assessment of Chronic Illness Therapy Fatigue Scale; HAQ-DI, Health Assessment Questionnaire Disability Index; LSM, least squares mean; M, number of patients with evaluation; n, number of subjects satisfying the criterion; N, total number of randomised patients (full analysis set); SEC, secukinumab; VAS, Visual Analogue Scale.

across multiple clinical and imaging endpoints were shown in a population with high activity of inflammatory back pain treated with secukinumab.

Treatment recommendations for axial PsA, extrapolated from AS and therapeutic interventions including new classes of biologics, have not reported efficacy in the axial manifestations of PsA in an RCT setting. However, in a previous study,[9] in a combined cohort of patients with either PsA or AS from a single centre, 24% of the patients fulfilled the classification criteria (modified New York (mNY) or CASPAR) for both conditions indicating overlapping features of axial PsA and AS.

Nevertheless, the axial involvement, represents an unmet clinical need in determining the treatment strategy across all PsA manifestations and ultimately supports informed treatment

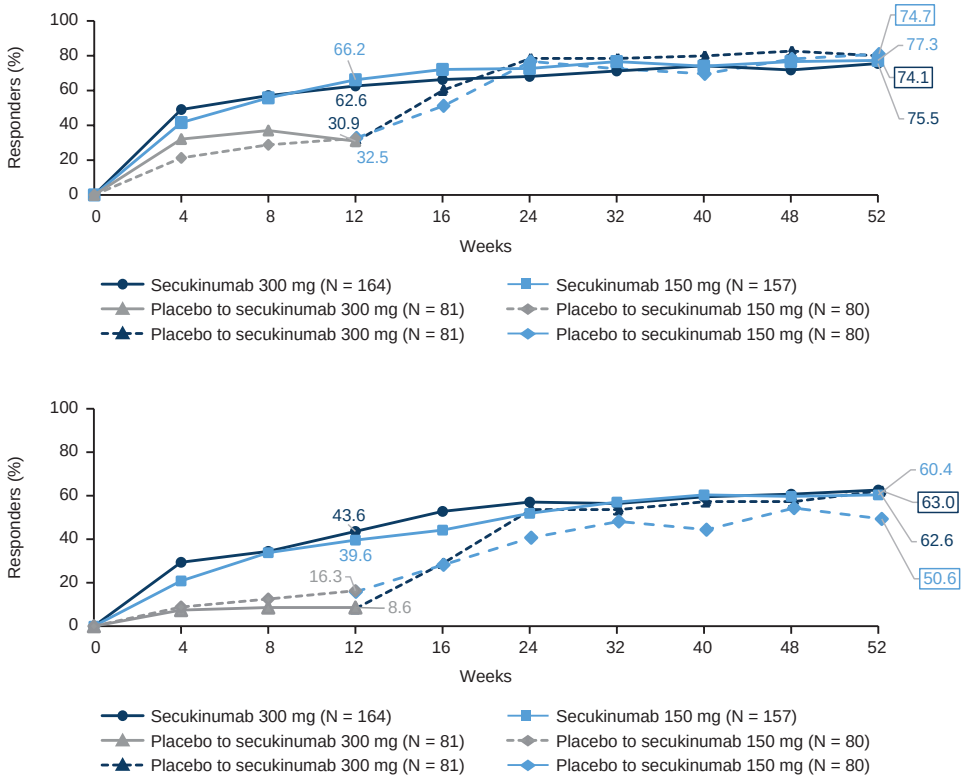

Figure 3 ASAS20 and ASAS40 responses through week 52*. (A) ASAS20. (B) ASAS40. *LOCF data in full analysis set. ASAS, Assessment of SpondyloArthritis international Society; LOCF, last observation carried forward.

decision-making. Additionally, patients with PsA tend to under-report axial symptoms and consequently the burden of disease might be underestimated for axial disease in such patients.[11 12] As a consequence, the efficacy of a biological treatment in managing axial symptoms in PsA has been investigated only in two observational studies to date, and never in a randomised controlled setting.[10–12]

In the MAXIMISE trial, secukinumab 300 mg and 150 mg demonstrated significant improvements across all primary, key secondary and secondary endpoints at week 12. Clinical

Table 3 Summary of secukinumab safety

	Treatment period 1 (week 12)			Entire treatment period (week 52)	
	Secukinumab 300 mg, s.c. (n=167)	Secukinumab 150 mg, s.c. (n=165)	Placebo (n=166)	Any Secukinumab 300 mg, s.c. (n=248)	Any Secukinumab 150 mg, s.c. (n=245)
Duration of exposure, days, Mean (SD)	84.6 (7.1)	84.9 (7.6)	84.9 (7.4)	313.4 (61.0)*	325.7 (39.4)*
Any AE, n (%)	67 (40.1)	61 (37.0)	80 (48.2.)	169 (68.1)	158 (64.5)
Any SAE, n (%)	4 (2.4)	1 (0.6)	4 (2.4)	13 (5.2)	14 (5.7)
AEs leading to study treatment discontinuation, n (%)	1 (0.6)	3 (1.8)	1 (0.6)	9 (3.6)	6 (2.4)
Death	0	0	0	1 (0.4)	0
Common AEs†	**n (%)**			**EAIR (95% CI)**	
Nasopharyngitis	9 (5.4)	4 (2.4)	11 (6.6)	14.8 (10.2 to 20.6)	9.4 (5.9 to 14.3)
URTI	3 (1.8)	5 (3.0)	5 (3.0)	4.9 (2.6 to 8.6)	5.9 (3.2 to 9.9)
Diarrhoea	4 (2.4)	2 (1.2)	4 (2.4)	6.7 (3.8 to 10.8)	2.9 (1.2 to 6.0)
AEs of special interest	**n (%)**			**EAIR (95% CI)**	
Candida infection‡	3 (1.8)	2 (1.2)	1 (0.6)	2.0 (0.7 to 4.7)	1.2 (0.3 to 3.6)
Crohn's disease	0	0	0	0.0 (0.0 to 1.5)	0.4 (0.0 to 2.3)
MACE	1 (0.6)	0	0	0.8 (0.1 to 2.9)	0.4 (0.0 to 2.3)
Malignancy§	0	0	0	0.8 (0.1 to 2.9)	0.4 (0.0 to 2.3)

*Exposure data for treatment period two in originally randomised groups (secukinumab 300 mg (n=167) and 150 mg (n=165)).
†AEs with an EAIR ≥5 in either of the secukinumab treatment groups over the entire treatment period.
‡Candida infections are reported as HLT (high level term).
§Malignancy are reported for standardised MedDRA query term malignant or unspecified tumours excluding basal cell carcinoma and squamous cell carcinoma.
AE, adverse event; EAIR, exposure adjusted incidence rate per 100-patient years; MACE, major adverse cardiovascular event; MedDRA, Medical Dictionary for Regulatory Activities; N, total number of randomised patients; s.c., subcutaneous; URTI, upper respiratory tract infection.

improvements were sustained through week 52 for the secukinumab arms; patients on placebo who switched to secukinumab 150 mg or 300 mg at week 12 improved rapidly and considerably across all assessed efficacy endpoints. In addition, MRI assessments demonstrated that secukinumab 300 mg and 150 mg significantly improved Berlin MRI scores versus placebo, providing objective evidence of reduced inflammation in both the spine and the SIJ for patients treated with secukinumab. Predefined exploratory analysis of the ASAS20/40 and BASDAI50 responses at week 12 by Baseline Berlin MRI score confirmed that MRI status at baseline did not have a significant effect on the outcome measures. The similar clinical responses in the MRI positive patients (for approximately 60% of the trial population) and the overall population regardless of MRI status at baseline further support the robustness of the clinical efficacy endpoints.

It is worth noting that the amount of active inflammation at baseline was lower compared with trials in active AS. However, the primary aim of the study was to assess the clinical outcomes of treatment with a bDMARD in axial PsA, and MRI positivity was not an inclusion criterion for the study. Nevertheless, since axial SpA and axial PsA may represent distinct disease entities although with overlapping features, such lower levels of objective signs of inflammation may be expected. Furthermore, the lack of a consensus in the definition of axial PsA has resulted in paucity of MRI data in axial PsA and hence there is no accurate benchmark of the expected levels of inflammation in terms of Berlin MRI score. It should also be noted that many studies have shown that MRI activity does not correlate with the burden of disease as measured by clinical assessments such as BASDAI, both for radiographic-axSpA and non-radiographic-axSpA) or, if at all, correlate very weakly with ASDAS before and after treatment.[21 22]

There is also an issue in PsA being a multifaceted condition as none of the available patient-reported outcomes (PROs) are specific to one domain, which is why MRI was assessed alongside the primary outcome of ASAS20 to allow an objective measurement of inflammation in the axial skeleton. However, it was decided not to mandate MRI changes to be included in the study to be as close as possible to the current clinical practice that is based on the clinical judgement of the treating physicians. Axial PsA is a poorly researched area, where further clinical insights, a universally accepted definition and disease specific endpoints are urgently needed. MAXIMISE, as the first randomised placebo-controlled study in this area, may provide clinically meaningful data on the treatment effects of a bDMARD on axial symptoms and a valuable data set to the research efforts on the classification and outcome measures of axial PsA.

The types and incidence of adverse events with secukinumab were comparable to placebo at week 12, with no apparent relation to dose. Over the entire treatment period, the rate of discontinuations due to adverse events and the rate of serious infections and *Candidiasis* was low for secukinumab and consistent with previously reported data for IL-17A inhibitors. One death occurred during the study. Overall, the safety profile of secukinumab was consistent with those published in previous reports.[16–18]

The limitations of the trial stem from the challenges in designing it; a major one being the lack of consensus in the clinical and/or imaging criteria to define this disease entity.[4 5] In addition, axial PsA is distinct from axSpA[3] and hence utilising mNY or ASAS criteria to determine the inclusion criteria for MAXIMISE would have been misinterpreted as having restricted its population to axSpA patients with psoriasis. Conversely, if stringent radiographic criteria had been applied, patients with clinical criteria of axial PsA without radiographic evidence would have been excluded and hence the results might have lacked generalisability to the whole axial PsA population.

Furthermore, the lack of axial PsA-specific outcome measures brought on the challenge of choosing the appropriate outcome measures. It is well-recognised that there is an unmet need for axial PsA specific outcome measures as ASAS and BASDAI although working well in AS trials, are not specific for axial inflammation in PsA. It should also be noted here that one of the ASAS response components is patient global assessment and BASDAI is impacted by the burden of peripheral arthritis, hence improvements in other domains of the disease may have influenced these results. The lack of randomised controlled trials and any precedent to aid the selection of axial PsA specific outcome measures, led to a general and inherent limitation. We therefore, selected ASAS20 as the primary endpoint as it was considered a valid option for a placebo-controlled randomised trial of this nature being the most frequently used outcome for assessing efficacy in axSpA trials. In addition, MAXIMISE included other assessments of axial symptoms such as ASAS40, BASDAI and ASDAS as secondary/exploratory outcomes and showed consistent results. Furthermore, greater improvements for both secukinumab 150 mg and 300 mg were shown versus placebo ($p<0.0001$) in the axial specific assessment of spinal pain indicating a clear effect of secukinumab on the axial skeleton. Finally, HLA-B27 data at baseline reported by the investigator was available for only 52% of the trial population.

In conclusion, secukinumab provided significant improvement in the signs and symptoms and objective signs of inflammation of axial disease in patients with psoriatic arthritis and inadequate response to NSAIDs. The clinical and imaging results from MAXIMISE provide valuable data that will support deepen the clinical understanding of axial PsA.

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Contributors All authors were involved in the drafting and critical review of the manuscript and approved the final version for submission. LG, SJ and AM-V were involved in the acquisition of clinical data and participated as investigators in the clinical study. XB, LG, LCC, BS, MR and CP were involved in the conception or design of the study. EP, BS and KN were involved in the analysis of the data. All authors were involved with the interpretation of the results. All authors agreed to be accountable for all aspects of the work and attest to the accuracy and integrity of the work.

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Competing interests XB: Grant/research support from: AbbVie, and Novartis, Consultant for: AbbVie, BMS, Celgene, Chugai, Galapagos, Gilead, MSD, Novartis, Pfizer, and UCB; Speakers bureau: AbbVie, BMS, Celgene, Chugai, MSD, Novartis, Pfizer, and UCB. LG: Research grants: Amgen, Lilly, Janssen, Pfizer, Sandoz, Sanofi, and Galapagos; consulting fees: AbbVie, Amgen, BMS, Biogen, Celgene, Gilead, Janssen, Lilly, Novartis, Pfizer, Samsung Bioepis, Sanofi-Aventis and UCB. EP: Employee of Novartis with Novartis stock. SJ: Research support/speaker: AbbVie, Pfizer, Roche, Novartis, MSD, Sandoz, Lilly, Egis, UCB and Celgene. AM-V: None declared. SD: Consultant for AbbVie, Biogen, BMS, Celgene, Lilly, MSD, Novartis and UCB; Speakers bureau: AbbVie, BMS and Celgene, Lilly, Novartis, Pfizer and Sanofi. BS: Employee of Novartis. MR: Employee of Novartis with Novartis stock. KN: Employee of Novartis. CP: Employee of Novartis with Novartis stock. LCC: Grant/research support: AbbVie, Janssen, Lilly, Novartis and Pfizer; Consultant for: AbbVie, Amgen, Biogen, Celgene, Pfizer, UCB, Boehringer Ingelheim, Novartis, Lilly, Janssen, Sun Pharma, Prothena and Gilead.

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TRANSLATIONAL SCIENCE

IL-23 skin and joint profiling in psoriatic arthritis: novel perspectives in understanding clinical responses to IL-23 inhibitors

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ABSTRACT

Objectives To determine the relationship between synovial versus skin transcriptional/histological profiles in patients with active psoriatic arthritis (PsA) and explore mechanistic links between diseased tissue pathology and clinical outcomes.

Methods Twenty-seven active PsA patients were enrolled in an observational/open-label study and underwent biopsies of synovium and paired lesional/non-lesional skin before starting anti-tumour necrosis factor (TNF) (if biologic-naïve) or ustekinumab (if anti-TNF inadequate responders). Molecular analysis of 80-inflammation-related genes and protein levels for interleukin (IL)-23p40/IL-23p19/IL-23R were assessed by real-time-PCR and immunohistochemistry, respectively.

Results At baseline, all patients had persistent active disease as per inclusion criteria. At primary end-point (16-weeks post-treatment), skin responses favoured ustekinumab, while joint responses favoured anti-TNF therapies. Principal component analysis revealed distinct clustering of synovial tissue gene expression away from the matched skin. While *IL12B, IL23A* and *IL23R* were homogeneously expressed in lesional skin, their expression was extremely heterogeneous in paired synovial tissues. Here, IL-23 transcriptomic/protein expression was strongly linked to patients with high-grade synovitis who, however, were not distinguishable by conventional clinimetric measures.

Conclusions PsA synovial tissue shows a heterogeneous IL-23 axis profile when compared with matched skin. Synovial molecular pathology may help to identify among clinically indistinguishable patients those with a greater probability of responding to IL-23 inhibitors.

Key messages

What is already known about this subject?

- Psoriatic arthritis (PsA) is a chronic heterogeneous inflammatory condition affecting patients with psoriasis, and the interleukin (IL)-23/IL-17 axis is believed to be key in psoriasis and PsA pathogenesis.
- Several drugs targeting the IL-23/IL-17 axis have been successfully tested in the context of psoriasis and PsA but, while 50%–60% of patients achieve almost complete psoriasis clearance on treatment, the joint disease improvement is modest. To date, the mechanism for the divergent skin-joint response remains largely unexplained.

What does this study add?

- It provides first-time detailed evidence of the expression of the IL-23 axis in matched skin and synovial tissue from active PsA patients demonstrating distinct gene expression clustering of the synovium away from paired skin. It reveals that, while *IL23A, IL12B* and *IL23R* are expressed at a high level in lesional skin, their expression in the synovium is hugely heterogeneous.
- It demonstrates that, while patients with diverse degrees of synovial inflammation could not be distinguished clinically by conventional clinimetric measures, the IL-23 axis signature is differentially expressed within the synovial tissue and strongly linked to high-grade synovitis.

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic heterogeneous inflammatory condition occurring in up to 30% of patients with skin and/or nail psoriasis (PsO), which variably affects the spine, peripheral synovial joints and entheses.[1] Although the mechanisms for such disease heterogeneity are not entirely clear, the interleukin (IL)-23/IL-17 axis is believed to be key in PsO and PsA pathogenesis.[2 3]

IL-23 is a proinflammatory cytokine composed of two subunits (p40, in common with IL-12, and p19, IL-23-specific) and mostly produced by keratinocytes, dendritic and myeloid cells. By binding its cognate receptors (IL-23R/IL-12Rβ1), it stabilises RAR-related-orphan-receptor-gamma-t (RORγt) in T-helper-17 cells, which, in turn, release their effector cytokines IL-17, IL-21 and IL-22 to initiate and amplify local autoimmune reactions and chronic inflammation.[2]

Several drugs targeting the IL-23/IL-17 axis have been successfully tested in PsO and PsA.[2] For example, ustekinumab and secukinumab, inhibitors of IL-12/IL-23p40 and IL-17A respectively, are recommended as a second-line biological treatment for PsA patients inadequate responders to conventional-synthetic (cs) disease-modifying antirheumatic drugs (DMARDs) who had failed at least one tumour necrosis factor (TNF) inhibitor (TNFi).[4 5] However, by blocking these pathways, while 47%–64% of patients achieve a 75%-improvement in skin disease (Psoriasis Area and

Key messages

How might this impact on clinical practice or future developments?

- This study demonstrates that psoriatic arthritis synovial tissue shows a heterogeneous interleukin 23 (IL-23) axis profile independently of its expression in paired-skin samples, thus providing a plausible mechanistic explanation for the divergent skin and joint clinical response to IL-23 inhibitors. It supports the need to test in larger appropriately designed and powered studies whether drug-target bioavailability correlates with the likelihood of response. Identifying biomarkers of joint response to therapy in patients clinically indistinguishable is going to be vital to improve disease outcomes, prevent disability and reduce healthcare and societal costs.

Severity Index (PASI75)), success in treating joints is more modest, and a mere 20% improvement (American College of Rheumatology (ACR20)) is observed in 35%–50% of patients.[6 7] The new IL-23p19 selective inhibitors have been shown to be more effective, and ACR20 is reached in approximately 60%.[8 9] However, while a similar proportion of patients achieve almost complete PsO clearance (PASI90), high hurdles joint disease ACR50/ACR70 is achieved in only 33%–36% and 13%–20% of patients, respectively.[8 9]

To date, the mechanism for such divergent skin-joint response, consistent across multiple trials, remains largely unexplained. Boutet *et al*[2] and Belasco *et al*[10] have postulated that different target expression levels in skin and joints contribute to the diverse clinical response. For example, Belasco *et al* reported that gene expression patterns in skin and synovium are distinct, showing a stronger IL-17 signature in skin than in synovium, and more equivalent TNF signal across both tissues.[10] Here, we present new evidence exploring the expression of the IL-12/IL-23 axis in psoriatic skin versus matched synovial tissue at both molecular and protein level.

METHODS

Full methods are included in online supplemental material. Briefly, 27 patients fulfilling the Classification Criteria for Psoriatic Arthritis (CASPAR)[11] with active peripheral joint disease despite csDMARDs and either biologic-naïve/ failing TNFi were recruited in this observational/open-label study (REC15/LO/0584). Patients underwent a baseline ultrasound (US)-guided synovial biopsy[12] and lesional/non-lesional skin punch-biopsies, and were then treated with TNFi/ustekinumab as per local guidelines. The chosen primary endpoint was 16 weeks. Gene expression was analysed by real-time PCR (Fluidigm). Paraffin-embedded skin/synovium samples were stained with H&E. Immune cells/IL-23-axis were quantified by immunohistochemistry. Synovial tissue were categorised in 'low-grade'(score 0–1) or 'high-grade'(score 2–7) synovitis[13] and in pathotypes (lympho-myeloid/diffuse-myeloid/pauci-immune).[14]

RESULTS

Patients' characteristics and treatment response

Baseline and 16 weeks demographic and clinical features are summarised in figure 1. The overall male to female ratio was ~1:1 (59% female), the average age was 45.4±12.5 and disease duration >10 years. Seventy-eight per cent of patients had concomitant skin involvement, with a mean PASI of 7.8. As per inclusion criteria, all patients had active joint disease (68-tender joints count 30.9±19.2, 66-swollen joints count 13±10.4, Disease Activity Score (DAS) 4.3±1.1) despite treatment with csDMARDs±anti TNF. Following the baseline biopsy, patients were treated with anti-TNF (n=18) if they were biologic-naïve or ustekinumab (n=9) if they had not responded to at least one TNFi. The higher number of females in the ustekinumab-arm (8/9) reflects the gender differences in TNFi-treatment outcomes observed in registries[15] (figure 1A). At 16 weeks, ESR, tender-joint scores, Ritchie Articular Index (RAI), Visual Analogue Scale (VAS)-pain, Likert-physician-assessment and DAS were significantly higher in the ustekinumab-treated group; PASI-scores improved from baseline in both groups (−4.7±7.5 in TNFi treated vs −8.9±14.3 in ustekinumab treated) and were comparable between the two treatment arms (2.3±2.6 in TNFi treated vs 2.3±2.3 in ustekinumab treated) (figure 1B). However, while significantly more patients in the anti-TNF group achieved EULAR(DAS)-response compared with ustekinumab-treated patients (70.6% vs 22.2%), there was a trend in favour of ustekinumab in terms of skin responses (figure 1C). Besides, as joint response to ustekinumab can be delayed up to 24–28 weeks, clinical responses were also assessed at 24 weeks. As shown in online supplemental figure S1, ustekinumab-treated patients maintained significantly higher tender joint scores, RAI, VAS-pain, Likert physician assessment and DAS; 50% and 68.8% of patients in the ustekinumab and TNFi arms achieved EULAR(DAS) response, respectively. Individual patient joint/skin responses are summarised in online supplemental table S1.

Gene expression profiles in paired skin and synovium reveal tissue-specific signatures and divergent expression patterns

Gene expression analysis was performed on 14 matched synovial tissue, lesional and adjacent non-lesional skin. As shown in figure 2A, principal component analysis (PCA), built on the expression of 80 inflammation-related genes (online supplemental table S2), showed that the synovium clusters away from the skin, with a partial overlapping of lesional and non-lesional skin. To further investigate the gene variance contributing to the diversity of expression within each anatomic site (skin/synovium), related PCA plots were covisualised with loading plots (biplots) (figure 2B and C). *IL17A/F*, *IL23R* and *IL21* were the major contributors of PC1/2 variation in lesional skin. In synovium, genes related to ectopic lymphoid structure (ELS) formation (*CXCL13*, *CXCR5*) and the IL-23 axis (*IL23A*, *IL12B*, *IL23R*) together strongly contributed to the PC variation. For instance, *CXCR5* and *IL23A* robustly aligned with PC1 in accounting for 35.4% of the variance within the synovium data set and *CXCL13* strongly and equally contributed to PC1 and PC2 variation. We next assessed the relative gene expression of the drug-targets of TNF- and IL-23/IL-12-inhibitors, that is, *TNF*, *IL23A* (encoding IL-23p19), *IL12B* (encoding IL-23p40) and *IL23R* (figure 2D). *TNF* was generally homogeneously expressed in both skin and synovial tissue. Conversely, *IL23A*, *IL12B* and *IL23R* showed higher expression in lesional skin compared with both non-lesional skin and synovium. Interestingly, we observed that while some patients did express IL-23 cytokines/receptor in both skin and joint, others had discordant expression, that is, active IL-23 pathway in the lesional skin but not in the synovium. To investigate potential mechanisms for the diverse expression of the IL-23-axis within the synovium, we stratified patients based on the degree of synovial inflammation.[13] Both *IL12B* and *IL23R* genes, but not *IL23A*, were significantly more expressed in patients with higher synovitis scores (figure 2E). Notably, despite the major variance in the degree of synovial inflammation and histological pathotypes, there were no significant clinical differences in the two patient groups (online supplemental table S3,S4).

A

	TOTAL POPULATION (27 PATIENTS)	ANTI-TNF (18 PATIENTS)	USTEKINUMAB (9 PATIENTS)	p-values
BASELINE				
Female % (n)	59% (16)	44.4% (8)	88.9% (8)	p=0.03
Age years (mean ± SD)	45.4 ± 12.5	42.2 ± 11.2	51.8 ± 12.9	ns
Disease duration years (mean ± SD)	10.2 ± 11.8	7.3 ± 7.2	15.3 ± 16.6	ns
ESR mm/hr (mean ± SD)	24 ± 16.7	22.8 ± 15.4	26.3 ± 19.8	ns
CRP mg/l mean (mean ± SD)	11.5 ± 21.1	11.4 ± 22.7	11.7 ± 18.6	ns
TJC/78 (mean ± SD)	34.2 ± 22.6	31 ± 25.1	40.7 ± 15.9	ns
TJC/68 (mean ± SD)	30.9 ± 19.2	27.7 ± 21.2	37.2 ± 13.1	ns
RAI (mean ± SD)	18.7 ± 9.8	16.6 ± 10.7	23.1 ± 5.9	ns
SJC/76 (mean ± SD)	13.6 ± 10.8	11.6 ± 8.5	17.7 ± 13.9	ns
SJC/66 (mean ± SD)	13 ± 10.4	11.3 ± 8.6	16.3 ± 13.1	ns
SJC/44 (mean ± SD)	10.3 ± 8.3	9.2 ± 7.8	12.6 ± 9.3	ns
Skin involvment, yes % (n)	78% (21)	83,3% (15)	66.7% (6)	ns
PASI (mean ± SD)	7.8 ± 10.7	6.5 ± 8.1	11 ± 16	ns
VAS tiredness (mean ± SD)	60 ± 30.2	59.1 ± 30.4	61.8 ± 31.6	ns
VAS pain (mean ± SD)	70.6 ± 21.7	70.3 ± 17.1	71.2 ± 30.2	ns
VAS GH Patient (mean ± SD)	70 ± 25.1	72.3 ± 20.8	65.4 ± 33.1	ns
VAS GH Physician (mean ± SD)	62.2 ± 16.2	61.2 ± 16.4	64.2 ± 16.5	ns
LIKERT Patient (mean ± SD)	3.8 ± 0.8	3.8 ± 0.8	3.9 ± 0.9	ns
LIKERT Physician (mean ± SD)	3.6 ± 0.6	3.4 ± 0.6	3.8 ± 0.7	ns
DAS (mean ± SD)	4.3 ± 1.1	4.1 ± 1.2	4.8 ± 1	ns
HAQ (mean ± SD)	1.9 ± 0.6	1.8 ± 0.6	2.1 ± 0.5	ns
Previous anti-TNF, yes % (n)	33.3% (9)	0% (0)	100% (9)	p<0.0001
Current DMARDs, yes % (n)	70.4% (19)	66.7% (12)	77.8% (7)	ns

B

	TOTAL POPULATION (26 PATIENTS)	ANTI-TNF (17 PATIENTS)	USTEKINUMAB (9 PATIENTS)	p-values
16 WEEKS				
ESR mm/hr (mean ± SD)	17.6 ± 14.6	12.2 ± 8.8	27.7 ± 18.4	p=0.05
CRP mg/l mean (mean ± SD)	6.3 ± 8.7	4.2 ± 6.7	10.4 ± 10.8	ns
TJC/78 (mean ± SD)	25.7 ± 22.1	28.2 ± 20.9	39.7 ± 17.9	p=0.01
TJC/68 (mean ± SD)	23.3 ± 19.5	16.1 ± 18.0	36.8 ± 15.0	p=0.007
RAI (mean ± SD)	13.9 ± 10.6	10.9 ± 11.1	19.4 ± 7.2	p=0.02
SJC/76 (mean ± SD)	8.3 ± 9.3	7.4 ± 8.0	10.1 ± 11.8	ns
SJC/66 (mean ± SD)	7.8 ± 9.0	7.0 ± 8.0	9.4 ± 11.0	ns
SJC/44 (mean ± SD)	5.8 ± 6.2	5.6 ± 6.0	6.3 ± 6.9	ns
PASI (mean ± SD)	2.2 ± 2.5	2.3 ± 2.6	2.3 ± 2.3	ns
VAS tiredness (mean ± SD)	54 ± 31.6	50.1 ± 31.9	61.3 ± 31.6	ns
VAS pain (mean ± SD)	46.7 ± 32.2	37.2 ± 30.2	64.6 ± 29.4	p=0.05
VAS GH Patient (mean ± SD)	50.2 ± 33.4	46.5 ± 33.9	57.1 ± 33.3	ns
VAS GH Physician (mean ± SD)	45.0 ± 26.0	38.8 ± 24.3	56.7 ± 26.1	ns
LIKERT Patient (mean ± SD)	2.9 ± 1.1	2.8 ± 1.1	3.1 ± 1.2	ns
LIKERT Physician (mean ± SD)	2.9 ± 1.3	2.5 ± 1.2	3.8 ± 1.1	p=0.01
DAS (mean ± SD)	3.4 ± 1.4	3.0 ± 1.5	4.1 ± 0.9	p=0.03
HAQ (mean ± SD)	1.5 ± 0.9	1.2 ± 1.0	2.0 ± 0.7	ns

C

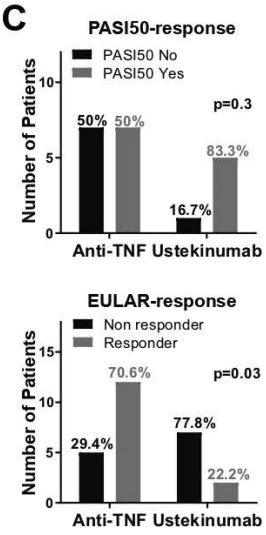

Figure 1 Baseline and 16 weeks characteristics of the patients included in the psoriatic arthritis pathobiology and its relationship with clinical disease activity (PsABRE) study. (A) Baseline features of the whole cohort (n=27) and comparison of variables between patients receiving anti-TNF (n=18) or ustekinumab (n=9). (B) Patients' characteristics at the chosen primary endpoint, that is, 16-weeks post-treatment (n=26, one patient lost to follow-up) and comparison between TNFi- (n=17) and ustekinumab-treated patients (n=9). (A, B) P values calculated using Mann-Whitney U test or Fisher's exact test as required (TNFi-arm vs ustekinumab-arm). (C) Skin (PASI50) and joints (EULAR(DAS) good/moderate vs none) response at 16 weeks. P values calculated using Fisher's exact test. CRP, C reactive protein; DAS, Disease Activity Score; DMARDs, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; N, number; NS, non-significant; PASI, Psoriasis Area and Severity Index; RAI, Ritchie Articular Index; SJC, swollen joints count; TJC, tender joints count; TNF, tumour necrosis factor; VAS, Visual Analogue Scale (0–100).

Synovial IL-23p40/p19 and IL-23R protein expression correlates with the histological inflammatory status

To confirm the molecular findings, we next evaluated protein expression levels of IL-23p40, IL-23p19 and IL-23R in skin and synovium by immunohistochemistry. As expected, the percentage of IL-23p40-, IL-23p19- and IL-23R-positive cells was significantly higher in lesional skin compared with paired non-lesional skin (figure 3A,B); within the synovium, it was greater in patients with higher degree of inflammation (figure 3C,D) and in lympho-myeloid and diffuse-myeloid pathotypes (online supplemental figure S2). This result was in line with the positive correlation observed between the synovial inflammatory score and the proportion of IL-23p40/IL-23p19/IL-23R-positive cells (figure 3E), as well as their correlation with each other's (data not shown). Of note, the percentage of IL-23p40/IL-23p19/IL-23R-positive cells at baseline was, on average, comparable between the treatment groups despite different drug exposure (online supplemental table S5). Except for the LIKERT patient score, we did not detect other significant correlations between IL-23-axis expression and clinical parameters at baseline, suggesting that patients with comparable disease severity may have, in fact, heterogeneous histopathological features and expression of drug

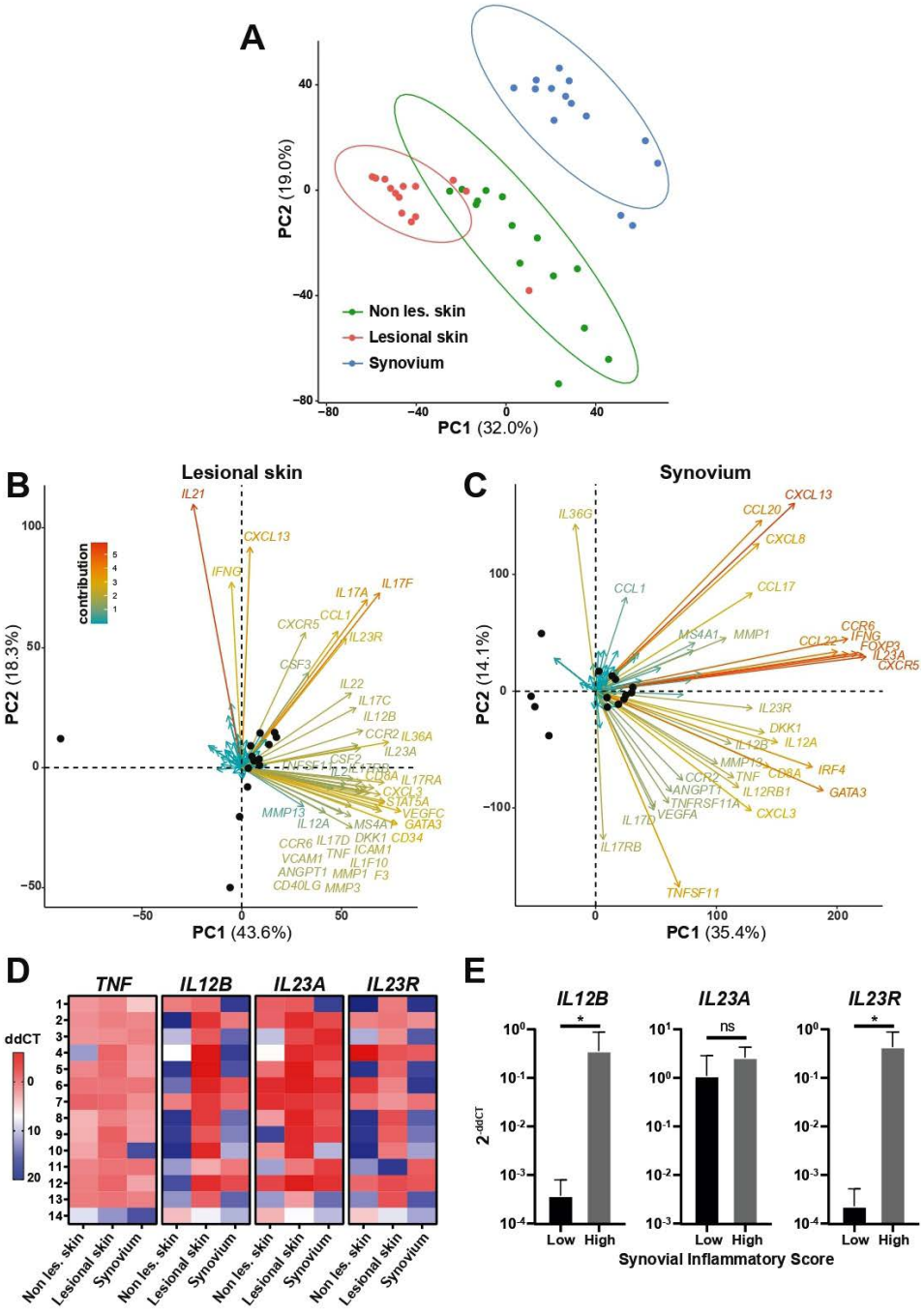

Figure 2 Gene expression analysis in matched skin and synovium from PsA patients. (A) Principal component (PC) analysis (PCA) performed on the expression data of a set of 80 selected genes in 14 matched non-lesional (non les.) and lesional skin and synovium. The first two eigenvalues were plotted with data ellipses for each tissue type using a CI of 0.95. The PCA clearly separates synovium (blue dots) from non-lesional skin (non les., green dots) and lesional skin (red dots). (B, C) Biplots showing individuals repartition in PC1 and 2 (black dots) and loading plots assessing the contribution of each of the 80 genes analysed in the PC, displayed for the lesional skin (B) and synovial tissues (C). Genes names are indicated if their contribution to the PC variance is >1. The PCA and biplots were created using function prcomp from the stats package within R statistics (version 3.5.3) and factoextra R package[20] (D) Heatmap representing *TNF*, *IL12B* (IL-23p40 protein), *IL23A* (IL-23p19 protein) and *IL23R* expression in 14 matched non-lesional (non les.) and lesional skin and synovium samples. dd-threshold cycles (ddCTs) are shown in colorimetric scale (low expression in blue, high expression in red). Lines 1–11 represent anti-TNF-treated patients, lines 12–14 ustekinumab-treated patients. E, *IL12B*, *IL23A* and *IL23R* gene expression in synovial biopsies classified as 'low' (0–1) and 'high' (2–7) synovial inflammatory score (Krenn's score). P values were calculated using Mann-Whitney U test, *P<0.05, mean and SD are shown. IL-23, interleukin 23; TNF, tumour necrosis factor.

targets within the diseased synovium (online supplemental table S6).

To further assess whether the IL-23-axis heterogeneity tracks across different stages of the disease, we analysed IL-23 expression pattern in the synovium of 21 treatment-naïve PsA patients with <12 months symptoms. As shown in online supplemental figure S3, overall, there was a positive correlation between IL-23p40/IL-23p19/IL-23R-positive cells and synovitis scores, and lower

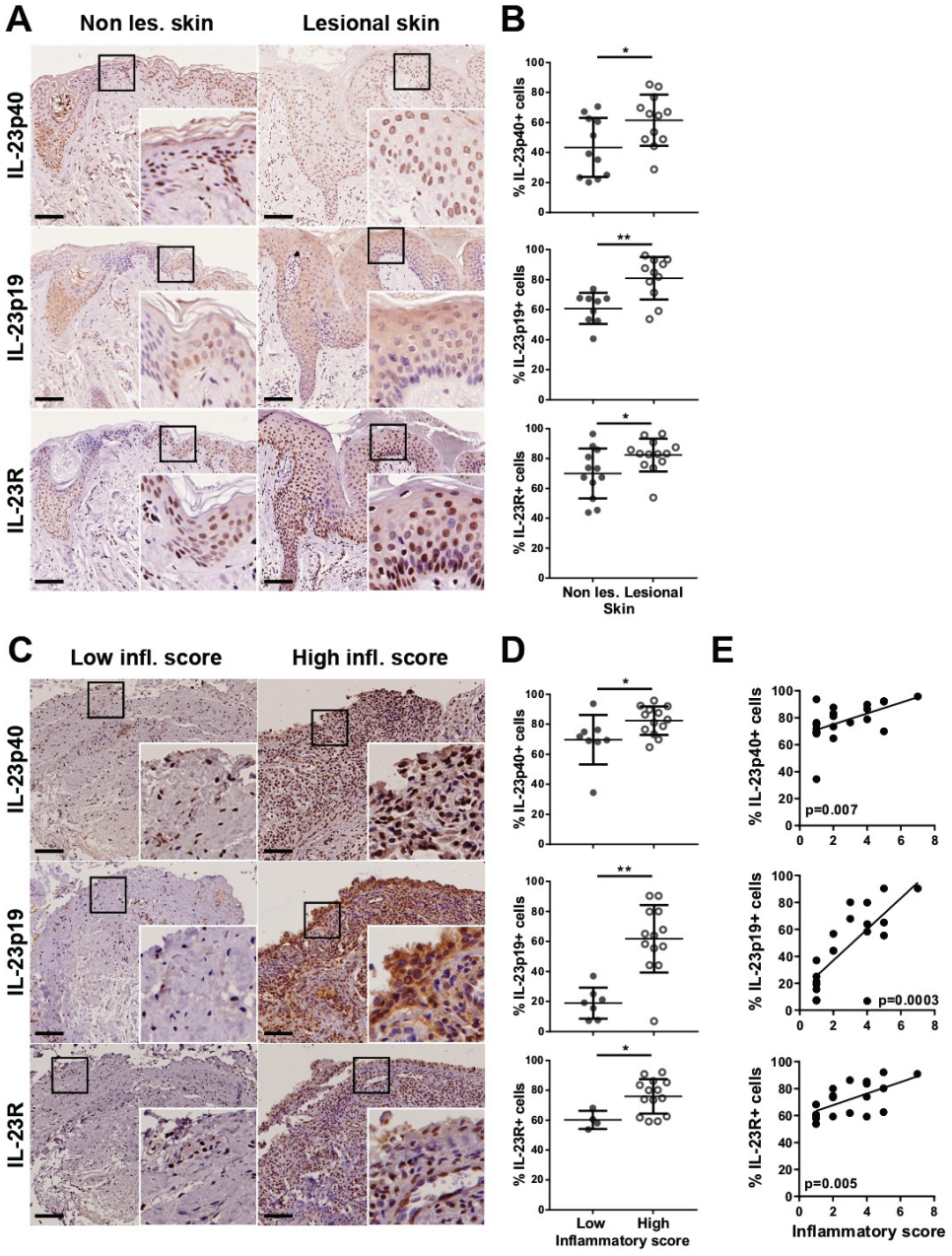

Figure 3 Expression of IL-23p40, IL-23p19 and IL-23R in skin and synovium from PsA patients. (A, C) representative images of sections of PSA non-lesional (non les.) and lesional skin (A) and synovial tissue of different degree of inflammatory scores (C) immunostained for IL-23p40, IL-23p19 and IL-23R. Scale bar=200 µm. Enlarged images correspond to the respective boxed areas. (B, D) Digital image analysis was performed on non-lesional and lesional skin (B) (n=11–12) and synovium (D) (low inflammatory score, n=4–8; high inflammatory score, n=13–14) sections. IL-23p40, IL23p19 and IL23R positive cells were determined using QuPath software[21] and are presented as % of the total number of cells. Results are shown as mean±SD. *P<0.05, **P<0.01 as assessed by Mann-Whitney U test. (E) Correlations between inflammatory scores and IL-23p40, IL-23p19 or IL-23R percentages of positive cells within the synovial tissue. P values, calculated by Spearman's bivariate correlation analysis, are indicated on each graph. IL-23, interleukin 23; PsA, psoriatic arthritis.

IL-23 cytokines/receptor tissue-availability in the pauci-immune compared with macrophage-rich pathotypes. Similarly to established PsA, we did not find significant correlations between clinical parameters and IL-23 axis expression. Finally, to investigate whether the differential IL-23-expression observed in PsA synovium was disease-specific or related to synovial histopathology, we quantified IL-23p40/IL-23p19/IL-23R in a cohort of 17 treatment-naïve rheumatoid arthritis (RA) patients spanning diverse degrees of synovial inflammation and histopathotypes, and confirmed that, at least in the early phases of RA, IL-23 expression pattern is pathology related and significantly associates with the presence of ELS (online supplemental figure S4).

DISCUSSION

To our knowledge, this study provides first-time detailed evidence of the expression of the IL-23 axis (IL-23p40/IL-23p40p19/IL-23R), both at transcript and protein level, in matched skin-synovium obtained from clinically active PsA patients before undergoing anti-TNF or ustekinumab.

Using a PCR-Fluidigm-assay of 80 inflammation-related genes, first, we demonstrated distinct synovial gene expression clustering away from paired skin but a partial overlapping between lesional and non-lesional skin profiles. We also showed that IL-17 and IL-23 cytokines together with CXCL13/CXCR5, key chemokines involved in ELS formation, significantly contribute to the gene expression variance within skin and joint sites, respectively. These results are in line with those reported by Belasco *et al*[10] demonstrating that IL-17 is a major contributor of the gene expression variability within the lesional skin, and Celis *et al*[16] who showed that in synovial biopsies (unmatched for skin samples) the expression of IL-23 correlates with ELS-positive samples.

The analysis of the expression profiles of biological DMARDs targets demonstrated that *TNF* was more homogeneously expressed in skin and synovial tissue, while *IL23A*/*IL12B*/*IL23R* were generally higher-expressed in lesional skin compared with both non-lesional skin and synovium. The synovial expression of *IL23A*/*IL12B*/*IL23R* was, in fact, greatly heterogeneous and could be either similar to or much lower than the paired lesional skin. Notably, *IL12B* and *IL23R* transcripts levels were dependent on the degree of tissue inflammation, being more expressed in the presence of higher synovitis scores. Similarly, we confirmed a preferential expression of IL-23p40/IL-23p19/IL-23R proteins in patients with high-grade synovitis and immune-cells-rich histopathotypes. Importantly, patients with variable degrees of synovial inflammation and diverse pathotypes, as well as different levels of IL-23-cytokines/receptor could not be phenotypically distinguished by conventional clinical scores. Furthermore, despite variable drug exposure, the pathology of the IL-23 axis in active patients was comparable at baseline. We confirmed that IL-23-axis expression relates to the synovial histopathology not only in PsA at different stages of the disease, including early treatment-naïve patients, but also in the early phase of RA, investigated as disease control. Therefore, the pattern of expression of the IL-23 axis does not seem to be disease-specific but rather dependent on the inflammatory status and histological features of the synovial tissue in both PsA and RA.

While it is generally accepted that patients with high disease activity respond better to biologics, clinimetric measures cannot determine the grade of histological synovitis or drug-target expression levels. Tissue bioavailability of the 'target', of course, does not guarantee clinical response; however, there is evidence to suggest that, for example, TNF levels in RA synovium are associated with better response to TNFi,[17] and other specific synovial tissue signatures are linked with different outcomes to anti-TNF[18] and anti-IL-6R therapy.[19] The results reported here support the concept that heterogeneous drug target bioavailability in the diseased tissue might also apply to the IL-23 axis. This prompted the hypothesis that different joint response rates in PsA, often divergent from the skin-response, might be explained, at least partially, by the preferential expression of the IL-23-axis by subsets of patients with higher histological synovitis but not necessarily higher disease activity.

PsABRE was an exploratory study, not designed to assess efficacy; thus, the relatively small sample size in each treatment-arm did not allow to test the above hypothesis. Moreover, no direct comparisons could be carried out between the anti-TNF- and the ustekinumab-treated cohorts: both populations failed to respond to csDMARDs, but while the former was biologic-naïve, the latter had inadequately responded to at least one TNFi representing, therefore, a more difficult-to-treat group. The trial took place in a real-life setting with no external or industry support; hence, the recruitment and treatment allocation had to follow the UK National Institute for health and Care Excellence prescription guidelines with consequent different drug exposure in the two groups. Despite these limitations, the main value of the study resides in its molecular pathology characterisation of paired skin and US-guided synovial biopsies of the most inflamed joint, including small joints, that demonstrates a divergent profile between the two diseased tissues and, generally, a lower level of expression of the IL-23 axis in the synovial tissue particularly in patients with low-grade synovitis.

The heterogeneous synovial expression of the IL-23-axis provides a plausible mechanistic explanation for the divergent outcomes consistently observed in clinical trials whereby IL-23i have better results in PsA skin than in joints. This hypothesis needs to be tested in larger, appropriately designed and powered studies. Identifying biomarkers of joint-response to therapy in patients clinically indistinguishable is going to be vital to refine PsA clinical classification and enrich for treatment response while reducing unnecessary exposure to costly and potentially toxic medications.

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EPIDEMIOLOGICAL SCIENCE

Vitamin K antagonist anticoagulant usage is associated with increased incidence and progression of osteoarthritis

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ABSTRACT

Objectives Vitamin K is hypothesised to play a role in osteoarthritis (OA) pathogenesis through effects on vitamin K-dependent bone and cartilage proteins, and therefore may represent a modifiable risk factor. A genetic variant in a vitamin K-dependent protein that is an essential inhibitor for cartilage calcification, matrix Gla protein (MGP), was associated with an increased risk for OA. Vitamin K antagonist anticoagulants (VKAs), such as warfarin and acenocoumarol, act as anticoagulants through inhibition of vitamin K-dependent blood coagulation proteins. VKAs likely also affect the functioning of other vitamin K-dependent proteins such as MGP.

Methods We investigated the effect of acenocoumarol usage on progression and incidence of radiographic OA in 3494 participants of the Rotterdam Study cohort. We also examined the effect of *MGP* and *VKORC1* single nucleotide variants on this association.

Results Acenocoumarol usage was associated with an increased risk of OA incidence and progression (OR=2.50, 95% CI=1.94–3.20), both for knee (OR=2.34, 95% CI=1.67–3.22) and hip OA (OR=2.74, 95% CI=1.82–4.11). Among acenocoumarol users, carriers of the high *VKORC1(BB)* expression haplotype together with the *MGP* OA risk allele (rs1800801-T) had an increased risk of OA incidence and progression (OR=4.18, 95% CI=2.69–6.50), while this relationship was not present in non-users of that group (OR=1.01, 95% CI=0.78–1.33).

Conclusions These findings support the importance of vitamin K and vitamin K-dependent proteins, as MGP, in the pathogenesis of OA. Additionally, these results may have direct implications for the clinical prevention of OA, supporting the consideration of direct oral anticoagulants in favour of VKAs.

Key messages

What is already known about this subject?

- Osteoarthritis (OA) is the most common form of arthritis worldwide, affecting 320 million people, and is a leading cause of disability. To date, there are no disease-modifying therapies available, and treatment development has been hampered by existence of only few recognised modifiable risk factors.
- Vitamin K and vitamin K-dependent proteins, such as matrix Gla protein (MGP), have been implicated in OA by epidemiological studies, genetic studies and subsequent in functional genomics studies, indicating vitamin K as possible modifiable risk factor for OA.

What does this study add?

- This study shows that the use of vitamin K antagonist anticoagulants (VKAs) significantly increases the risk of progression of hip and knee OA, by inhibiting the vitamin K pathway.
- This study also demonstrates that known OA genetic risk variants in *MGP* and pharmacogenetic variants known to affect vitamin K metabolism increase the risk of OA progression when using VKAs.

How might this impact on clinical practice or future developments?

- The findings suggest the consideration of novel (or direct) oral anticoagulants in favour of VKAs, such as acenocoumarol and warfarin, in people with OA.

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INTRODUCTION

Osteoarthritis (OA) is a chronic disabling joint disease that also increases in prevalence with age. It is the most common form of arthritis, one of the fastest growing chronic diseases worldwide,[1] and is the fourth leading cause of years lived with disability globally.[2] To date, there are no known therapies that can alter its progression or prevent its occurrence. Apart from obesity and knee injury, very few other modifiable risk factors have been identified. Vitamin K has been hypothesised to play a role in OA pathogenesis through its effects on several vitamin K-dependent bone and cartilage proteins,[3] and therefore may represent a modifiable risk factor. A number of observational studies reported an association between vitamin K status and prevalence and incidence of OA.[4–6] There has been one modestly sized clinical trial studying the effect of vitamin K supplementation on OA progression. This ancillary study, originally designed to study vascular calcification, reported no overall beneficial effects of vitamin K supplementation. However, in individuals with insufficient vitamin K levels at baseline, a beneficial effect was observed.[7] No studies to

date have evaluated the relation between vitamin K antagonist anticoagulants (VKAs) and OA, which can be expected to result in low vitamin K functioning, which may lead to increased OA incidence or progression.[4–6]

Vitamin K is an essential cofactor in the post-translational γ-carboxylation of glutamic acid to form γ-carboxyglutamic acid (Gla) residues, which confer functionality to Gla proteins. VKAs deplete the active form of vitamin K by inhibiting the enzyme vitamin K epoxide reductase complex 1 (*VKORC1*). Genetic variants of *VKORC1* account for approximately 25% of the variance in VKA dose.[8] Matrix Gla protein (*MGP*) is a vitamin K-dependent Gla protein that is an essential inhibitor of cartilage and vascular mineralisation.[9 10] Recently, genome-wide association study (GWAS) and functional studies identified *MGP* to be causally involved in OA.[11 12]

VKAs such as warfarin and acenocoumarol are primarily prescribed for the prevention of thromboembolic events in patients with atrial fibrillation (AF).[13] With ageing-related increases in prevalence of AF, the projected number of individuals with AF needing anticoagulation is predicted to rise to 17.9 million by 2060 in the European Union.[14] While a new class of anticoagulants are available, the direct oral anticoagulants (DOACs), VKAs are still widely prescribed, particularly to older adults.[15] Whether long-term VKA use with resultant impairment of vitamin K-dependent proteins such as *MGP* increases risk of OA incidence or progression is not known. Given the high prevalence of VKA users in addition to the high prevalence of OA globally, clarifying this relationship would have substantial public health impact by identifying a potentially modifiable risk factor for OA.

We therefore examined the relation of VKA use to progression and incidence of hip and knee OA in two subcohorts of the large prospective population-based cohort of the Rotterdam study (RS). We additionally examined how the impact of VKA use varies by the presence of the *MGP* risk allele that influences *MGP* expression and single nucleotide variations (SNVs) affecting *VKORC1* gene expression, which impact VKA dosage.

METHODS

Study population and clinical data

The Rotterdam Study (RS) is a large prospective population-based cohort study ongoing since 1990 to study determinants of chronic disabling diseases in the elderly.[16] It consists of separate subcohorts (RS-I, RS-II, RS-III). All RS cohort participants live in the Ommoord district of the city of Rotterdam, the Netherlands. Residents of 55 years and older were first recruited in 1990. In 2000, a second cohort, RS-II, was started with individuals who had become 55 years of age or moved into the study district since the start of the study. Follow-up data were collected at follow-up visits every ~5–6 years. Details of the design and rationale of the RS have been published elsewhere.[16] Participant measurements at baseline and follow-up were obtained during visits to the research centre for physical examinations, computerised pharmacy records and from home interviews. Our study included participants of RS-I and RS-II for whom radiographs of knee and hip joints at baseline and follow-up visit were present, obtained and scored (online supplemental figure S1). Additional information included sex, age at baseline visit, body mass index (BMI, kg/m^2), physical activity (metabolic equivalent of task/hours per week), smoking (never, former and current smoker), locomotor disability, education level (UNESCO education classification), diabetes mellitus, hypertension, femoral neck bone mineral density (FN-BMD), HDL/total cholesterol ratio and the Stanford Health Assessment Questionnaire (see online supplemental text for details).

The RS has been approved by the institutional review board (Medical Ethics Committee) of the Erasmus Medical Center and by the review board of The Netherlands Ministry of Health, Welfare and Sports. The approval has been renewed every 5 years (MEC 02.1015). All participants provided written informed consent for participation in the RS.

Incidence and progression of OA

Our study included participants of RS-I and RS-II for whom radiographs of knee and hip joints at baseline and follow-up visit were obtained and scored by trained medical professionals for OA severity using Kellgren and Lawrence Grade (KLG)[17 18] (online supplemental figure S1). Individuals who had at baseline locomotor disability were excluded from our study population[19] (online supplemental figure S1). We analysed OA incidence and progression together, as both definitions cannot be accurately defined based on radiographic examination alone, and by combining both into one definition reduces this bias.[20] We evaluated any OA progression, defined as an increase of KLG between baseline and follow-up of ≥1 and/or joint replacement; if baseline KLG was 0, progression was defined as an increase of KLG ≥2 (incidence).[21] Joints with a baseline KLG of 4 or baseline joint replacement were excluded from analysis (online supplemental figure S1). OA progression was defined in a joint-specific and side-specific manner (knee, hip; left and right). Joints with no progression of OA comprised the referent group. Joints with missing data were excluded (online supplemental figure S1), with the exception of joints with missing baseline data and a KLG of ≤1 at follow-up, which were included in the referent group (online supplemental figure S1).

Vitamin K antagonist anticoagulants

For each participant, we extracted the usage of VKAs (acenocoumarol) for the period between baseline visit (RS-I-1, RS-II-1) and follow-up visits (RS-I-3, RS-II-2), from computerised pharmacy data (online supplemental text). Acenocoumarol is the main prescribed VKA in the Netherlands as warfarin is not registered for use as a drug. All participants taking VKAs attended an anticoagulation clinic, which is standard practice in the Netherlands.[22] We excluded participants who were taking VKA (n=148) during the baseline visit to avoid prevalent user bias. We defined VKA usage as any acenocoumarol usage during the period between the baseline (RS-I, RS-II) and follow-up visit (RS-I-3, RS-II-2), regardless of duration or dosage. To examine the effects of increasing duration of use, we defined duration of use by tertiles: ≤180 days, between >180 days and ≤556 days of use, and >556 days of use.

Genetic data and haplotype analysis

Methods for DNA isolation, genotyping, quality control and data processing have been described elsewhere.[11] Data from 11 SNVs were extracted from the genotyped and imputed genetic dataset: the *MGP* SNV previously found associated with OA[11] and 10 SNVs needed for the *VKORC1* H-haplotypes as described on the PharmGKB database[23] (online supplemental table S1). Haplotypes were inferred from all available genotypes (N=8448), using imputed genotype dosage data (HRC panel v.1.1[24]) and the R-package haplo.stats.[25] Haplotypes were grouped based on VKA maintenance dose/*VKORC1* expression association: (A) low-dose VKA requirement/low *VKORC1* expression and (B) high-dose VKA requirement/high *VKORC1* expression.[26] Study

Table 1 Study population characteristics

	Study population (RSI and RSII)	
	Non-users (N=3255)	Acenocoumarol users (N=240)
General characteristics		
RSI	2394 (73.5%)	207 (86.6%)
RSII	861 (26.5%)	33 (13.4%)
Age, years	64.2 (6.3)	66.6 (6.8)
Females (%)	1778 (54.6%)	117 (49.0%)
Follow-up period, months, median (IQR)	76.1 (55.2, 78.5)	76.7 (75.1, 79.4)
Smoking status		
Never	1028 (31.6%)	65 (27.2%)
Former smoker	1538 (47.3%)	119 (49.8%)
Current smoker	689 (21.2%)	55 (23.0%)
Education		
Primary education	385 (11.7%)	34 (14.2%)
Intermediate general education	1433 (44.0%)	103 (43.1%)
Higher general education	1026 (31.5%))	69 (28.9%)
Higher vocational education/University	411 (12.6%)	33 (13.8%)
Physical activity (MET/week)	89.9 (44.69)	78.27 (41.5)
Body mass index (BMI), kg/m^2	26.3 (3.5)	26.9 (3.5)
Total cholesterol/HDL ratio	5.0 (1.6)	5.3 (1.5)
Hypertension (%)	1709 (52.3%)	143 (59.8%)
Diabetes mellitus (%)	386 (11.9%)	41 (17.2%)
Femoral neck BMD (g/cm^2)	0.88 (0.13)	0.88 (0.12)
Osteoarthritis status*		
Hip OA (%)	65 (2.0%)	10 (4.6%)
Knee OA (%)	262 (8.5%)	26 (12.0%)
MGP risk allele (rs1800801)		
Non-risk allele (A/A)	1168 (38.6%)	79 (36.3%)
Risk allele carrier (T/*)	1856 (61.4%)	137 (63.4%)
VKORC1 Haplotype groups†		
Low—AA	447 (14.8%)	42 (19.5%)
Intermediate—AB	1425 (47.3%)	92 (42.8%)
High—BB	1142 (37.9%)	81 (37.7%)
MGP risk allele carrier and *VKORC1* BB-haplotype	684 (22.7%)	51 (23.7%)

*Osteoarthritis at baseline was defined as radiographic OA, Kellgren-Lawrence score ≥2 in either the left or right or both investigated joints.
†*VKORC1* groups based on *VKORC1* H-Haplotypes and their association with *VKORC1* expression/VKAs maintenance dosage; low: low *VKORC1* expression and associated with lower required dosage; high: high *VKORC1* expression and associated with higher required dosage, also see online supplemental table S1.
BMD, bone mineral density; HDL, high-density lipoprotein; MET, metabolic equivalent of task hours; OA, osteoarthritis; RS, Rotterdam Study.

participants were further stratified into: low expression/dose (AA), intermediate expression/dose (AB) and high expression/dose (BB) groups (table 1 and online supplemental table S1).

Statistical analysis

We evaluated the relation of VKA to the risk of overall progression of OA of either the knee or hip using logistic regression with generalised estimating equations (GEE) to account for correlations between joints within an individual.[21] We repeated the analyses stratified by *VKORC1* and *MGP* genotype/haplotype. The following covariates were included in all analyses: sex, age, BMI, physical activity, smoking, education level, diabetes mellitus, hypertension, FN-BMD, HDL/total cholesterol ratio, baseline OA severity, time between follow-up visits, joint modelled and RS cohort (online supplemental text).

RESULTS

Relation of acenocoumarol use to OA progression

A total 3494 of participants of two large prospective older-age population-based cohorts, the Rotterdam Study (RS), were included in this study, with RS-I contributing 2601 individuals, while RSII contributed 894 participants.[16] See table 1 for the general characteristics of the study population. At baseline, there were 363 individuals with OA (KLG ≥2), 75 with hip OA and 288 with knee OA. We identified 239 new users of acenocoumarol (VKA) (RS-I n=207, RS-II n=33) in our study population in our follow-up period.

When we examined the incidence/progression of OA in acenocoumarol users and non-users, there was a >2-fold higher risk for overall OA incidence/progression (ie, OA of the knee or hip) in acenocoumarol users compared with non-users (OR=2.50, 95% CI=1.94–3.20; table 2, online supplemental table S2). This association was also observed in each subcohort (RS-I and RS-II) separately (online supplemental table S3) and for OA incidence and OA progression separately (online supplemental table S4). Overall OA incidence/progression risk estimates remained similar for longer duration of acenocoumarol use (tertiles): ≤180 days (OR=2.82, 95% CI=1.90–4.20), >180 days and ≤556 days (OR=2.94, 95% CI=2.00–4.32), with only a reduction of risk with long-term use, >556 days of acenocoumarol use (OR=1.74, 95% CI=1.10–2.76) (table 2).

Increased risk of overall OA incidence/progression in acenocoumarol users was also observed in the knee and hip joints separately (table 2) (knee OA (OR=2.34, 95% CI=1.69–3.22) and hip OA (OR=2.74, 95% CI=1.82–4.11)), as well as in each subcohort separately (online supplemental table S3). Interestingly, longer duration of acenocoumarol use does seem to have slight different effects on overall knee OA incidence/progression than on hip OA. Knee OA risk seems to increase for longer duration of acenocoumarol use, whereas risk for hip OA seems to decrease for longer durations of acenocoumarol use. These differences may represent true biological differences between the joints. However, this is more likely the effect of low statistical power (tertiles analysis), the statistical power difference between the joints as there were more cases of overall knee OA incidence/progression (n=385 joints) in our study cohorts than for hip OA (n=216 joints). In addition, the absence of a stronger effect in long-term acenocoumarol users could be explained by depletion of susceptible bias.[27] This bias suggests that the cohort is depleted of all its susceptible subjects who had the event, OA incidence/progression, early on. Seemingly decreasing the risk with longer acenocoumarol (exposure) use. Thus, the remaining acenocoumarol users will include fewer OA incidence/progression predisposed subjects over the longer follow-up, causing the risk to decrease over time.

MGP, VKORC1 genetics and acenocoumarol affect OA progression

Maintenance dosages of VKAs are dependent on genetic variants (SNVs) affecting *VKORC1* expression activity.[22 28] This altered *VKORC1* expression can also affect MGP γ-carboxylation, as *VKORC1* is needed for γ-carboxylation by vitamin K, a process that is needed to activate MGP. We therefore examined the extent to which acenocoumarol users with a genetic predisposition for decreased *MGP* and/or altered *VKORC1* expression had an altered risk for overall incidence/progression of OA. The study population was stratified in *MGP* risk allele carriers (T/*) and non-carriers (A/A) (table 1).

Acenocoumarol users among both *MGP* genotype groups had a significantly higher risk of overall OA incidence/progression than non-users (table 3). Using the *VKORC1*-Haplotypes, the study population could be also stratified into low (AA), intermediate (AB) and high (BB) *VKORC1* expression/VKA dose groups[26] (table 1 and online supplemental table S1). Similar to the *MGP* genotypes, we observed no effect of the VCORC1 genotypes on the risk for overall OA incidence/progression (table 3), although

Table 2 Association between acenocoumarol use and risk of OA incidence and progression in RSI and RSII

	Overall osteoarthritis progression						Overall progression of knee osteoarthritis						Overall progression of hip osteoarthritis					
	Joints N*	Incidence/Progression N (%)	OR adj.†	95% CI	P value		Joints N*	Incidence/Progression N (%)	OR adj.†	95% CI	P value		Joints N*	Incidence/Progression N (%)	OR adj.†	95% CI	P value	
Non-users	12 594	506 (4.0%)	1	–	–		6162	329 (5.3%)	1				6432	177 (2.8%)	1	–	–	
Users	863	94 (10.9%)	2.5	1.94 to 3.20	>0.001		426	55 (12.9%)	2.34	1.69 to 3.22	>0.001		437	39 (8.9%)	2.74	1.82 to 4.11	>0.001	
Duration of acenocoumarol usage																		
Non users	12 594	506 (4.0%)	1	–	–		6162	329 (5.3%)	1	–	–		6432	177 (2.8%)	1	–	–	
≤180 days	279	35 (12.5%)	2.82	1.90 to 4.20	>0.001		144	15 (10.4%)	1.78	1.01 to 3.18	4.8×10^{-02}		135	20 (14.8%)	4.84	2.73 to 8.56	>0.001	
>180 days and ≤556 days	285	36 (12.6%)	2.94	2.00 to 4.32	>0.001		135	20 (14.8%)	2.69	1.62 to 4.46	>0.001		150	16 (10.7%)	3.33	1.79 to 6.18	>0.001	
>556 days	299	23 (7.7%)	1.74	1.10 to 2.76	1.9×10^{-02}		147	20 (13.6%)	2.65	1.57 to 4.46	>0.001		152	3 (2.0%)	0.27	0.18 to 1.85	0.35	

Incidence and progression of osteoarthritis (OA) in RS-I and RS-II within the follow-up time associated with acenocoumarol use. Model used is a GEE (generalised estimated equations) multivariate logistic regression model including acenocoumarol use and adjusted for age, sex, BMI, smoking, time between baseline and follow-up visit, baseline OA severity in Kellgren-Lawrence score, joint modelled, femoral neck BMD, HDL/total cholesterol ratio, physical activity, education level, hypertension, diabetes mellitus and Rotterdam Study cohort.
Progression: number of joints showing overall progression of either hip or knee joints or both. Acenocoumarol usage examined by tertiles: first: ≤180 days, second: >180 and ≤556 days, third: >556 days of acenocoumarol use.
*Number of individual knee and/or hip joints studied from RSI and RSII (online supplemental figure S1 for exclusions).
†Unadjusted (raw) ORs are reported in online supplemental table S2.
BMI, body mass index; HDL, high-density lipoprotein; RS, Rotterdam Study.

the *VKORC1*-BB group had the highest risk of overall OA incidence/progression (OR=3.35, 95% CI=2.22–5.05, table 3).

As individuals can be both carriers of *MGP* risk alleles and *VKROC1* haplotypes, we examined the combined effects of the *VKORC1-BB* haplotype and *MGP* risk allele carriers (T/*). We stratified our study population into *VKORC1* BB-haplotype or AA/AB carriers, which was then further stratified into carriers (T/*) and non-carriers (A/A) of the *MGP* risk allele, rs1800801 (figure 1 and online supplemental table S5). Acenocoumarol users whom either were carriers of the *MGP* risk alleles (T/*) or carriers of the *VKORC1 BB*-haplotype had a significant increased risk of overall OA incidence/progression. Individuals whom were carriers of both the *VKORC1 BB-haplotype* and the *MGP* risk allele had a fourfold increased risk of OA incidence/progression (OR=4.18, 95% CI=2.69–6.50, figure 1). Interestingly, *VKORC1* AA/AB-haplotype carriers who were not carriers of the MGP risk allele (A/A) did not have a significant increased risk of overall OA progression/incidence when using acenocoumarol (OR=1.72, 95% CI=0.93–3.19, figure 1).

DISCUSSION

We demonstrated that use of the acenocoumarol was associated with a higher risk of overall OA incidence/progression in non-users. We observed that the increased OA risk in acenocoumarol users varied based on genetic variants affecting the vitamin K cycle with VKA use and *MGP* risk allele status. Acenocoumarol users with the *MGP* risk allele and *VKORC1* BB-haplotype had a fourfold higher risk for overall OA incidence/progression.

The *VKORC1* BB-haplotype is associated with a higher expression of *VKORC1*, associated with greater vitamin K activity; however, individuals who are *VKORC1* BB-haplotype carriers also require and receive higher dosages of VKA for the desired anticoagulation effect.[26] Intuitively, the anticoagulation, amount of VKORC1 inhibition, should be similar in all users regardless of *VKORC1* haplotype since the amount of anticoagulation is the dosing measurement, not *VKORC1* expression. Previous research in animal models has indicated that vitamin K availability levels differ significantly between tissues[29 30] and warfarin affects vitamin K inhibition differently in liver compared with bone.[31–33] In liver, another enzyme in addition to *VKORC1* is available for the recycling of vitamin K into its active form, *NQO1* (*NAD(P)H quinone oxidoreductase 1*). This enzyme is not present in bone tissue, causing the bone tissue to be potentially more susceptible to VKA dosages than liver tissue.[32–34] Thus, we propose the following hypothesis: that the higher VKA dosages in *VKORC1* BB-haplotype carriers, needed for desired inhibition of vitamin K-dependent blood coagulation proteins in the liver, might be too high of a dosage for *VKORC1* functioning in the joint. This hypothesis, however, needs to be further examined in functional studies, particularly in human functional studies.

Oral VKAs, which include acenocoumarol and warfarin, were the only oral anticoagulants available for decades.[35] New oral anticoagulant drugs developed over the past decade target thrombin (IIa) or factor X (Xa) instead of vitamin K. These are known as direct oral anticoagulants (DOACs). Recent years have seen a rise in the use of DOACs,[15] which have an improved efficacy-to-safety ratio over VKAs. Additionally, they do not need routine coagulation monitoring and have fewer food and drug interactions compared with VKAs; however, DOACs are more costly and difficult to reverse.[36–38] Nonetheless, VKAs continue to be commonly prescribed and are the only indicated anticoagulant class for certain indications (eg, antiphospholipid antibody syndrome, mechanical heart valves). With ageing of the population, the number of people with OA and

Table 3 Acenocoumarol use interacts with *MGP* OA risk variants and *VKORC1* haplotype groups, leading to increased risk of overall incidence/progression of osteoarthritis

	Joints*	Incidence/Progression			OR	95% CI	P value
Acenocoumarol use	N	N (%)	OR	95% CI	Adj.	Adj.	Adj.
MGP rs1800801 alleles							
Non-users MGP non-risk allele carriers (A/A)	4332	205 (4.7%)	1	–	1	–	–
Non-users MGP risk allele carriers (T/*)	7211	274 (3.8%)	0.83	0.69 to 1.00	0.86	0.71 to 1.04	0.11
Users MGP non-risk allele carriers (A/A)	286	26 (9.1%)	2.11	1.37 to 3.24	2.01	1.29 to 3.13	2.0×10^{-03}
Users MGP risk allele carriers (T/*)	492	58 (11.8%)	2.82	2.08 to 3.84	2.57	1.87 to 3.54	1.1×10^{-08}
VKORC1 haplotype groups							
Non-users low VKORC1 group (AA)	1735	75 (4.3%)	1		1	–	–
Non-users intermediate VKORC1 gGroup (AB)	5525	203 (3.7%)	0.84	0.64 to 1.11	0.84	0.64 to 1.10	0.2
Non-users high VKORC1 group (BB)	4448	201 (4.5%)	1.05	0.80 to 1.37	1.04	0.79 to 1.36	0.8
Users low VKORC1 group (AA)	152	16 (10.1%)	2.6	1.48 to 4.59	2.31	1.28 to 4.17	5.3×10^{-03}
Users intermediate VKORC1 group (AB)	317	24 (7.6%)	1.81	1.13 to 2.92	1.53	0.34 to 2.51	9.1×10^{-02}
Users high VKORC1 group (BB)	305	42 (13.8%)	3.53	2.37 to 5.27	3.35	2.22 to 5.05	7.2×10^{-09}

Overall progression of osteoarthritis (OA) in RSI and RSII within the follow-up time associated with acenocoumarol use effect *MGP* rs1800801 OA risk variant and *VKORC1* expression/VKA dosage haplotypes. Model used is a GEE (generalised estimated equations) multivariate logistic regression model including acenocoumarol use adjusted (Adj.) for age, sex, BMI, smoking, time between baseline and follow-up visit, baseline OA severity in Kellgren-Lawrence score, joint modelled, femoral neck BMD, HDL/total cholesterol ratio, physical activity, education level, hypertension, diabetes mellitus and Rotterdam Study cohort. *VKORC1* haplotype groups are based on the H haplotypes, see online supplemental table S1. For *MGP* risk variants and carriers, see Table 1. Progression: number of joints showing overall OA progression; T/*: MGP osteoarthritis risk variant carrier (T/A) or (T/T).
*Number of individual knee and hip joints included in the analysis.
BMD, bone mineral density ; BMI, body mass index.

requiring anticoagulation medication will continue to rise. Given our findings of increased risk of OA incidence/progression with VKA use and the lack of effective treatment options for OA, it may be reasonable to consider DOACs over VKAs for medical indications in which N=DOACs can be used. This may be particularly the case for those who are carriers of the *MGP* risk allele and the *VKORC1* BB-haplotype, which is an estimated 21% of individuals of European ancestry.

The strengths of our study include the robust underlying biological hypothesis, large sample size and high-quality prospectively

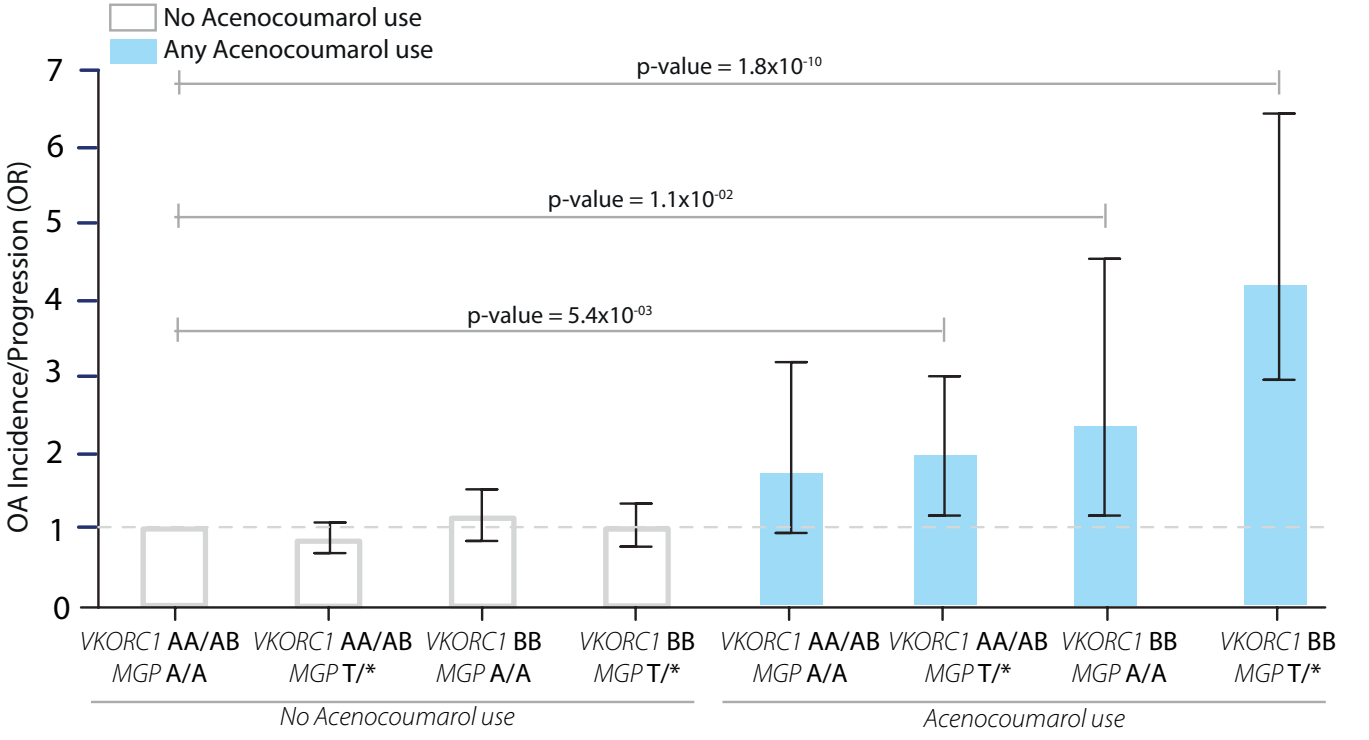

Figure 1 Acenocoumarol use interacts with *MGP* osteoarthritis (OA) risk single nucleotide variants (SNVs) and *VKORC1* haplotype groups, leading to increased risk of overall progression of OA. *VKORC1* haplotype groups are based on the *VKORC1* H-haplotypes, which can be divided into three groups based on their VKA dosage/*VKORC1* expression association: AA=low VKA dose/*VKORC1* expression; AB=intermediate VKA dose/*VKORC1* expression; and BB=high VKA dose/*VKORC1* expression. Acenocoumarol use in *VKORC1* BB haplotype carriers and *MGP* risk allele carriers and OA risk. OR, CI and p value based on GEE multivariate logistic regression adjusted for age, sex, BMI, smoking, time between baseline and follow-up visit, baseline OA severity in Kellgren-Lawrence score, joint modelled, femoral neck BMD, HDL/total cholesterol ratio, physical activity, education level, hypertension and diabetes mellitus. A/A: non-carrier of *MGP* risk allele; T/*: carrier of *MGP* risk allele; *VKORC1 AA*: homozygous *VKORC1 A* haplotype carriers; *VKORC1 BB*: homozygous *VKORC1 BB* haplotype carriers. error bars indicate 95% CI for the OR. P values belong to depicted OR. See online supplemental table S3 for the exact values depicted in this graph.

collected data. However, some limitations of our study should be acknowledged. First, while we found similar results in RS-I and RS-II, analyses in other independent and even larger cohorts are warranted, as in our large sample size, the numbers of acenocoumarol users is still relatively low. Specifically in RSII, which has a much smaller sample size and number of acenocoumarol users compared with RSI. Also replication in non-Central European ancestry-based cohorts is warranted. Second, the association we noted in this study may be due to a shared disease pathology between OA and VKA indications.[39 40] We addressed this issue by adjusting for multiple cardiovascular disease risk factors in our analysis (hypertension, HDL/total cholesterol ratio, diabetes mellitus, BMI, physical activity, smoking and age). However, we cannot rule out possible confounding by indication. This potential bias needs to be addressed more directly. This could be done by examining direct (new) oral anticoagulants (DOAC/NOAC) users as a comparator group, as these oral anticoagulants do not inhibit the vitamin K cycle. Unfortunately, our study population contains too few DOAC/NOAC users for such an analysis (n=9). Third, as we only have radiographs available of participants whom were able, healthy and survived long enough to come to our research centre at baseline and follow-up visits, our study may contain health and survivor bias. However, this could also possibly indicate that our found effect may even be larger.[20] Last, as with all observational studies, we cannot rule out residual confounding.

In summary, we found an increased risk of overall OA incidence/progression in users of the VKA acenocoumarol, which was further increased in *VKORC1* BB-haplotype and *MGP* risk allele carriers. These findings are consistent with the known biology of MGP and vitamin K, and are in keeping with prior studies of vitamin K in OA. Taken together, these studies, including the current one, highlight the importance of vitamin K and vitamin K-dependent Gla proteins such as MGP in the pathogenesis of OA. Importantly, our results may also indicate a role for other vitamin K-dependent proteins which occur and function in cartilage and bone tissues, such as osteocalcin and Gla-rich protein.[3] Given that there are as yet no treatment options for preventing OA onset or progression, vitamin K may represent a modifiable risk factor. These data provide strong rationale for a properly powered randomised clinical trial of vitamin K in an appropriate patient population, such as those with insufficient vitamin K levels and/or MGP risk allele carriers. Additionally, these data lend support to the consideration of DOACs in favour of VKAs when appropriate for a supported indication, and highlight the future possibility of genetic screening to identify individuals at high risk of OA incidence/progression.

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Contributors CGB designed the study, performed the analyses, made the figures and tables, and wrote the manuscript. IS contributed to study design. NLN performed analysis. TN and IM contributed to study design. AGU and MAI provided access to the Rotterdam study dataset. SB-Z contributed to study design. BS provided pharmacological data, contributed to study design and analysis. JBvM designed the study and supervised this work. All authors critically assessed the manuscript.

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Data availability statement Data are available upon reasonable request. All relevant data supporting the key findings of this study are available within the article and its supplementary data. Due to ethical and legal restrictions, individual-level data of the Rotterdam Study (RS) cannot be made publicly available. Data are available upon request to the data manager of the Rotterdam Study Frank van Rooij (f.vanrooij@erasmusmc.nl) and subject to local rules and regulations. This includes submitting a proposal to the management team of RS, where upon approval, analysis needs to be done on a local server with protected access, complying with GDPR regulations.

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EPIDEMIOLOGICAL SCIENCE

Warfarin use and risk of knee and hip replacements

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ABSTRACT

Background Identification of modifiable risk factors and treatments for osteoarthritis (OA) are needed. Warfarin, a vitamin K antagonist, causes fetal and animal model skeletal abnormalities. Vitamin K insufficiency has been associated with OA, but whether warfarin is also detrimental to OA is not known.

Methods We conducted a nested case–control study using a UK general practitioner electronic medical records database. We identified cases of knee or hip replacement (KR or HR) from among adults with atrial fibrillation newly prescribed either warfarin or direct oral anticoagulants (DOACs). Cases were matched with four controls by age and sex. We assessed the relation of warfarin compared with DOAC use to risk of joint replacement using conditional logistic regression. We also evaluated different durations of warfarin use.

Results We identified 857 subjects with KR or HR (cases), of whom 64.6% were warfarin users, and 3428 matched controls, of whom 56.1% were warfarin users (mean age 75, 47% female). Warfarin users had a 1.59 times higher risk of joint replacement than DOAC users (adjusted OR 1.59, 95% CI 1.31 to 1.92). Longer duration of warfarin use was associated with higher risk of joint replacement in comparison with <1 year of warfarin use.

Conclusion Warfarin, a vitamin K antagonist, was associated with greater risk of KR and HR (an indicator for end-stage knee OA) than DOAC use, supporting the importance of adequate vitamin K functioning in limiting OA progression.

Key messages

What is already known about this subject?

► Vitamin K deficiency is associated with incidence and progression of osteoarthritis (OA). However, it is unclear whether vitamin K antagonism through warfarin is also detrimental to OA.

What does this study add?

► In this study, use of warfarin, a vitamin K antagonist, was associated with greater risk of knee and hip replacement (KR and HR; an indicator for end-stage knee OA) than direct oral anticoagulant (DOAC) use, suggesting that vitamin K antagonism may also be detrimental to OA.

How might this impact on clinical practice or future developments?

► These data raise the consideration of using DOACs over warfarin when appropriately indicated in people with OA.

INTRODUCTION

Warfarin is a commonly prescribed anticoagulant that is known to have adverse effects on the skeletal system in the context of human fetal embryopathy and in rat models characterised by abnormal skeletal mineralisation.[1–4] These effects could have implications for osteoarthritis (OA), the most common form of arthritis, for which no effective treatments exist. Thus, identifying modifiable risk factors remains a high priority.

Warfarin's anticoagulant effects occur through inhibition of the functioning of vitamin K.[5] Vitamin K, in turn, is an essential cofactor in the post-translational gamma carboxylation of Gla proteins, a step required for these proteins to be functional.[6] Gla proteins play an important role in blood coagulation, and also in the bone and cartilage, including matrix Gla protein (MGP), osteocalcin and Gas-6.[7–9] Thus, warfarin's inhibition of vitamin K leads to inadequate functioning of Gla proteins. Low vitamin K status has been associated with both incidence and progression of knee OA in observational studies.[10–13] Furthermore, in a randomised controlled trial of vitamin K supplementation versus placebo, those with insufficient vitamin K at baseline had trends towards less joint space narrowing on hand radiographs.[14]

Taken together, these data highlight the potentially detrimental effects of warfarin via vitamin K antagonism on joint tissues that could contribute to OA. We therefore sought to determine the relation of warfarin use to risk of knee and hip replacements (KR, HR), as a reflection of end-stage OA, in a large population-based cohort.

METHODS

Study design

We performed a nested case–control study using data from the IQVIA Medical Research Data (IMRD; incorporating The Health Improvement Network (THIN)). IMRD is a general practitioner (GP) electronic medical records database from the United Kingdom (UK) that is representative of the general UK population. This database has been validated for use in pharmacoepidemiological research.[15]

The nested case–control study was assembled from among a cohort of adults with atrial fibrillation, a common indication for long-term anticoagulation, to minimise confounding by indication. Because atrial fibrillation can be managed with warfarin or direct oral anticoagulants (DOACs), which do not antagonise vitamin K,[16] we used an active comparator approach to further minimise

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confounding by indication. Since DOACs were first introduced to the market in the UK in 2008, we identified eligible study participants from 2009 onwards to allow time for market uptake. Study entry criteria included adults aged between 40 and 89 with atrial fibrillation who had been enrolled with a GP for at least 1 year. Participants were further required to be incident warfarin or DOAC users, defined as those who were newly prescribed an anticoagulant after 2009, having ≥1 prescription after study entry and also within 1 year before the index date (defined below) to ensure a relevant time frame of use. From among this cohort, we identified cases as those patients with KR or HR between 2014 and 2018. The index date for cases was defined as the date of surgery. Each case was matched with four controls by age and sex; if more than four controls were eligible for matching, the four controls were selected randomly. The matched controls were assigned the same index date as that of their matching case's surgery date (figure 1).

We excluded participants with KR or HR prior to 2014, those with warfarin or DOAC use prior to study entry (criteria defined above) and those who used both warfarin and DOAC within 1 year prior to the index date. We also excluded those with high-risk cancer (oesophageal, gastric, pancreatic and metastatic cancer), body mass index (BMI) >40 kg/m^2, joint infection and oxygen therapy, as these are severe comorbidities that would limit surgical candidacy.

Analytic approach

For our primary analysis, we assessed the relation of warfarin use compared with DOAC use, both within 1 year prior to the index date, to risk of KR and HR. Because the biological effects of warfarin may become evident only after a period of use, in a secondary analysis, we assessed the relation of duration of warfarin use to risk of KR and HR, defined as ≥4 years, 2–<4 years, and 1–<2 years, compared with warfarin use of <1 year prior to the index date. Duration of use was calculated based on the sum of each prescription duration between study entry and the index date.

We considered the following potential confounders for adjustment in our models: BMI, renal disease, severe liver disease, prior gastrointestinal bleeding, prior intracranial haemorrhage, mitral stenosis, presence of prosthetic heart valve, prior falls, cancer, chronic obstructive pulmonary disease, dementia or cognitive impairment, diabetes, heart failure, hypertension, hyperlipidaemia, ischaemic heart disease, stroke, venous thromboembolism, medication use (antihypertensive drugs, oral hypoglycaemic drugs, insulin, lipid-lowering drugs, non-steroidal anti-inflammatory drugs and paracetamol), GP visits and hospitalisations. Confounders were assessed by Read codes for medical conditions and with prescription records for medication. Of these, severe liver disease, prior intracranial haemorrhage, mitral stenosis and presence of prosthetic heart valve had a prevalence of <1% and were subsequently not included in multivariable adjusted models. We assessed the relation of warfarin compared with DOAC use, and duration of warfarin use, to risk of KR or HR using conditional logistic regression in separate models, adjusting for these potential confounders.

We performed two additional sensitivity analyses. Because there may be variation across GP practices in both choice of anticoagulant and referral for surgery, we matched cases and controls according to GP practice, and adjusted for age and sex in addition to the other potential confounders listed above. Each GP practice typically serves the same geographical area but could include multiple GPs. In a second set of sensitivity analyses, we repeated the primary analysis stratified by type of joint replacement, with the recognition that 97% of knee replacements are performed for knee OA, whereas hip replacements can be performed for other indications, such as hip fracture.[17]

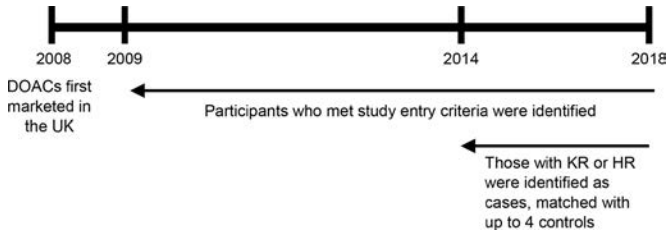

Figure 1 Study design and timeline. DOACs, direct oral anticoagulants; HR, hip replacement; KR, knee replacement.

Table 1 Characteristics of participants

Participants	Cases (KR/HR) (n=857)	Controls (n=3428)
General demographics		
Age (years), mean±SD	75.4±7.2	75.4±7.2
Female	403 (47.0%)	1612 (47.0%)
BMI 25–<30	313 (36.5%)	1297 (37.8%)
BMI ≥30	365 (42.6%)	1088 (31.7%)
Comorbidities		
Cancer	154 (18.0%)	648 (18.9%)
COPD	214 (25.0%)	825 (24.1%)
Dementia/cognitive impairment	6 (0.7%)	106 (3.1%)
Diabetes	170 (19.8%)	782 (22.8%)
Heart failure	113 (13.2%)	600 (17.5%)
Hyperlipidaemia	153 (17.9%)	702 (20.5%)
Hypertension	590 (68.8%)	2303 (67.2%)
IHD	191 (22.3%)	959 (28.0%)
Mitral stenosis	8 (0.9%)	17 (0.5%)
Prior falls	153 (17.9%)	576 (16.8%)
Prior GI bleeding	26 (3.0%)	98 (2.9%)
Prior intracranial haemorrhage	4 (0.5%)	37 (1.1%)
Prosthetic valve	3 (0.4%)	6 (0.2%)
Renal disease (CKD 1–3)	203 (23.7%)	895 (26.1%)
Renal disease (CKD 4–5 and renal transplant)	10 (1.2%)	54 (1.6%)
Severe liver disease	5 (0.6%)	17 (0.5%)
Stroke	135 (15.8%)	702 (20.5%)
Venous thromboembolism	36 (4.2%)	154 (4.5%)
Medication use		
Antihypertensive drugs	775 (90.4%)	3076 (89.7%)
Insulin	13 (1.5%)	110 (3.2%)
Lipid-lowering drugs	492 (57.4%)	2090 (61.0%)
NSAIDs	337 (39.3%)	1275 (37.2%)
Oral hypoglycaemic drugs	87 (10.2%)	463 (13.5%)
Paracetamol	581 (67.8%)	1340 (39.1%)
GP visits (assessed within 1 year before first warfarin/DOAC prescription)		
0–5	330 (38.5%)	1606 (46.8%)
>5	527 (61.5%)	1822 (53.2%)
Hospitalisations (assessed within 1 year before first warfarin/DOAC prescription)		
0–2	820 (95.7%)	3189 (93.0%)
≥3	37 (4.3%)	239 (7.0%)

Results are shown as N (%) unless stated otherwise.
BMI, body mass index; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; DOAC, direct oral anticoagulant; GI, gastrointestinal; GP, general practitioner; HR, hip replacement; IHD, ischaemic heart disease; KR, knee replacement; NSAIDs, non-steroidal anti-inflammatory drugs.

Table 2 Warfarin use and risk of knee and hip replacements

A. Warfarin versus DOAC use within 1 year of index date, regardless of duration	Cases (KR/HR)	Controls
Participants (n)	857	3428
Warfarin use	554 (64.6%)	1923 (56.1%)
DOAC use	303 (35.4%)	1505 (43.9%)
Odds ratio (95% CI), matched by age and gender	1.57 (1.32 to 1.86)	
Adjusted* odds ratio (95% CI)	1.59 (1.31 to 1.92)	
B. Warfarin versus DOAC use, matched by practice	**Cases (KR/HR)**	**Controls**
Participants (n)	857	3422
Warfarin use	554 (64.6%)	2077 (60.7%)
DOAC use	303 (35.4%)	1345 (39.3%)
Odds ratio (95% CI), matched by practice	1.25 (1.05 to 1.50)	
Adjusted† odds ratio (95% CI)	1.36 (1.11 to 1.66)	
C. Warfarin versus DOAC use, stratified by anatomic location of joint replacement	**Cases (KR only)**	**Controls**
Participants (n)	497	1988
Warfarin use	324 (65.2%)	1139 (57.3%)
DOAC use	173 (34.8%)	849 (42.7%)
Odds ratio (95% CI), matched by age and gender	1.52 (1.21 to 1.92)	
Adjusted* odds ratio (95% CI)	1.58 (1.22 to 2.04)	
	Cases (HR only)	**Controls**
Participants (n)	485	1940
Warfarin use	304 (62.7%)	1129 (58.2%)
DOAC use	181 (37.3%)	811 (41.8%)
Odds ratio (95% CI), matched by age and gender	1.27 (1.01 to 1.60)	
Adjusted* odds ratio (95% CI)	1.33 (1.03 to 1.72)	

*Adjusted for the same variables as in table 1 excluding age and sex, which were matching variables.
†Adjusted for age and gender in addition to variables in table 1.
DOAC, direct oral anticoagulant; HR, hip replacement ; KR, Knee replacement.

Patient and public involvement

Patients and the public were not involved in this study.

RESULTS

We identified 857 cases with KR or HR and matched them to 3428 controls. The mean age of both groups was 75 years and 47% were female. Other baseline characteristics are listed in table 1. Notable differences in comorbidities included a higher prevalence of diabetes, heart failure, stroke and ischaemic heart disease among controls, probably because individuals with these comorbidities were less likely to be surgical candidates. As expected, obesity (BMI ≥30) was more commonly seen among cases (42.6% vs 31.7%).

Of the 857 cases, 64.6% were warfarin users and the remaining 35.4% were DOAC users. Among the 3428 controls, 56.1% were warfarin users and 43.9% were DOAC users. Warfarin use was associated with 59% higher risk of having a KR or HR than DOAC use (adjusted OR 1.59, 95% CI 1.31 to 1.92; table 2A).

Longer durations of warfarin were associated with higher risk of KR or HR compared with <1 year of warfarin use (figure 2 and online supplemental table 1). Participants with warfarin use for ≥4 years had 86% higher risk of KR or HR compared with new warfarin users (<1 year) (95% CI 1.35 to 2.57).

When analyses were repeated with matching by GP practice, the magnitude of the association was slightly diminished but remained statistically significant (adjusted OR 1.36, 95% CI 1.11 to 1.66) (table 2B). When we stratified analyses by type of joint replacement (knee or hip), the results were similar to the primary analysis for KRs, and slightly diminished for HRs. Warfarin use was associated with 58% higher risk of KR (95% CI 1.22 to 2.04) and 33% higher risk of HR (95% CI 1.03 to 1.72) compared with DOAC users (table 2C).

DISCUSSION

In this population-based case–control study of older adults with atrial fibrillation, warfarin use was associated with a higher risk of knee and hip replacements, an indicator of end-stage OA, compared with DOAC use. Furthermore, longer duration of warfarin use was associated with greater risk of joint replacement compared with shorter duration of its use.

The mechanism for this observed association of warfarin on risk of end-stage OA as assessed by joint replacement is probably related to warfarin's role as a vitamin K antagonist. Warfarin's antagonism of vitamin K would be expected to recapitulate effects of insufficient vitamin K. Because vitamin K confers functionality to Gla proteins through gamma-carboxylation, insufficient vitamin K or inhibition of vitamin K's functioning through warfarin leads to undercarboxylation of vitamin K-dependent proteins, limiting their functionality. An important vitamin K-dependent protein that has been specifically linked to abnormalities in soft tissue mineralisation and OA is MGP. Genetic deficiencies of MGP in humans, known as Keutel syndrome, and in transgenic mice result in cartilage calcification, highlighting the role of MGP as an inhibitor of mineralisation.[18–21] Of specific

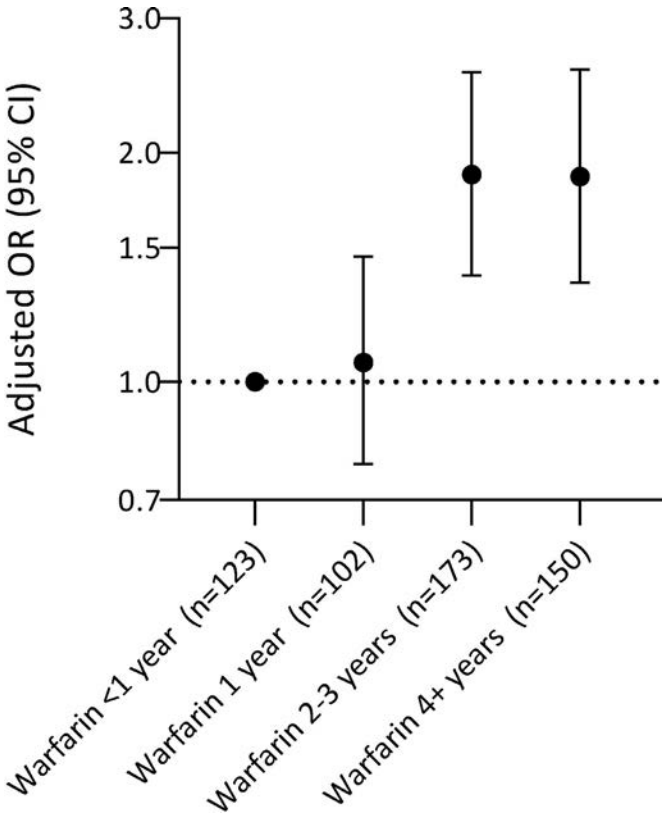

Figure 2 The relation of duration of warfarin use to risk of knee or hip replacement. Analyses adjusted for potential confounders in table 1, with the exception of age and sex, which were matching variables.

relevance to OA, MGP is primarily uncarboxylated in human OA cartilage, whereas it is primarily carboxylated (and therefore functional) in healthy cartilage.[22] Furthermore, a genome-wide association study identified coding variants of *MGP* as associated with hand OA, and complementary functional studies demonstrated that *MGP* RNA expression of the hand OA allele was higher than that of the reference allele in human OA cartilage.[23] These findings complemented a smaller study that also identified *MGP* single nucleotide polymorphism in hand OA.[24] Thus, the detrimental effects of warfarin through inhibition of vitamin K's activities may be further exacerbated in those with genetic polymorphisms of *MGP*.

Our results add to the existing literature, extending insights into the importance of vitamin K and its dependent proteins in OA. Low levels of plasma phylloquinone, the major form of circulating vitamin K, were associated with prevalence of both radiographic hand and knee OA in the Framingham Offspring cohort, while low dietary vitamin K intake was associated with radiographic knee OA in a Japanese population-based cohort.[10 11] Complementing those radiographic findings, two longitudinal studies also demonstrated an association of low plasma phylloquinone with incidence[12] and progression[13] of cartilage lesions on knee MRI, providing more direct support for a role of vitamin K in cartilage pathology. To more definitively evaluate the role of vitamin K in OA, a randomised controlled trial of vitamin K supplementation versus placebo was conducted in 378 participants who were enrolled without regards to their baseline vitamin K status. There was no difference overall in the prevalence of hand OA between the two arms.[14] However, in a post hoc analysis limited to patients who were vitamin K insufficient at baseline, those in the vitamin K supplementation arm had 47% significantly less joint space narrowing than those receiving placebo, suggesting that for hand OA those with insufficient vitamin K could derive benefit from vitamin K supplementation.[14]

In addition to vitamin K's role in OA through Gla proteins in the bone and cartilage, it might have direct effects on inflammation, which could have relevance for OA.[25] Higher plasma phylloquinone was associated with lower inflammatory burden in two separate cohorts cross-sectionally.[26 27] In contrast, undercarboxylated osteocalcin, a Gla protein, was not associated with inflammation.[26] Since these effects appear to be unrelated to vitamin K's role in gamma-carboxylation, it is unlikely that warfarin would play a role in vitamin K's effects on inflammation.[28] Thus, there might be potential additional benefit to targeting vitamin K in OA beyond warfarin alone.

We recognise that this observational study cannot provide definitive causal insights. However, it is unlikely that a randomised trial of warfarin versus a DOAC for an OA end point would be performed. We dealt with confounding by indication by limiting our sample to adults with atrial fibrillation as this diagnosis warrants anticoagulation, and by including an active comparator arm of DOACs, which are anticoagulants used for the same indication but do not antagonise vitamin K. Our study also has limitations. As with all observational studies, there is potential for residual confounding. We identified exposure to warfarin and DOACs through prescriptions, but these do not necessarily reflect medication adherence. Joint replacement was used as a proxy for end-stage OA. While approximately 97% of KRs are performed for knee OA, HRs can be performed for other reasons, such as hip fracture.[17] We are unable to disentangle putative effects of warfarin on bone density and risk of osteoporotic fracture[29] versus end-stage OA as the reason for HR in this study. Nonetheless, in stratified analyses, warfarin use was associated with risk of KR with a similar magnitude as in the main analysis, and with HR, though with a slightly lower magnitude. Overall, our study provides support for a detrimental effect of warfarin in OA, complementing prior studies examining the effects of vitamin K in OA, and supports the inference that warfarin's effects are due to its role as a vitamin K antagonist.

Given the worldwide prevalence and impact of OA and lack of effective disease-modifying therapies, our study supports the need for a well-powered randomised control trial evaluating vitamin K supplementation in OA. Our study also raises the consideration of preferentially using DOACs rather than warfarin, when appropriately indicated, in people with OA.

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Contributors PB was involved in study design, code selections, drafting the manuscript and critical revision. CP was involved in study design, data analysis and critical revision. CGB was involved in critical revision. TN was involved in conception, study design, data analysis, critical revision and final approval of the manuscript.

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Competing interests None declared.

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Data availability statement Data may be obtained from a third party and are not publicly available. Deidentified data were used for this work. The IMRD dataset used in this work is a subscription-based dataset with a legal contract requiring data to remain onsite and analysed at Boston University Medical Center. We are therefore legally unable to make these data publicly available. We would be able to collaborate with potential external investigators to deal with research questions of interest if appropriate resources are provided. Investigators may contact IMRD for further information about obtaining data.

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EPIDEMIOLOGICAL SCIENCE

Geospatial clustering of childhood IgA vasculitis and IgA vasculitis-associated nephritis

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ABSTRACT

Objectives Research on spatial variability of the incidence of IgA vasculitis (IgAV) in children and its potential implications for elucidation of the multifactorial aetiology and pathogenesis is limited. We intended to observe spatial variability of the incidence of IgAV and IgA vasculitis-associated nephritis (IgAVN) using modern geostatistical methods, and hypothesised that their spatial distribution may be spatially clustered.

Methods Patients' data were retrospectively collected from 2009 to 2019 in five Croatian University Hospital Centres for paediatric rheumatology, and census data were used to calculate the incidence of IgAV. Using spatial empirical Bayesian smoothing, local Morans' I and local indicator of spatial autocorrelation (LISA), we performed spatial statistical analysis.

Results 596 children diagnosed with IgAV were included in this study, of which 313 (52.52%) were male. The average annual incidence proportion was estimated to be 6.79 per 100 000 children, and the prevalence of IgAVN was 19.6%. Existence of spatial autocorrelation was observed in both IgAV and IgAVN; however, clustering distribution differed. While IgAV showed clustering in Mediterranean and west continental part around cities, IgAVN was clustered in the northern Mediterranean and eastern continental part, where a linear cluster following the Drava and Danube river was observed.

Conclusion IgAV incidence in Croatia is similar to other European countries. Spatial statistical analysis showed a non-random distribution of IgAV and IgAVN. Although aetiological associations cannot be inferred, spatial analytical techniques may help in investigating and generating new hypotheses in non-communicable diseases considering possible environmental risk factors and identification of potential genetic or epigenetic diversity.

Key messages

What is already known about this subject?

- IgA vasculitis (IgAV) is the most common vasculitis in children, affecting boys more than girls. The incidence of IgAV varies worldwide.

What does this study add?

- The most accurate estimate of IgAV incidence is in Croatia, which is estimated to be 6.79 with a 95% CI between 6.26 and 7.36 per 100 000 children.
- The prevalence of IgAV-associated nephritis in Croatia is 19.6%, with a 95% CI between 16.49% and 23.01%.
- Both IgAV and IgAV-associated nephritis may not be randomly distributed in space, but clustered more around cities.

How might this impact on clinical practice or future developments?

- Besides estimating the incidence of IgAV, the results help to illustrate the spatial distribution of IgAV, which can serve an illustrative example for public health policies regarding non-communicable diseases.
- Spatial analytical techniques may help in investigating and generating new hypotheses in non-communicable diseases considering possible environmental risk factors and identification of potential genetic or epigenetic diversity.

INTRODUCTION

IgA vasculitis (IgAV) or Henoch-Schönlein purpura is the most common vasculitis in children with an incidence ranging from 3 to 27 cases per 100 000 children.[1 2] According to the European League Against Rheumatism/Paediatric Rheumatology International Trials Organisation/Paediatric Rheumatology European Society (EULAR/PRINTO/PRES) classification, it is characterised as a non-granulomatous leucocytoclastic small vessel vasculitis.[3] Five major clinical groups of signs and symptoms are characteristic to IgAV: (1) palpable non-thrombocythopenic purpuric skin lesions, (2) arthritis or arthralgia, (3) glomerulonephritis, (4) diffuse abdominal pain and (5) biopsy-proven predominant IgA deposition. In most cases, IgAV is a self-limiting disease; however, between 20% and 60% of children with IgAV develop nephritis (IgA vasculitis-associated nephritis (IgAVN)) and among them, in 1%–15%, chronic kidney disease has been reported, making the renal aspect of the disease the main prognostic factor.[4–7]

While dominantly being observed in childhood, it can also rarely occur in adulthood, but with different clinical manifestations and higher rate of progression to end-stage renal disease.[8] Male children tend to be more frequently affected than female children.[9] No single causal agent was identified. Multifactorial aetiology is proposed, combining genetic

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predispositions, and a variety of environmental exposures.[10–13] Microbial agents are also associated with IgAV, since a significant proportion of patients have had preceding infections.[9 14 15]

Spatial and geostatistics are emerging tools that combine statistical, geographical and epidemiological methodologies in describing the spatial characteristics of both infectious and non-infectious diseases.[16]

Most of the previous work on the pathogenesis of IgAV and IgAVN explores potential risk factors using observational studies, adopting classical statistical techniques; however, the literature lacks in the application of geospatial analyses in IgAV. On a global scale, differences in the incidence of IgAV are known to exist; however, the generalisation of incidences on a country level reduces information which may be used to deepen the knowledge of potential risk factors involved in the pathomechanism of IgAV. A deterministic approach from a global to local scale may provide useful information of genetic, socioeconomic and environmental risk factors while simultaneously taking into account their spatial diversity.

The motivation for this study is our observation that there is a lack of application of geostatistical methods in the field of paediatric rheumatology, specifically, IgAV and IgAVN. Based on Tobbler's first law of geography: 'Everything is related to everything else, but near things are more related than distant things',[17] if indeed near things are more related than distant things, we hypothesise that the incidence of childhood IgAV and IgAVN may be spatially clustered.

METHODS

Study area, data collection and management

The country of interest is Croatia, which is divided into 21 counties and further subdivided into 128 towns and 428 municipalities, covering a land area of 56 594 km^2 (figure 1). Both towns and municipalities served as the basic unit of spatial analysis (n=566).

In this retrospective study, patients under the age of 18 years diagnosed with IgAV, without previous chronic inflammatory diseases and chronic kidney disease between Jaunary 2009 and December 2019, were included. The diagnosis of IgAV and IgAVN were defined based on the EULAR/PRINTO/PRES-endorsed Ankara 2008 criteria.[3] This research is a part of a larger study where patients' data were collected using an in-house collection instrument which includes IgAV and IgAVN relevant clinical, laboratory and histological data at all included hospital centres. The data were collected directly from the source documentation of patients from five tertiary teaching hospital centres in Croatia where IgAV patients are referred to. Location data were identified based on the patients' address and connected to towns and municipalities. A total of 611 patients were treated in this period, out of which 15 foreign patients were excluded. Thus, the final number of domestic patients used in the study is 596 patients.

Several epidemiological measures of interest are needed for further analysis:

1. The average annual number of cases=the total number of cases/11 years.
2. The average annual incidence=the average annual number of cases/the total population.

To estimate the incidence proportion, population data were obtained from the 2011 census. According to the Croatian Bureau of Statistics, Croatia has a population of 4 284 889 citizens. The total population of children under the age of 18 years was 797 855.[18]

Spatial smoothing using empirical Bayesian analysis

A major issue that occurs when calculating raw average annual incidence across the study is due to the differences in the population sizes.[19] This may result in spurious outliers, random errors and variance instability.[20] A low number of cases (ie, rare events) in municipalities with a small population size tends to produce a high incidence. To overcome this problem, we applied the spatial empirical Bayes (SEB) smoothing techniques.

After connecting the raw incidence with the polygonal area data, a neighbourhood matrix was calculated based on the first-order contiguity Queen criteria, for identifying and calculating the median number of neighbours (k=5). The median number of neighbours was further selected to create a k-nearest neighbour weight matrix with five neighbours. The rationale for this approach is the existence of islands, which, due to their distance from the mainland (ie, lack of neighbours), would not be included in the analysis. The empirical Bayesian (EB) analysis has the effect of smoothing over raw incidence rates by borrowing information from neighbouring spatial units. EB rates are the weighted sum between the raw and global mean rate.[19 20] SEB further uses the strengths of local neighbouring spatial units. Therefore, instead of having raw count data, SEB smoothed incidences were used, which make the results more reliable and stable, and helps to find true outliers.

Spatial autocorrelation analysis (SAC)

SAC was performed to determine the spatial heterogeneity and clustering of IgAV.[21 22] The process of SAC can be performed using global and local autocorrelation. The global SAC can be used to investigate whether a particular attribute at a global

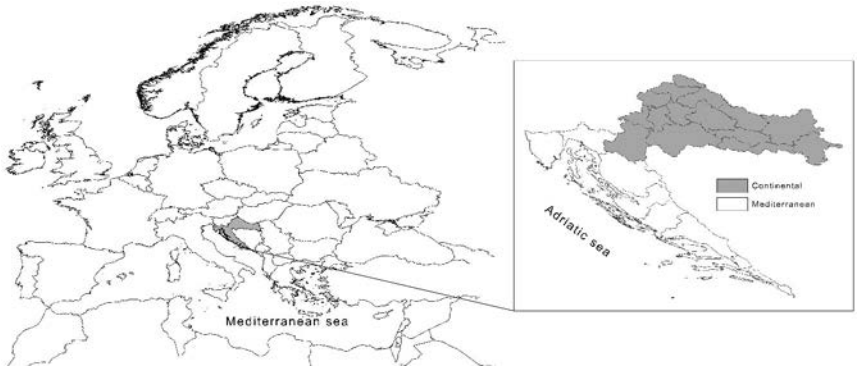

Figure 1 Location of Croatia in Europe.

(country) level exists, while the local SAC investigates if attributes are locally clustered, and can reveal spatial distribution patterns.

Moran's I index is a measure of spatial autocorrelation, ranging from −1 to +1, and can be similarly interpreted like other classical statistics correlation indices.[23] Values close to the boundaries −1 and +1 indicate a strong positive or negative autocorrelation. In this study, local Moran's I was chosen as a measure of autocorrelation due to our interest in the identification of local clusters. Statistical significance testing was performed using the permutation test, under the null hypotheses that the incidence of IgAV and IgAVN was randomly distributed. The number of the permutations was set to 99 999, and pseudo p values less than 0.05 are considered as statistically significant, meaning that the incidences are not randomly distributed. Besides using a large number of permutations, to increase the robustness of the study, a significance plot based on different p value thresholds was plotted.

To increase the interpretability of the local Moran autocorrelation results, local indicators of spatial autocorrelation (LISAs) are additionally used. The Moran scatterplot is a graphical representation where the original variable is represented on the x axis, and its spatial lag is represented on the y axis. The resulting slope of the linear fit to the scatter plot is Moran's I. After splitting the scatterplot with a horizontal and vertical axis, four different spatial autocorrelation relationships are obtained. As a result, this combined information allows classification of clusters into four categories: high–high and low–low spatial clusters and high–low and low–high spatial outliers.[24]

Of particular interest are hot spots (high–high), significant clusters of higher incidences and cold spots (low–low), significant clusters of lower incidences. It should be noted that the autocorrelation analysis was performed on the smoothed SEB incidence, not on raw incidence data. Besides, it should be noted that the meaning of high and low is not absolute but relative to the mean of the variable of interest.

The analysis was performed with the QGIS3, and GeDA software V.1.14.0.

Patient and public involvement

Patients were not involved.

RESULTS

A total of 596 children diagnosed with IgAV were included in this study, of which 313 (52.52%) were male and 283 (47.48%) were female, with a median age of 6.33 (IQR 4.50–8.92) years. Based on the provided census data, the estimated average annual incidence proportion equals 6.79 with a 95% CI between 6.26 and 7.36 per 100 000 children. Sex-adjusted average annual incidence for male children was estimated to be 6.96 (95% CI 6.21 to 7.77) per 100 000, and that for female children was 6.61 (95% CI 5.87 to 7.43) per 100 000. Among the diagnosed patients, the prevalence of IgAVN was 19.6% (95% CI 16.49% to 23.01%, n=117). The average annual incidence proportion of IgAVN was 1.33 (95% CI 1.1 to 1.6) per 100 000 children.

The raw count map as well as the SEB estimated incidences are presented in figure 2. As expected, cities with larger population sizes have a higher number of patients with IgAV. Based on the SEB results, few areas with higher incidences can be seen. Those areas are around the largest cities, Zagreb, Rijeka and Split.

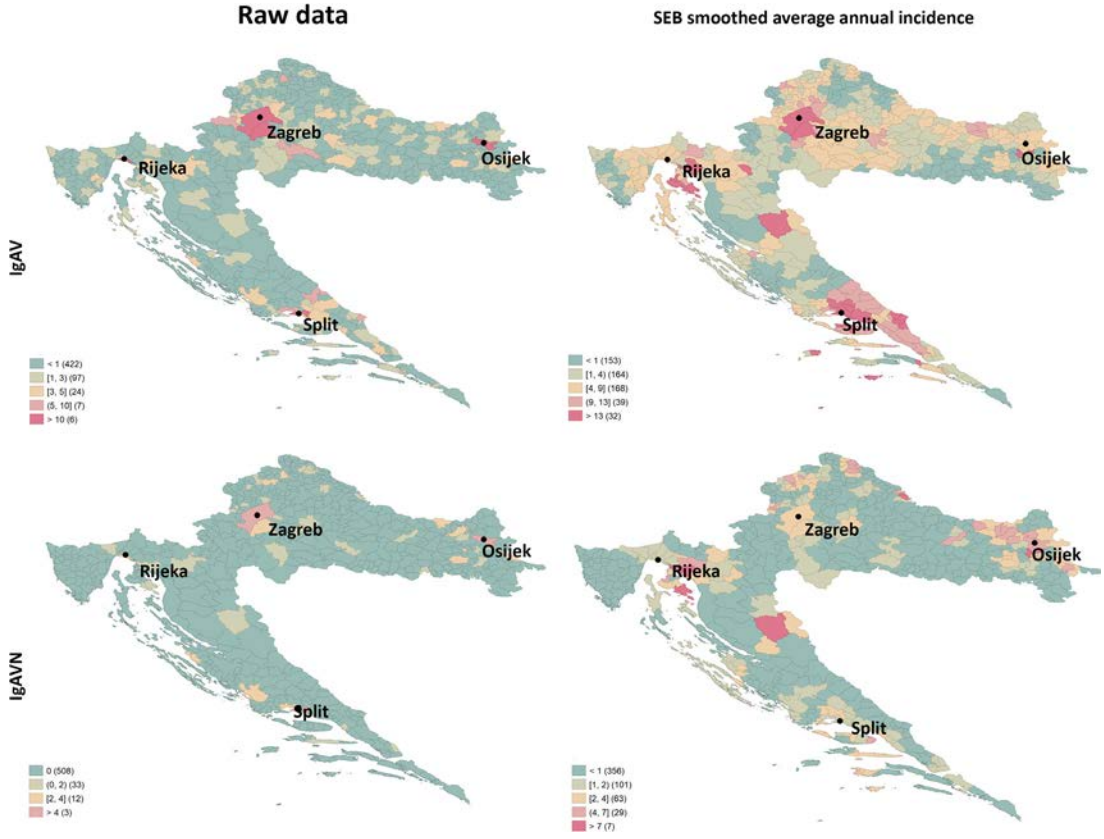

Figure 2 Raw data of IgAV (first row) and IgAV-related nephritis (second row) are presented on the left, and SEB smoothed average annual incidences per 100 000 children are on the right map. Numbers in parentheses represent the number of administrative units belonging to each category. IgAV, IgA vasculitis; IgAVN, IgA vasculitis-associated nephritis; SEB, spatial empirical Bayes.

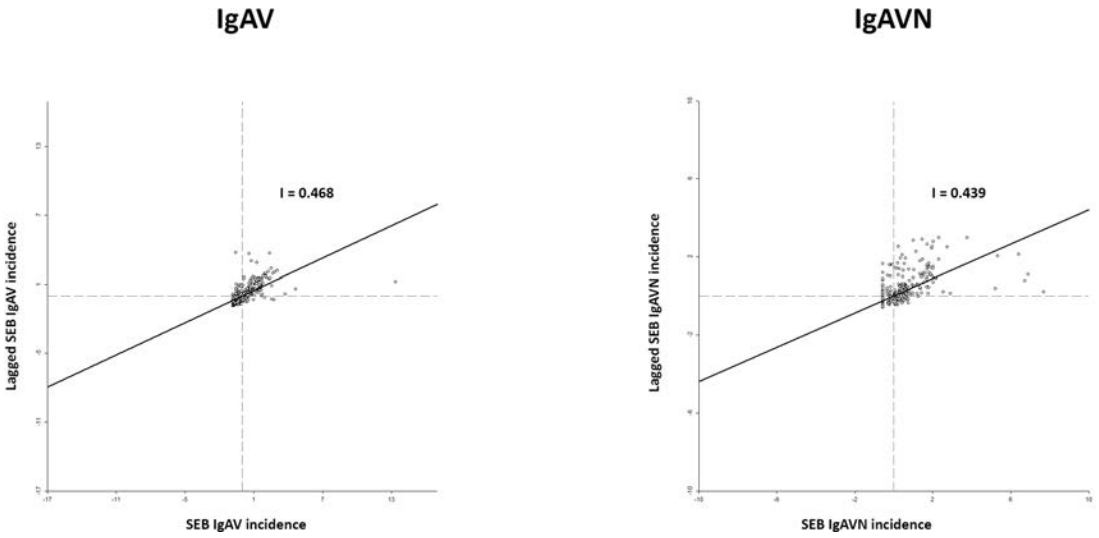

Figure 3 Moran scatter plot of SEB smoothed average annual incidences of IgAV (left) and IgAV-associated nephritis (right). IgAV, IgA vasculitis; IgAVN, IgA vasculitis-associated nephritis; SEB, spatial empirical Bayes.

Both a raw count and SEB estimated incidences of IgAVN are presented in figure 2. Similar to the raw IgAV, cities with larger population sizes have a higher number of patients with IgAV. However, higher SEB average annual incidences are found to be in the eastern part of Croatia, near Osijek, and few scattered areas.

To assess the spatial autocorrelation, the calculated Moran's I for IgAV was 0.468 and that for IgAVN was 0.439 (figure 3). Observations in the right upper quadrant have a larger than average incidence and are surrounded by observations with larger incidences. The lower left quadrant contains observations that have lower than average incidences and are surrounded by others with lower incidences. These results are visually represented in figure 4, where significant clusters are highlighted based on the LISA analysis results. Higher incidence clusters (high–high clusters or hot spots) can be seen in the southern and northern coast sides, as well as in the western continental part around the capital city, Zagreb. Lower incidence clusters (low–low clusters or cold spots) tend to be more in the eastern continental and mid-Mediterranean part of Croatia. Several low–high outliers were found nearby high-incidence clusters, while no high–low cluster was found.

A different pattern was observed with IgAVN. Besides several dispersed smaller ones, two larger high incidence clusters were

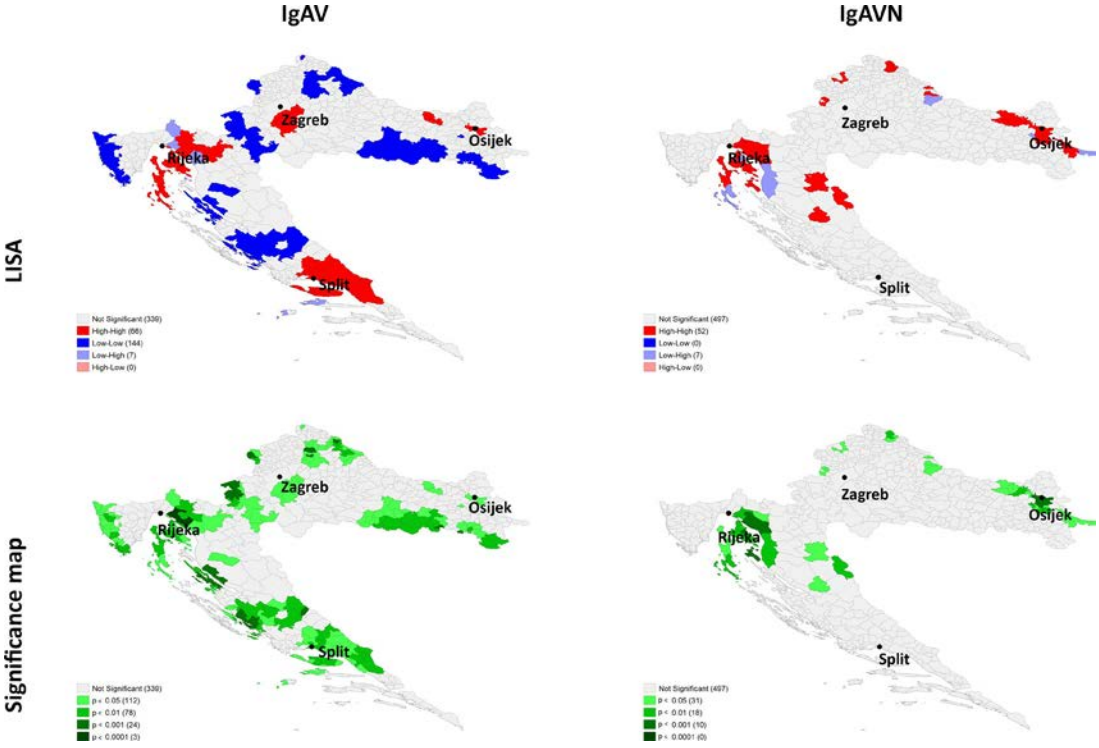

Figure 4 Significant clusters of IgAV (left) and IgAV-associated nephritis (right) according to the LISA analysis (first row), and its significance map (second row). Numbers in parentheses represent the number of administrative units belonging to each category. IgAV, IgA vasculitis; IgAVN, IgA vasculitis-associated nephritis; LISA, local indicator of spatial autocorrelation.

found: one linear in the eastern part of Croatia near Osijek and one in the northern Mediterranean part, near Rijeka.

DISCUSSION

The purpose of this study was to examine the spatial distribution of the incidence of IgAV in Croatia. To our best knowledge, this is the very first study investigating the spatial distribution and relationship of childhood IgAV applying modern geostatistical methods, as well as the largest study estimating the incidence of IgAV in Croatia. We hypothesised and found out that IgAV and IgAVN incidences are not randomly distributed but clustered in space.

The annual incidence of IgAV in children differs worldwide. Our results found an average annual incidence of 6.79/100 000 children, which is similar to other European countries. For example, in Denmark, an annual incidence in children under 14 years was reported to be 18, and in England, 6.21–20.4/100 000 children were at risk. The estimated annual incidence in the Czech Republic was found to be 10.2, while that in Netherlands was 6.1/100 000. In Asia, the annual incidence was found to be 12.9 in children under 17 in Taiwan and 55.9 per 100 000 in South Korea.[1 14 25–29] As a consequence, two questions naturally arise and should be discussed: where do events happen and why do they happen where they happen?

When observing infectious diseases epidemiology, due to the spread of the microbial agent causing the disease, clustering is expected. Thus, spatio-temporal distributions of infectious diseases have been extensively studied.[30–33] However, it seems that several non-communicable diseases in humans also show clustering in space. Such is the case of inflammatory polyarthritis, heart diseases and diabetes.[34–36] On the contrary, rheumatoid arthritis showed no evidence of spatial clustering.[37]

The four largest cities in Croatia are dispersed across the country. In the western inner part lies the capital city, Zagreb; in the eastern part is Osijek, while in the northern Mediterranean part lies Rijeka, and in the southern, Split. Morran's I showed significant positive spatial autocorrelation, indicating that both IgAV and IgAVN are not randomly distributed in space. Based on the LISA analysis, three high–high clusters were found around Zagreb, Rijeka and Split, showing higher incidence positive autocorrelations around those areas. Interestingly, despite the highest incidences clustering around large cities, higher incidences were not found in the eastern part, near Osijek. Low–low clusters were found dispersed partially in the eastern and northern continental, as well as in the mid-Mediterranean part of Croatia.

To the best of our knowledge, there is a very small amount of precedent work investigating the spatial relationship and using spatial analysis of IgAV incidence. Nielsen found suggestive but not conclusive evidence of space–time clustering in an epidemiological study of IgAV in Denmark.[14] However, there is a significant difference in the methodology provided compared with ours. The authors applied the methodology proposed by Knox,[38] where data were represented in a three-dimensional coordinate system: geographical coordinates of the patients' addresses and the date of admission. The authors extended their approach using municipality of residence as units, reducing the geographical coordinates into a single number, further applying simulation with random permutations with the coordinates of the experimental data.

In another study, Farley *et al* investigated the epidemiology of a cluster of IgAV, providing weak support for infectious causes.[39] It should be noted that the word cluster is used in a different context. The authors identified children with IgAV in a geographical area and described a case cluster which was clearly defined in space and time.

One particularly interesting finding in our study is the linear clustering of IgAVN in the eastern part of Croatia. It should be noted that this linear pattern follows the course of the Drava and, partially Danube, rivers. Parts of eastern Croatia near the Sava and Danube rivers are known to be locations where Balkan endemic nephropathy occurs. Although this disease is a completely different entity, it has also been associated with diverse aetiological agents, ranging from viral infection, genetic predisposition, water contamination with lignite toxins and ochratoxin in food.[40]

If a parallel is drawn with chronic kidney disease, the IgAV and IgAVN hotspot clusters appear where genetic and environmental factors overlap substantially.[41] It can be hypothesised whether these hotspots are primarily caused by environmental factors with possible genetic influences or are hotspots that are primarily caused by genetic factors, while environmental factors are triggers that contribute to a wide range of clinical manifestations. For example, the aforementioned Balkan endemic nephropathy is known to be an example of a disease in which hotspots appear primarily due to environmental factors (aristolochic acid), although clusters have been observed within individual families, speculating that different gene polymorphisms that may activate or detoxify may be responsible for this clusters.[41] On the other hand, there is IgA nephropathy, which is associated with different variants of innate immunity genes, as well as with genes important for defence against parasitic infections.[42] This explains the occurrence of hotspots of this disease in East Asia. Environmental factors, such as various infections, especially respiratory, can be triggers in this disease.

Geospatial analysis may serve as a stepping stone for further research. A potential causal risk assessment would take a multivariate approach correlating potential risk factors which range from sources of environmental contaminations, socioeconomics to genetics factors. Considering resource and time limitations, instead of a population-based screening approach, when a higher incidence cluster is identified, researchers might consider to investigate what the risk factors specific for this cluster may be. From an environmental perspective, a multilayer analysis considering possible environmental risk factors (ie, pollution sources). A socioeconomic-driven investigation would use factors related to higher or lower socioeconomic status. Investigating the genetic aspect would consider comparing subpopulations between higher incidence clusters, or comparing them to subpopulations in regions of lower incidence to identify potential genetic or epigenetic diversity. Identifying spatial clusters may not only serve for research purposes but also for healthcare planning of target interventions. Healthcare policy providers might especially pay attention and allocate resources to support interventions if a higher incidence cluster was found in remote regions, which have less access to specialist care. Besides planning healthcare interventions on a national level, identifying local clusters may be beneficial for local healthcare providers by providing useful information on the distribution of chronic diseases and building partnerships between community health centres, organisations, public health centres and regional clinics.

It should be noted that there are several limitations in this study. The first is being a retrospective study. Another limitation is the possibility of not including all of the children with IgAV, particularly the ones with milder symptoms that might have been treated in local county hospitals, which may lead to potential ascertainment bias. Although, based on our knowledge, area coverage and consultations with colleagues from local county

hospitals, and low average annual incidences of IgAV and IgAVN, we estimate that the number of not included patients should not be large. Mainly, since almost all Croatia's teaching hospital centres were included in this research, using the Bayesian interpolation approach may be a reasonable compensation for this limitation. Besides, we used a stationary approach relying on spatial analyses, not investigating the spatiotemporal distributions. However, due to the nature of the analysis, the research may suffer from the problem of multiple comparisons, which may increase the type I error. Due to the lack of a completely satisfactory solution, we added different p value thresholds on the significance map. In this case, the term interesting observations of clusters may be more suitable rather than an explicit statement of significant clustering.[43]

Spatial statistics is still a relatively new field, and its applications in medicine are being further developed. We believe that combining epidemiological surveillance with spatial analyses may help to identify populations at risk and to generate aetiological hypotheses and serve as a tool for identifying causes of IgAV and IgAVN.

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Contributors MSa and MJ designed the research and prepared and analysed the data. MJ is the project Leader of Croatian Science Foundation Research Project IP-2019-04-8822: 'Histological, Clinical, Laboratory and Genetic Predictors of Outcome in Patients with Henoch-Schönlein Purpura and Nephritis (PURPURAPREDICTORS)'; she reviewed and revised the manuscript and supervised the research. MSa wrote the first draft, interpreted the results, reviewed the literature and revised the draft. MF, MSe, SS, AO, MBV and AG contributed to the acquisition and interpretation of data for the study: they examined the patients, performed rheumatological assessments, created a database which includes patients with IgAV and contributed in interpreting the study data and in writing the manuscript. KK, DB and KM contributed to the analysis and interpretation of data for the study: they performed the statistics, developed the figures, analysed and interpreted the study data and contributed in writing the manuscript. All authors read and approved the final manuscript to be published. All coauthors are fully responsible for all aspects of the study and the final manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Competing interests None declared.

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

Patient consent for publication Not required.

Ethics approval The study was approved by the ethics committee of the University of Zagreb School of Medicine, Zagreb, Croatia (date: 18 September 2019; protocol number - Class: 641-01/19-02/01).

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Data availability statement Data are available upon reasonable request. The data that support the findings of this study are available upon request from the corresponding author.

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TRANSLATIONAL SCIENCE

Monocyte and bone marrow macrophage transcriptional phenotypes in systemic juvenile idiopathic arthritis reveal TRIM8 as a mediator of IFN-γ hyper-responsiveness and risk for macrophage activation syndrome

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ABSTRACT

Objectives Systemic juvenile idiopathic arthritis (SJIA) confers high risk for macrophage activation syndrome (MAS), a life-threatening cytokine storm driven by interferon (IFN)-γ. SJIA monocytes display IFN-γ hyper-responsiveness, but the molecular basis of this remains unclear. The objective of this study is to identify circulating monocyte and bone marrow macrophage (BMM) polarisation phenotypes in SJIA including molecular features contributing to IFN response.

Methods Bulk RNA-seq was performed on peripheral blood monocytes (n=26 SJIA patients) and single cell (sc) RNA-seq was performed on BMM (n=1). Cultured macrophages were used to define consequences of tripartite motif containing 8 (TRIM8) knockdown on IFN-γ signalling.

Results Bulk RNA-seq of SJIA monocytes revealed marked transcriptional changes in patients with elevated ferritin levels. We identified substantial overlap with multiple polarisation states but little evidence of IFN-induced signature. Interestingly, among the most highly upregulated genes was TRIM8, a positive regulator of IFN-γ signalling. In contrast to PBMC from SJIA patients without MAS, scRNA-seq of BMM from a patient with SJIA and MAS identified distinct subpopulations of BMM with altered transcriptomes, including upregulated IFN-γ response pathways. These BMM also showed significantly increased expression of TRIM8. In vitro knockdown of TRIM8 in macrophages significantly reduced IFN-γ responsiveness.

Conclusions Macrophages with an 'IFN-γ response' phenotype and TRIM8 overexpression were expanded in the bone marrow from an MAS patient. TRIM8 is also upregulated in SJIA monocytes, and augments macrophage IFN-γ response in vitro, providing both a candidate molecular mechanism and potential therapeutic target for monocyte hyper-responsiveness to IFNγ in cytokine storms including MAS.

Key messages

What is already known about this subject?

- Children with systemic juvenile idiopathic arthritis (SJIA) are at risk for the cytokine storm macrophage activation syndrome (MAS). Hyper-responsiveness of SJIA monocytes to interferon (IFN)-γ is a key driver of MAS, but the molecular mechanisms that promote this are unknown.

What does this study add?

- SJIA monocytes display both proinflammatory and anti-inflammatory properties, in an attempt to compensate for systemic hyperinflammation.
- Overexpression of the IFN regulator tripartite motif containing 8 (*TRIM8)* distinguishes circulating monocytes in SJIA and haemophagocytic bone marrow macrophage subpopulations in one SJIA patient with early MAS.
- TRIM8 increases macrophage responsiveness to IFN-γ, the pivotal cytokine in MAS, and thus may promote this complication in SJIA.

How might this impact on clinical practice or future developments?

- TRIM8 represents both a molecular mechanism and novel therapeutic target for monocyte responsiveness to IFN-γ in cytokine storms including MAS.

INTRODUCTION

Systemic juvenile idiopathic arthritis (SJIA) is the most severe subtype of JIA, notable for marked systemic immune activation with features of auto-inflammation.[1 2] The pathophysiology of SJIA is driven by continuous activation of innate immune pathways especially by the monocyte/macrophage lineage, although the precise cellular source of the pivotal IL-1 and IL-6 cytokines in SJIA remains unclear.[3 4] Gene expression signatures in peripheral blood mononuclear cells (PBMCs) during active SJIA reveal increased expression of monocyte and macrophage activation markers, genes induced by TLR/interleukin (IL)-1 signalling pathways, and genes involved in negative regulation of innate inflammatory responses.[5–7] About 15% of SJIA patients will also develop macrophage activation syndrome (MAS), a life-threatening episode of

hyperinflammation driven by excessive activation and expansion of T cells and haemophagocytic macrophages.[8–12] This cytokine storm leads to extreme hyperferritinaemia, cytopenias, liver dysfunction and coagulopathy.[10 12] For reasons poorly understood, while widespread use of biologics targeting IL-1 and IL-6 has markedly improved overall disease control, children with SJIA remain at risk for MAS.[12–14]

MAS bears close clinical resemblance to haemophagocytic lymphohistiocytosis (HLH), a constellation of life-threatening cytokine storm syndromes due to both primary HLH (pHLH) and secondary acquired causes.[15 16] pHLH is a group of rare disorders linked to genetic defects affecting the perforin-mediated cytolytic pathway.[15] MAS in SJIA is widely viewed as a distinct form of secondary HLH occurring in the setting of inflammatory and rheumatic disorders.[12] Interestingly, up to 40% of SJIA patients who develop MAS carry hypomorphic mutations in pHLH genes.[17 18] Substantial evidence in pHLH supports interferon (IFN)-γ blockade as novel therapy for this cytokine storm,[19] and the anti-IFN-γ antibody emapalumab has been recently approved for this condition.[20] Interestingly, while IFN-γ does not play a major role in the pathogenesis of SJIA itself,[21] in several studies the development of MAS in SJIA patients paralleled activation of IFN-induced pathways in monocytes, and distinguished acute MAS versus a conventional flare of SJIA.[22 23] These observations combined with the fact that neutralisation of IFN-γ reverted MAS in a murine model,[24] led to the phase II clinical trial of emapalumab in MAS complicating SJIA (NCT 03311854), the preliminary results of which are promising.[25] IL-18 has been identified as another key cytokine in MAS pathophysiology, with elevated IL-18 distinguishing both SJIA and adult-onset Still's disease and associated with risk for MAS, presumably through augmenting IFN-γ production.[4 26 27]

Another intriguing observation by us[21] and others[28] is that monocytes in SJIA exhibit hyper-responsiveness to IFN-γ in vitro that may be further exaggerated by IL-1 and IL-6 inhibiting biologics, which could explain the persistent risk for MAS in SJIA treated with these agents. The mechanistic reasons for such hyper-responsiveness remain unclear, but may be determined by the subtype and polarisation status of monocytes and macrophages.[29] While previously considered as a dichotomy between classically activated 'M1' and alternatively activated 'M2' macrophages, recent work has shown that macrophages are activated towards a diverse spectrum of distinct polarisation phenotypes.[30] Previous cell-surface immunophenotyping has demonstrated that monocytes in SJIA do not align with a single polarisation state, but rather exhibit features reflecting multiple activation phenotypes.[31 32]

The objective of this study was to further characterise the polarisation phenotype of both circulating monocytes and bone marrow macrophages (BMM) from SJIA patients using transcriptional profiling, to identify factors that may influence cellular responsiveness to IFN-γ and risk for development of cytokine storm.

METHODS

Patients and peripheral blood samples

Written informed parental consent was obtained for each subject prior to participation, and child assent was obtained where appropriate. Fresh whole blood was collected from SJIA patients (table 1 and online supplemental table S1) with active new onset or established disease; clinically inactive disease (CID) as defined by the Wallace criteria,[33] and healthy age-matched controls obtained separately from children undergoing routine phlebotomy. All enrolled patients satisfied the ILAR classification criteria for SJIA.[1] MAS patients met the 2016 MAS classification criteria.[11] Monocytes were isolated as described.[32]

Table 1 Summary of clinical and laboratory characteristics for patients in this study

	Active SJIA	Inactive SJIA	Controls
N	16	10	11
Female:male	13:3	8:2	5:6
Median age, years (IQR)	8.5 (3.25–11.75)	6.5 (3–12.5)	12 (7–16)
Median disease duration, months (IQR)	15.5 (3–48)	80 (9–103)	NA
Active arthritis (%)	14 (88)	0	NA
Median active joints (IQR)	5 (2.25–16.25)	0	NA
Fever (%)	8 (50)	0	NA
Rash (%)	10 (63)	0	NA
HSM (%)	7 (44)	0	NA
Adenopathy	5 (31)	0	NA
Median Ferritin, ng/mL (IQR)	194 (42–5327)	30 (7–161)	ND
Median CRP, mg/dL (IQR)	8.79 (<0.29–13.53)	0.35 (<0.29–0.74)	ND
Median ESR, mm/hour (IQR)	36 (6–65)	7 (5.25–9.25)	ND

CRP, C reactive protein; ESR, erythrocyte sedimentation rate; HSM, hepatomegaly and/or splenomegaly; NA, not applicable; ND, not determined; SJIA, systemic juvenile idiopathic arthritis.

In vitro macrophage polarisation

THP-1 human monocytic cell line (American Type Culture Collection (ATCC)) and primary human monocytes was maintained in RPMI and polarised as described.[32]

Bulk and single-cell RNA-seq gene expression profiling

Methods regarding bulk RNA-seq analysis of peripheral blood monocytes and single-cell RNA-seq analysis of BM macrophages are in online supplemental methods.

Tripartite motif containing 8 knockdown via siRNAs

THP-1 was transfected with either ON-TARGETplus siRNA against tripartite motif containing 8 (*TRIM8*) (Dharmacon) or ON-TARGETplus Non-targeting Pool (Dharmacon) using our established protocol.[34] *TRIM8* primers were: 'Forward': 5'-CCTATCTGCCTGCACGTTTT-3'; 'Reverse': 5'-GTTGTAGGCCTGGTTGCACT-3'. Primers for *GAPDH* have been previously reported.[32]

Monocyte responsiveness to IFN-γ

STAT1 phosphorylation assays were performed as described.[21] *CXCL9*/*CXCL10* primers have been previously reported,[32] and expression was assessed by RT-PCR.

RESULTS

SJIA monocyte transcriptomes reflect multiple polarisation states but lack prominent features of IFNγ-response

Bulk RNA-seq of purified peripheral blood monocytes was performed in 26 patients with SJIA (table 1). This revealed marked transcriptional changes between cells from SJIA patients and healthy controls, regardless of disease activity (figure 1A). Control samples as a group did show some increase in mitochondrial reads and decrease in ribosomal reads, which may reflect some differences in cell quality from patients. However,

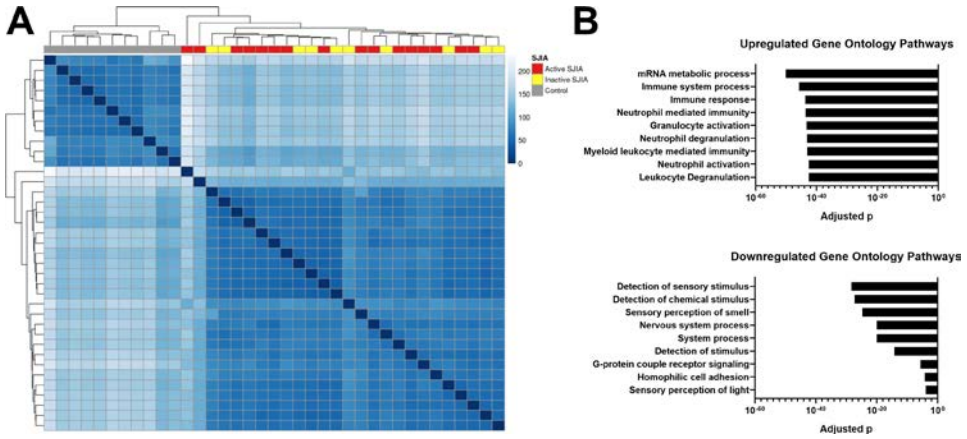

Figure 1 Differential gene expression in freshly isolated peripheral blood monocytes from patients with systemic JIA (SJIA) versus healthy controls. (A) Distance matrix based on all transcripts where red, yellow and grey colours indicate patients with active SJIA, inactive SJIA, and healthy controls respectively. Colours represent distance matrices calculated by computing the euclidean distance between all sample pairs. (B) Over-representation analyses of gene ontology terms for the upregulated (top) and downregulated (bottom) differentially expressed genes. JIA, juvenile idiopathic arthritis.

pathway analysis revealed that the most enriched gene ontology pathways among upregulated genes in SJIA patients included those involved in immune system processes ($p=21.9\times10^{-46}$) and myeloid-mediated immunity (5.08×10^{-43}) (figure 1B). Although not among the top 500 enriched pathways, pathways reflecting response to pro-inflammatory cytokines including IL-1 ($p=1.04\times10^{-7}$), TNF (6.94×10^{-6}), and IFN-γ (4.95×10^{-5}), but not IL-6 or IFN-β, were also significantly enriched. Together these data suggest that SJIA monocytes broadly exhibit altered transcriptional activity reflecting an activated phenotype. Strikingly, no clear separation was observed between monocytes from SJIA patients with active disease versus those with CID (figure 1A).

Since this comparison considered as the 'active SJIA' group a highly heterogeneous collection of all patients with variable disease duration who failed to meet the Wallace criteria for CID,[33] we next examined more specific markers of inflammatory activity. Patients with SJIA and particularly those with features of MAS are characterised by hyperferritinaemia. Stratifying SJIA patients with high (≥210 ng/mL) vs normal serum ferritin levels (a cut-off that best paralleled SJIA patient clustering in our previous gene expression study[6]), showed clear separation of monocytes into two groups, one including 7/8 'high ferritin' samples and the other exclusively 'normal ferritin' samples (figure 2A). Interestingly 4/6 'normal ferritin' samples that clustered with the 'high ferritin' group had mild elevations in inflammatory markers without hyperferritinaemia. We also noted that there was significant overlap between the 'high ferritin' samples and untreated, new-onset SJIA (6/8 'high ferritin' patients; online supplemental table S1). Differential expression analysis revealed 686 upregulated and 418 downregulated genes between 'high ferritin' and 'normal ferritin' monocytes (figure 2B and online supplemental table S2). The gene set enrichment analysis revealed upregulation of pathways including immune response (4.76×10^{-44}), vesicle-mediated transport (5.06×10^{-41}), myeloid cell activation (3.42×10^{-40}) and secretion (1.26×10^{-35}) (figure 2C). While not among the top 200 enriched pathways, the 'high ferritin' signature did show mild enrichment in pathways reflecting response to both IL-1 (3.6×10^{-3}) and IFN-γ (1.94×10^{-6}), supporting the association between elevated ferritin and MAS. There was no significant enrichment in pathways reflecting specific polarisation phenotypes. Among the downregulated genes there were no pathways with adjusted $p<0.1$. Together, this suggests that monocytes from SJIA patients with elevated ferritin show proinflammatory transcriptional activation.

Previous work has suggested that monocytes in SJIA display features of multiple polarisation phenotypes. To further characterise the polarisation properties of SJIA monocytes, we first empirically determined transcriptional signatures of primary monocytes from healthy individuals polarised towards well described in vitro phenotypes to generate M(LPS+IFN-γ), M(IL-4), M(LPS+immune complexes (IC)) and M(IL-10) signatures (online supplemental figure S1 and table S3). When comparing the 'high ferritin' SJIA monocyte signature to these empirically determined polarisation signatures, we found the highest enrichment in the alternatively activated M(IL-4) and M(IL-10) signatures, with less enrichment with classically activated M(LPS+IFN-γ) signature. Together, these demonstrate that monocytes in SJIA reflecting either a mixed polarisation phenotype, or multiple distinct cell populations (figure 2D).

Active SJIA is also associated with markedly elevated levels of serum IL-18.[4 26 27] Since increased free (unbound) serum IL-18 is proposed to promote MAS by enhancing IFN-γ production, we assessed expression of both IL-18 and its natural antagonist IL-18 binding protein (IL-18BP). As shown in figure 2E, compared with healthy controls, SJIA monocytes were expressing significantly higher levels of *IL18* and significantly lower levels of *IL18BP*.

Elevated expression of IFN gamma receptors and *TRIM8*

Since we and others have shown that SJIA monocytes show increased responsiveness to IFN-γ, we then examined these signatures to identify factors that could modulate IFN signalling. Both 'high ferritin' and 'normal ferritin' SJIA monocytes expressed significantly higher levels of IFN-γ receptors (*IFNGR1 and IFNGR2*) compared with monocytes from healthy controls (figure 3A). Increased expression of IFN receptors has been previously shown to contribute to increased IFN responsiveness in human monocytes and macrophages.[29 35] To further explore this finding, we examined protein levels of IFNGR (CD119) on the surface of monocytes from patients with active SJIA. As shown in figure 3B,C, we find significantly increased surface expression of CD119 on SJIA patient monocytes, compared with control monocytes.

Interestingly, our gene expression data also identified marked overexpression of *TRIM8* compared with control monocytes, regardless of SJIA disease activity (figure 3A). As shown in

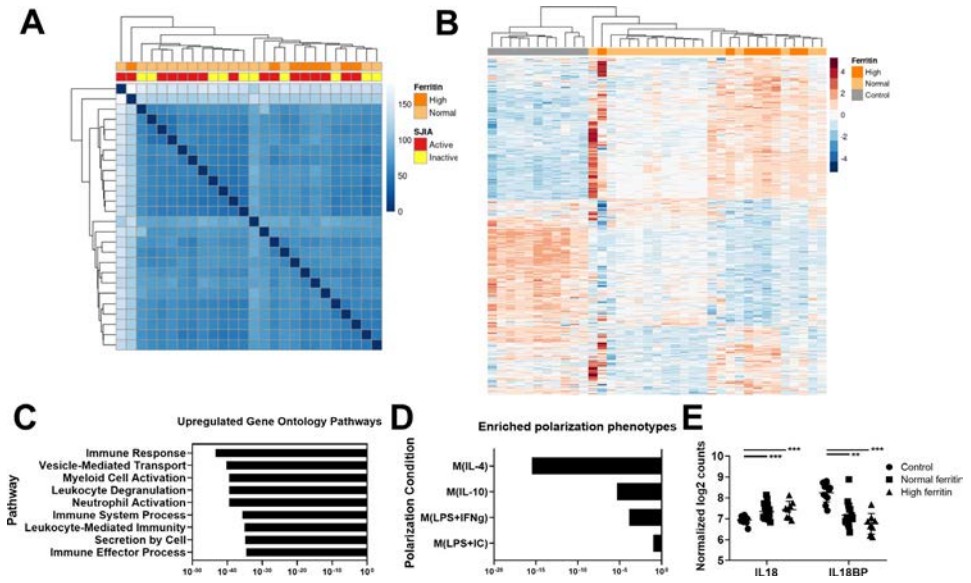

Figure 2 Analysis of the genes differentially expressed in freshly isolated peripheral blood monocytes from SJIA patients with high versus normal serum ferritin levels. The list of differentially expressed genes were generated using *limma* moderated t-tests, with FDR 5%. (A) Distance matrix based on all transcripts where red and yellow colours indicate patients with active SJIA and inactive SJIA, respectively, with colours representing distance matrices calculated by computing the euclidean distance between all sample pairs. Dark orange and light orange colours indicate patients with high and normal ferritin levels, respectively. (B) Hierarchical clustering of genes differentially expressed between high and normal ferritin groups. The complete linkage clustering algorithm, in which distance is a measure of similarity, was used to generate the hierarchical clustering tree. In this tree, each row represents a separate gene and each column represents a separate individual. Dark orange, light orange and grey colours indicate patients with high ferritin, normal ferritin and healthy controls, respectively. The scaled normalised expression level for each gene is indicated by colour. (C) Over-representation analysis of gene ontology terms for genes differentially expressed between high and normal ferritin groups (D) Polarisation signature analysis that reflects overlapping patterns of gene expression between SJIA monocytes and in vitro polarised M(LPS+IFN-γ), M(IL-4), M(LPS+IC) and M(IL-10) macrophages. (E) Expression levels of *IL18* and *IL18BP* in peripheral blood monocytes from patients with high ferritin (n=8), normal ferritin (n=18) and controls (n=11). **P<0.01. ***p<0.001 by ANOVA with follow-up Dunnett's multiple comparisons test. ANOVA, analysis of variance; IFN-γ, interferon-γ; IL-8, interleukin 8; SJIA, systemic juvenile idiopathic arthritis.

figure 3D, TRIM8 is an E3 ubiquitin-protein ligase and a positive regulator of IFN-signalling,[36 37] which participates in the activation of IFN-γ signalling by promoting proteasomal degradation of negative regulators including the suppressor of cytokine signalling 1 (SOCS1).[36] TRIM8 has also been reported to positively regulate NF-κB signalling pathways.[38] Indeed, we find that in addition to increased *TRIM8* expression, SJIA monocytes from patients with high ferritin upregulate more than 10% of genes in the 'I-kappaB kinase/NF-kappaB signalling' GO pathway (adjusted p=0.03). To confirm this observation of increased *TRIM8* expression, we assessed the whole blood gene expression profiles obtained during the clinical trial of canakinumab in SJIA.[7] As shown in figure 3E, *TRIM8* expression was upregulated in all SJIA patients compared with controls prior to canakinumab treatment (day 1). By day 3 of treatment, *TRIM8* expression significantly decreased in most responders and was comparable to controls, but remained elevated in non-responders. In contrast to SJIA, examination of our previously published gene expression data sets[39 40] revealed only subtle trend towards higher expression of *TRIM8* in whole blood in active polyarticular JIA (Log FC 0.11, p=0.05) and pHLH (Log FC 0.057, p=0.63). Taken together, these findings demonstrate that monocytes from patients with SJIA demonstrate several gene expression changes that could affect IFN-γ responsiveness, including *TRIM8* overexpression that is rather specific to SJIA.

Elevated *TRIM8* and IFN-γ-induced signature in haemophagocytic BMM in MAS

Circulating monocytes are recruited to inflammatory sites, where in the context of a specific cytokine milieu, they mature into resident macrophages. As such, blood monocytes may not reflect the phenotype of myeloid cells during SJIA and emergence of MAS. We utilised single cell (sc) RNA-seq to better understand the specific gene expression signatures of BMM in SJIA (figure 4A). Three independent control samples yielded 180 single BMM. While there was substantial interindividual variability, a core set of approximately 1400 genes were identified that contributed to the heterogeneity of normal BMM population (online supplemental figure S2A). Control macrophages formed three primary cellular clusters, which were distinguished based on expression of genes associated with inflammatory responses including IFNGR2 (cluster 1), granulocyte-monocyte colony stimulating factor (GM-CSF) signalling (cluster 2) and aurora B signalling (cluster 3) (figure 4B). We also noted that these profiles represent a dominant signature (spanning all three clusters and >10 000 genes), obscuring identification of other subclusters. We, thus, performed an unsupervised analysis, excluding this dominant signature, in ICGS2 (see online supplemental methods),[41] which identified 11 additional sub-clusters, including a small IFN- response enriched cell population (online supplemental figure S2B).

To assess changes in BMM populations in SJIA, we profiled a BMM sample from a patient with newly diagnosed SJIA with histologic findings on BM biopsy of mild histiocytic hyperplasia with rare haemophagocytosis (figure 4C), consistent with early or subclinical MAS (further patient description in online supplemental methods). Indeed, the sorted BM aspirate demonstrated a twofold increase in BMM than control aspirates (figure 4D). BMM expression profiles from this SJIA/MAS patient were largely distributed

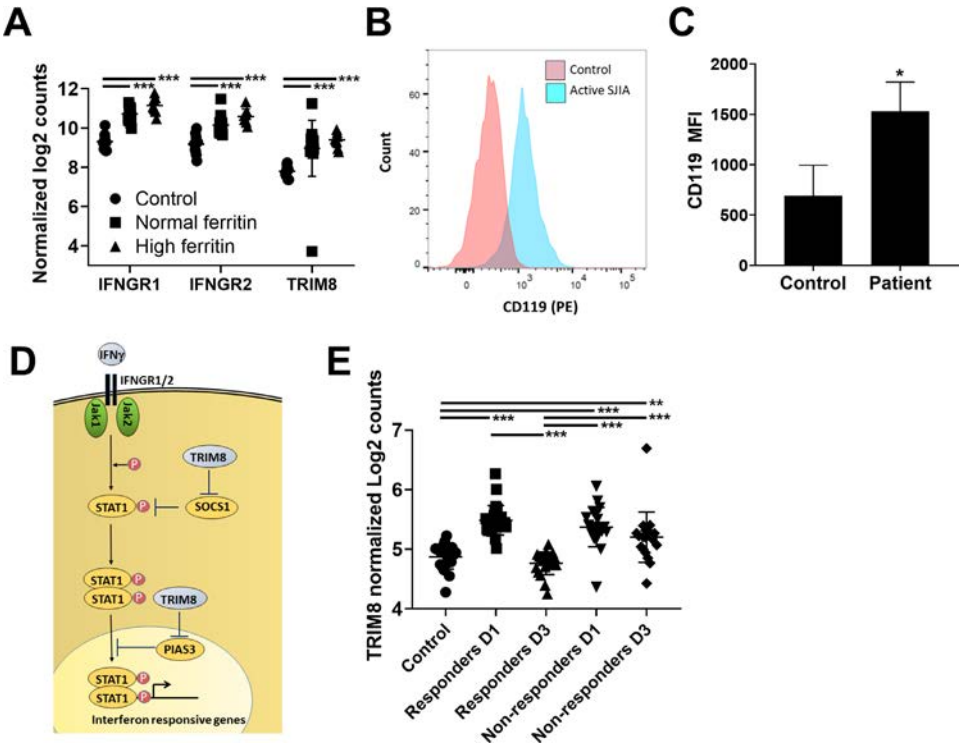

Figure 3 Differential expression of genes modulating IFNγ signalling pathway. (A) Normalised expression levels of *IFNGR1*, *IFNGR2* and *TRIM8* in peripheral monocytes from SJIA patients in the high-ferritin group (n=8), normal-ferritin group (n=18) and healthy controls (n=11). Expression levels of each individual gene for each patient were normalised against the mean expression level in the entire set of samples. ***P<0.001 by ANOVA with follow-up Dunnett's multiple comparisons test. (B) Representative histograms showing CD119 intensity in monocytes from control (red) and patients with active SJIA (blue) as determined by flow cytometry. (C) Median fluorescence intensity of CD119 as determined by flow cytometry in control and SJIA patient monocytes, pooled from three independent samples. *P<0.05 by t-test. (D) Schematic representation of IFNγ signalling pathway. TRIM8 degrades SOCS1 and PIAS3 levels, both of which negatively regulate STAT1 activity (adapted from reference 37). (E) Normalised expression levels of TRIM8 in whole blood from patients before (day 1) and after (day 3) initiation of canakinumab treatment, stratified by those achieving adapted ACR 50 response. **P<0.01. ***p<0.001 by ANOVA with follow-up Dunnett's multiple comparisons test. ANOVA, analysis of variance; IFNγ, interferon-γ; SJIA, systemic juvenile idiopathic arthritis; TRIM8, tripartite motif containing 8.

among control donor BMM clusters identified above (figure 4E). However, two clusters were identified with distinct subpopulations of BMM from the SJIA/MAS patient that exhibited markedly altered transcriptional profiles (figure 4E), and *TRIM8* was among the top marker genes of the smaller of these clusters. To understand the broader molecular impact of this subtype, we identified the cells with this *TRIM8*-associated signature to identify differentially expressed genes vs control macrophages (see online supplemental methods). In total this signature was present in 20% (12/61) of SJIA patient cells. In addition to upregulation of *TRIM8* (3.8-fold change), this SJIA/MAS macrophage population showed a strong *IFNγ*-induced signature ('cellular response to IFN gamma', adjusted $p=4.9\times10^{-3}$). In addition, this signature also demonstrated significant upregulation of gene pathways including response to cytokines ($p=3.7\times10^{-4}$) and innate immune response ($p=1.5\times10^{-3}$), and a large activated transcription-factor network (figure 4F,G). Notably this macrophage population signature included significant upregulation of pathways involved in intracellular granule movement ($p=2.7\times10^{-4}$) including the MAS-associated gene *STXBP2* ($p=3.1\times10^{-5}$), suggesting that we correctly identified the population of haemophagocytic macrophages (figure 4G, online supplemental table S4 and S5). There were no other specific cytokine response pathways that were significantly enriched. Crayne *et al*[42] recently suggested that haemophagocytic macrophages may have anti-inflammatory properties including heme-oxygenase (HO-1) production and secretion of IL-10 and IL-4. Although *HO-1* was expressed in this population of macrophages, *IL4* and *IL10* were not on the list of DEGs. In contrast, overexpression of several DAMPs capable of serving as endogenous TLR agonists (*HMGB1*, *HMBG2*, *S100A12* and *S100 A8/9*) was prominent. The presence of a strong *IFN-γ-induced signature* on the other hand was consistent with work demonstrating that IFN-γ alone can act directly on macrophages to induce haemophagocytosis leading to consumptive anaemia of inflammation.[43] Together, this suggests that there exists distinct and activated proinflammatory BMM subpopulations during early/subclinical MAS, with the potential for exaggerated responses to IFN-γ.

Previously, Cepika *et al*[44] identified decreased aryl hydrocarbon receptor (AHR) expression in SJIA monocytes as a factor promoting differentiation of monocytes into macrophages in SJIA patients. The authors felt that this was an important factor contributing to the risk for MAS. Although in our data set *AHR* was not on the list of the DEGs in the population of haemophagocytic BMM, there were two other genes from the same signalling pathway: *AIP* and *AHRR*, both overexpressed. *AHRR* encodes AHR repressor that functions as a feedback modulator by repressing AHR-dependent gene expression.[45] The *AIP* gene (AHR interacting protein) has also been implicated in negative regulation of AhR signalling.[46] Overexpression of these two genes in proinflammatory likely haemophagocytic macrophages would lead to downregulation of the AHR pathway in transition to MAS. Therefore, our data does support the observation made by Cepika *et al*.

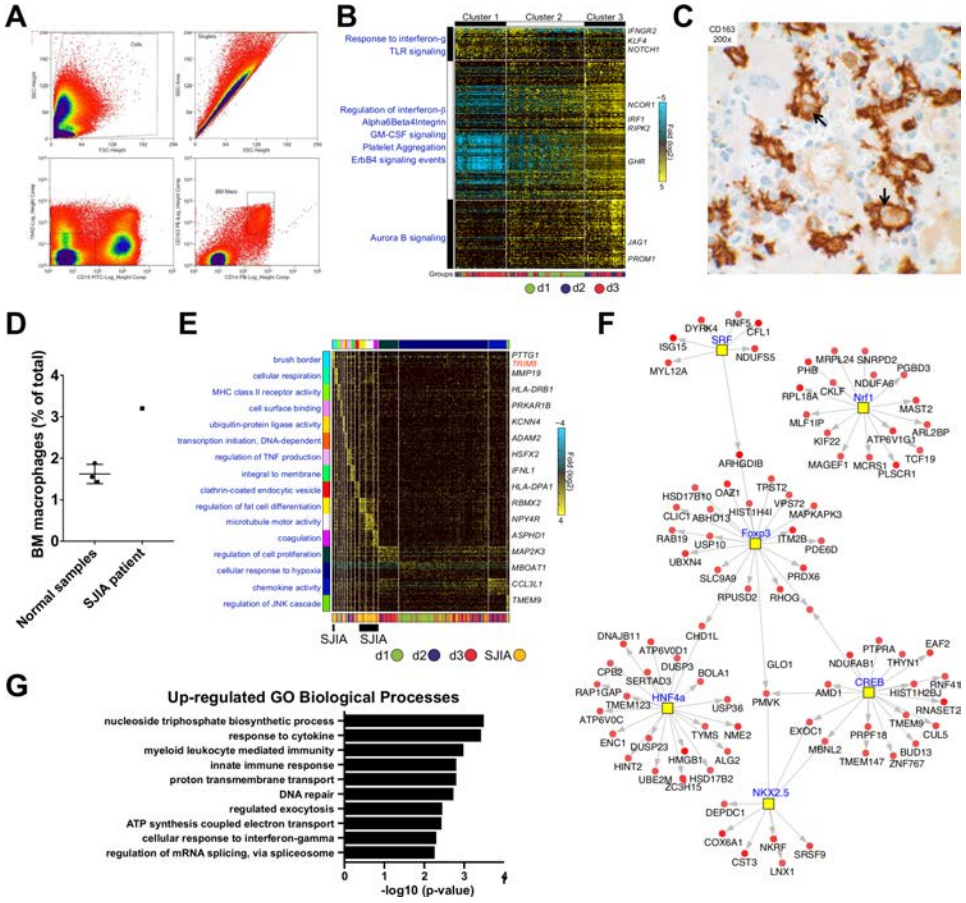

Figure 4 Single-cell RNA sequencing of BMM identifies distinct subpopulations in MAS with features of interferon response. (A) Isolation of BMM by flow cytometry. Cells were gated for 7AAD- (live)/CD15−, and then for CD14+CD163+ macrophages. (B) Identification of macrophage populations in normal BM samples (d1–3), using HOPACH clustering of the most highly variable genes. This clustering shows significant variability in expression in BMM from three independent normal clinical biopsy samples. At least three distinct cluster of macrophages and at least three groups of genes could be discriminated. Along left side of the plot are enriched functional pathways within the gene cluster (PathwayCommons), and representative genes listed along right edge. (C) BM biopsy from patient with new-onset SJIA. Immunohistochemical staining with CD163 shows increased macrophages/histiocytes with rare haemophagocytosis. (D) Proportion of CD14 +CD163+CD15- macrophages isolated from SJIA patient compared with those from normal BM samples. (E) Distinct macrophage population with altered transcriptional profile in MAS. Unsupervised clustering of scRNA-seq (ICGS version 2) from three normal samples (d1–3) and one patient with SJIA and early MAS. The black bars at the bottom of the plot denote SJIA specific or highly enriched clusters. The top marker gene is shown to the right of the plot and *TRIM8* is denoted in red. (F) Network representation of statically enriched transcription factor regulated targets from the software GO-Elite (TFTarget database) for upregulated genes in the *TRIM8* MAS BM expanded cell cluster. Red circles denote upregulated genes and yellow boxes denote the predicted regulatory transcription factor (G) Gene-set enrichment of differentially expressed genes in systemic JIA BM macrophage subpopulation compared with all control BMM using the ToppFun website.[49] d1, d2, d3 represent control BMM donor samples. 7AAD, 7-aminoactinomycin D; BMM, bone marrow macrophage; ICGS, iterative clustering and guide-gene selection; MAS, macrophage activation syndrome; scRNA, single cell RNA; SJIA, systemic juvenile idiopathic arthritis; TRIM8, tripartite motif containing 8.

TRIM8 knockdown via siRNAs in THP-1 macrophages led to decreased production of *CXCL9* and *CXCL11* in response to stimulation with IFN-γ in vitro

Based on prior work, we hypothesised that *TRIM8* overexpression will decrease repression of IFN-induced signalling in SJIA monocytes and macrophages, leading to exaggerated responsiveness to IFN-γ and contributing to the development of MAS. We assessed the effects of *TRIM8* knockdown in THP-1 macrophages on expression of the IFN-induced genes *CXCL9*/*CXCL11* on IFN-γ stimulation in vitro. *TRIM8* siRNA was used to reduce expression of TRIM8 at both mRNA and protein levels (figure 5A,B). As shown in figure 5C, on IFN-γ treatment, macrophages with reduced levels of TRIM8 demonstrated significantly reduced upregulation of *CXCL9* and *CXCL10*. Notably, *TRIM8* knockdown did not simply change the kinetics of IFN-γ response, as diminished IFN-γ-induced expression of CXCL9/CXCXL11 was observed at 4, 16 and 24 hours post-treatment (figure 5D).

TRIM8 knockdown decreases STAT1 phosphorylation in response to IFN-γ in vitro

Finally, to assess whether decreased production of *CXCL9/11* was associated with decreased IFN-induced signalling, phosphoflow was used to measure STAT1 phosphorylation in THP-1 cells in response to stimulation with IFN-γ in vitro. As shown in figure 6, pretreatment with *TRIM8* siRNA decreased the intensity of pSTAT1 signal assessed 30 min after IFN-γ stimulation. Taken together, we show that TRIM8 expression is required for full macrophage responsiveness to IFN-γ, and could represent a key and targetable pathway in MAS pathogenesis.

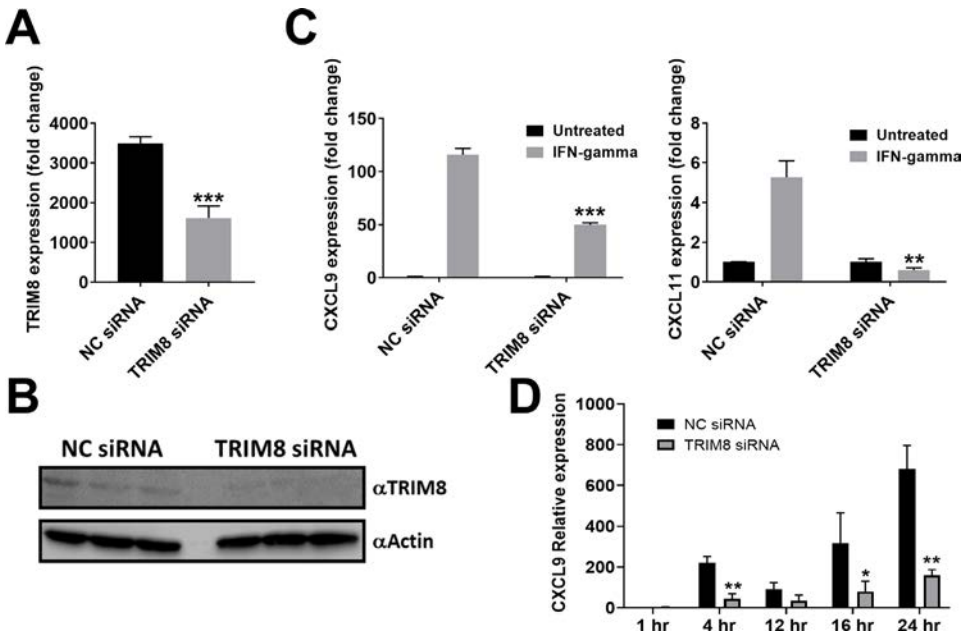

Figure 5 Effects of *TRIM8* knockdown on CXCL9 and CXCXL11 production in THP-1-derived macrophages stimulated with IFN-γ in vitro. Macrophages were incubated with either negative control (NC) or TRIM8 siRNAs. (A) *TRIM8* mRNA levels in macrophages treated with either NC or *TRIM8* siRNAs assessed by RT-PCR. ***P<0.001 by t-test. (B) TRIM8 protein levels in triplicate samples of macrophages treated with either NC or *TRIM8* siRNAs assessed by Western Blot. (C) Fold increase in *CXCL9* and *CXCL11* mRNA levels as determined by RT-PCR in macrophages pretreated with either NC or *TRIM8* siRNAs at 4 hours after stimulation with IFN-γ in vitro. **P<0.01, ***p<0.001 by ANOVA with follow-up Dunnett's multiple comparisons test. (D) Expression of *CXCL9* as determined by RT-PCR relative to *GAPDH* in macrophages pretreated with either NC or *TRIM8* siRNAs at 1,12,16 and 24 hours after stimulation with IFN-γ in vitro. All experiments were performed in triplicates. *P<0.05. **p<0.01 by ANOVA with follow-up Dunnett's multiple comparisons test. ANOVA, analysis of variance; IFN-γ, interferon-γ; TRIM8, tripartite motif containing 8.

DISCUSSION

Children with SJIA demonstrate continuous activation of monocytes and macrophages.[3] However, the precise function of these cells in systemic hyperinflammation remains poorly understood. Several gene expression, immunophenotyping and microRNA analysis studies suggest that monocytes in SJIA display a mixed polarisation state, with markers reflecting both classical activation and multiple alternatively activated phenotypes.[5 6 28 31 32] Here, we report extensive transcriptional profiling of purified monocytes from patients with SJIA. First, we find that SJIA monocytes show dramatically distinct transcriptomes from control monocytes, regardless of disease activity. This is in agreement with our previous work showing persistently altered microRNA profiles in monocytes during inactive disease, and may suggest persistent and more durable epigenetic changes in these cells. Second, we identify a robust transcriptional signature of myeloid cell activation present in monocytes from SJIA patients with elevated serum ferritin levels. Third, we show that this 'high ferritin' signature was enriched for genes representing multiple polarisation phenotypes, but most enriched for alternatively activated conditions such as M(IL-4) and M(IL-10). Together, these data suggest that SJIA monocytes are functioning in an attempt to compensate for systemic hyperinflammation, and display both proinflammatory and anti-inflammatory properties.

MAS remains a critical complication of 10%–15% of SJIA patients despite introduction of IL1- and IL6-inhibiting biologics. IFN-γ, a cytokine not considered a major player in SJIA itself, is increasingly recognised as a pivotal driver of MAS.[12 23 24] Consistent with this concept, preliminary results of the ongoing Phase II clinical trial of the anti-IFN-γ antibody emapalumab in MAS/SJIA

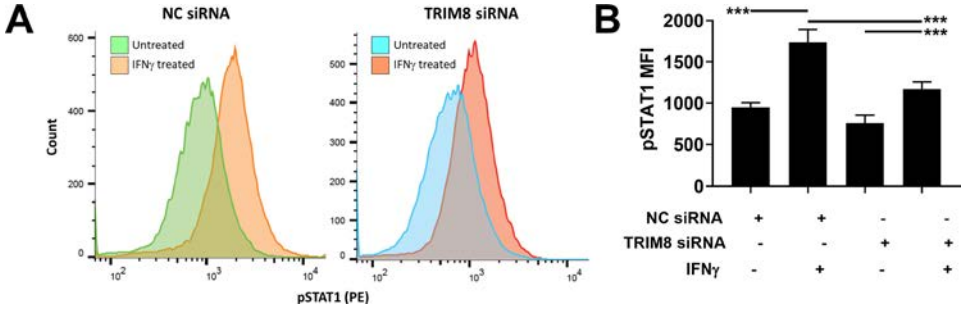

Figure 6 TRIM8 knockdown reduces IFNγ-mediated STAT1 phosphorylation. THP-1-derived macrophages were incubated with either negative control (NC) or *TRIM8* siRNA. Intracellular STAT1 phosphorylation was assessed by flow cytometry at 30 min after in vitro stimulation with IFNγ. (A) Representative histograms showing pSTAT1 intensity in macrophages treated with NC (left) and TRIM8 (right) siRNA at baseline and 30 min after stimulation with IFN-γ in vitro. (B) Median fluorescence intensity of pSTAT1 indicating the amount of pSTAT1 produced in cells, pooled from three independent experiments. ***P<0.001 by ANOVA with follow-up Dunnett's multiple comparisons test. ANOVA, analysis of variance; IFN-γ, interferon-γ; TRIM8, tripartite motif containing 8.

(NCT03311854) are very promising.[25] Interestingly, and consistent with prior work,[5 6] we found little evidence of IFN-γ-mediated activation in circulating SJIA monocytes. However, during MAS circulating monocytes are recruited to inflammatory sites where they mature into activated tissue macrophages. To explore that, we report the first transcriptional profile of haemophagocytic BMM during MAS at the sc level. These cells showed upregulated gene pathways that would be predicted for haemophagocytes, including cytokine response, granule secretion, and MAS-associated genes. They also exhibited a strong IFN-γ-induced signature, which is among the most significantly enriched gene ontology pathways. These findings are consistent with a model where in MAS, inflammatory monocytes rapidly traffic to tissue on IFN-γ activation. It also highlights the importance of studying key effector cells in tissue in conjunction with the periphery.

Overall our data support the concept that increased IFN-γ activity observed during MAS could be facilitated by two factors. The first is strikingly high levels of free IL-18, a cytokine that augments production of IFN-γ in response to various stimuli.[4 26 27] Our data here confirm that monocytes in SJIA show a progressive increase in *IL18* expression, and decrease in *IL18BP* expression, when stratified by disease activity and degree of hyperferritinaemia. Notably, the primary cellular source of IL-18 in SJIA remains uncertain and may include epithelial cells.[4] However, a second key factor is the exaggerated responsiveness of monocytes and macrophages to IFN-γ which we have previously noted.[21] Indeed, markedly increased expression of the IFN gamma receptors (*IFNGR1* and *IFNGR2*) both transcriptionally and on the surface SJIA monocytes and macrophages may serve as one mechanism to exagerate these cell's responsiveness to IFN-γ. Similar IFN hyper-responsiveness has been recently reported in lupus, where increased expression of *IFNAR1* was found in monocytes from both mouse models and human patients, and linked to higher IFN-α-stimulated gene expression.[29 35]

More importantly, our study identified TRIM8 as a likely contributor to the exaggerated responsiveness of monocytes and macrophages to IFN-γ. This observation was confirmed using the publically available blood gene expression profiles obtained during the clinical trial of canakinumab in SJIA.[7] Separately, our scRNA-seq of BMM identified *TRIM8* overexpression as one of the features distinguishing multiple populations of proinflammatory macrophages with IFN-γ response signatures from other macrophages in the BM during SJIA. Of note, this patient had features of early/subclinical MAS without clinically overt disease; whether more extensive transcriptional changes are seen during progression to 'full-blown' MAS remains to be seen. TRIM8 is an E3 ubiquitin-protein ligase that plays important roles in innate immune pathways.[36–38] Thus, TRIM8 plays a positive role in the TNF- and IL-1β signalling pathways. Little is known regarding the transcriptional regulation of TRIM8. The main TRIM8 regulatory region contains ChIP-seq peaks from multiple transcription factors including the vital hematopoietic transcription factor GATA2. It likely has complex cytokine regulation, as it contains both a STAT1 peak suggesting induction by IFN-γ, and TRIM22, an IFN-γ-induced epigenetic repressor.[47 48] Mechanistically, TRIM8 induces the lys-63 polyubiquitination of MAP3K7/TAK1 component leading to the activation of NF- κB,[38] and was associated here with upregulation of NF-κB-induced genes in SJIA monocytes. TRIM8 also activates IFN-γ signalling by promoting proteasomal degradation of the IFN-γ repressors SOCS1 and PIAS3.[36 37] SOCS1 is induced by various proinflammatory cytokines including IFN-γ and negatively regulates IFN-signalling by inhibiting IFN-induced JAK-STAT activation.[35] Indeed, we show that *TRIM8* knockdown resulted in decreased STAT1 phosphorylation and decreased expression of *CXCL9* and *CXCL11* in response to stimulation with IFN-γ in vitro. The observed effects of TRIM8 knockdown on STAT1-phosphorylation may suggest that TRIM8 functions primarily through degradation of SOCS1 (figure 3B), but the mechanisms by which TRIM8 overexpression potentiates IFN responsiveness remains to be investigated. In addition, the effect of TRIM8 knockdown on IFN-induced responses in primary SJIA monocytes still needs to be assessed

We note that this study is limited by the BMM experiments being derived from a single patient. However, these experiments address the question of localisation of observed IFN-γ pathway associated gene expression responses at the single-cell level. A broader single-cell study would be needed to assess single-cell variability in a larger cohort. In addition, the specificity of TRIM8 elevation to SJIA monocytes remains to be tested in similarly isolated cells from other inflammatory disorders, as well as a critical need to test the impact of TRIM8 knockdown on primary SJIA monocytes and defining the mechanisms by which TRIM8 mediates IFN hyperresponsiveness. We also note that our SJIA cohort has a female predominance that is not observed in the disease more broadly. Finally, we note that the collection and processing of control samples as separate batches may introduce unmeasured variance in the gene expression profiles.

Augmented production of IFN-γ facilitated by IL-18, combined with exaggerated responsiveness to IFN-γ, that is, at least partially, caused by increased TRIM8, may be two pathophysiological features that explain strikingly high rates of MAS in SJIA. The in vitro experiments demonstrating the effects of TRIM8 inhibition on macrophage responsiveness to IFN-γ provide a rationale for using TRIM8 as a biomarker for risk for MAS. Indeed, future work will explore the potential of TRIM8 as a possible therapeutic target both in acute episodes of cytokine storm including MAS, as well as a long-term prophylactic for SJIA patients at risk for recurrent MAS.

DNA sequencing datasets

Bulk and single-cell RNA-seq datasets have been deposited in gene expression omnibus (GSE147608 and GSE147795, respectively).

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Contributors GS and AAG designed the study. GS, TD, SD, NF and ST performed the experiments. GS, AP, DS, MM, NS and AAG performed gene expression analysis. GS and AAG wrote the first draft of the manuscript. All authors contributed to the final manuscript and approved its submission.

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Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as online supplemental information. Bulk and single-cell RNA-seq datasets have been deposited in gene expression omnibus (GSE147608 and GSE147795, respectively).

regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

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TRANSLATIONAL SCIENCE

Association of novel rare coding variants with juvenile idiopathic arthritis

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ABSTRACT

Objective Juvenile idiopathic arthritis (JIA) is the most common type of arthritis among children, but a few studies have investigated the contribution of rare variants to JIA. In this study, we aimed to identify rare coding variants associated with JIA for the genome-wide landscape.

Methods We established a rare variant calling and filtering pipeline and performed rare coding variant and gene-based association analyses on three RNA-seq datasets composed of 228 JIA patients in the Gene Expression Omnibus against different sets of controls, and further conducted replication in our whole-exome sequencing (WES) data of 56 JIA patients. Then we conducted differential gene expression analysis and assessed the impact of recurrent functional coding variants on gene expression and signalling pathway.

Results By the RNA-seq data, we identified variants in two genes reported in literature as JIA causal variants, as well as additional 63 recurrent rare coding variants seen only in JIA patients. Among the 44 recurrent rare variants found in polyarticular patients, 10 were replicated by our WES of patients with the same JIA subtype. Several genes with recurrent functional rare coding variants have also common variants associated with autoimmune diseases. We observed immune pathways enriched for the genes with rare coding variants and differentially expressed genes.

Conclusion This study elucidated a novel landscape of recurrent rare coding variants in JIA patients and uncovered significant associations with JIA at the gene pathway level. The convergence of common variants and rare variants for autoimmune diseases is also highlighted in this study.

Key messages

What is already known about this subject?

► Juvenile idiopathic arthritis (JIA) is the most common type of arthritis among children, however, a few studies have focused on the contribution of rare variants to the aetiology of JIA.

What does this study add?

► This study elucidated the genomic landscape of recurrent rare coding variants in JIA patients by RNA-seq and whole-exome sequencing data.
► We identified novel genes harbouring rare coding variants in pathways significantly associated with JIA.

How might this impact on clinical practice or future developments?

► Results from rare variant analysis may help disease risk assessment and identification of new drug targets.

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most common type of arthritis among children and is an autoimmune disorder which can result in severe complications. JIA is highly heritable and there is also evidence of an increased incidence of other autoimmune diseases among JIA probands' relatives, demonstrating the familial autoimmunity in JIA,[1] suggesting shared genetic basis for JIA and other autoimmune diseases.

The predisposition to JIA involves complex genetic components which are polygenic and pleiotropic.[2] Genome-wide association studies have identified more than 30 common-variant loci associated with JIA,[3 4] and the heritability of JIA attributable to common genomic variations (SNP-h2) have been quantified to be 0.727 (±SE.e. 0.037).[5] It has been hypothesised that rare variants with minor allele frequency <0.5% are likely to make a major part of contribution to the missing heritability.[6 7]

It is known that rare variants play an important role in the pathogenesis of complex human diseases.[6] Several causative mutations in the *LACC1* gene have been found in families of severe systemic-onset JIA, oligoarticular JIA and enthesitis-related arthritis subtypes.[8 9] Variants in *LACC1* like p.Cys284Arg are also present in other autoimmune diseases, such as Crohn's diseases.[10] It has also been shown that the rare loss of function mutations of *UNC13D* were detected in patients of autoimmune lymphoproliferative syndrome.[11] Case data suggest that the mutations of the *UNC13D* gene may be related to the pathogenesis of systemic JIA, which provides a new perspective for the study of JIA.[12] At the same time, *UNC13D* was also associated with macrophage activation syndrome, the complication of JIA.[13] However, a few studies have systematically

assessed the contribution of rare variants to the development of JIA beyond family study.

We examined the association of rare coding variants with JIA based on RNA-seq datasets and whole-exome sequencing (WES) data of JIA patients. We further assessed the impact of recurrent rare coding variants on gene expression and conduced pathway enrichment analysis.

METHODS

Detailed description of analytical methods in this study is available as online supplemental methods file.

RESULTS

The pipeline of detecting rare coding variants in RNA-seq datasets

To fully use the sequencing information harboured in RNA-sequencing, we set up a pipeline of variant-calling based on the GATK best practice to detect rare coding variants from RNA-seq data (online supplemental figure S1) and applied additional extensive quality control filters (online supplemental figure S1) to eliminate false positives due to technical errors introduced during sequencing, alignment and biological artefacts arising from RNA-editing (online supplemental methods). To test the performance of our pipeline, we carried out comparison between variant calls from RNA-seq data and the matched WES data of the same individuals on 100 randomly selected GTEx blood samples. Results from the comparison showed that an average of 77.46% (95% CI75.54% to 79.38%) of rare variants called from RNA-seq was detected by WES of the same samples (online supplemental table S1), figure 1, which means the overall false discovery rate (FDR) is 22.54%. We further performed another method validation study on an independent set of three samples from which we collected their blood and processed RNA sequencing. We conducted variant calling, filtering for functional rare variants and then randomly selected 10 rare variants from each sample for Sanger sequencing. The results showed that seven, eight and eight variants were validated for the three samples respectively, resulting in an overall FDR of 23.6%. (online supplemental table S2,figure 1, online supplemental figure S2). Thus, both validations by WES and Sanger sequencing showed an average FDR of 23% for the rare variants that we called from RNA-seq through a rigorous variant calling and filtering pipeline.

Rare coding variant association analysis on JIA RNA-seq datasets

In this study, we first identified recurrent JIA rare coding variants on three RNA-seq datasets (GSE79970, GSE81259 and GSE112057) in the Gene Expression Omnibus database with the rigorous variant calling and filtering pipeline on 228 JIA patients and 42 healthy individuals (online supplemental methods,online supplemental figure S1,online supplemental table S3). There were 56, 47 and 115 JIA samples which passed sample QC in the dataset GSE79970, GSE81259 and GSE112057, respectively (online supplemental table S4, online supplemental figure S3). GSE81259 contains 41 polyarticular and 6 oligoarticular JIA subjects, but without disease subtype at the individual level. Because of the small number of oligoarticular subtype and the similarity of the pathogenicity between the oligoarticular and polyarticular subtypes,[4] we considered all of the samples in GSE81259 as of polyariticular JIA subtype in further analysis. The dataset GSE112057 contains three subgroups: polyarticular, oligoarticular and systemic; together with the datasets GSE79970 (polyarticular), GSE81259 (polyarticular) there are five subgroups in our study. A total of 63 variants passed the conservative filtering for recurrent variants (online supplemental table S4–S7). We examined the relationship between the number of samples in each of the five subgroups and the number of distinct variants identified from each group, the correlation is statistically significant (Pearson correlation test $p=0.017$, $r=0.943$), suggesting that the number of distinct rare coding variants per sample in each subgroup is at a similar level. Further homogeneity test of the distribution of recurrent rare variants in each polyarticular subgroup yielded a p of 0.753, which did not indicate any significant heterogeneity between the three polyarticular subgroups. This result demonstrated that our method of rare-variant calling and filtering was robust to cohort heterogeneity. The 63 SNVs included 62 missense mutations and one stop-gain with gene truncating effect (online supplemental figure S4, online supplemental table S5–S7). Among these variants, 13 variants are predicted as deleterious by both Polyphen and SIFT, and another 16 variants are predicted as deleterious by Polyphen or SIFT (online supplemental table S5–S7).

Previously reported JIA variants in the literature were also called in the JIA patients. Two patients with polyarticular JIA carried compound heterozygous mutations p.A995P and p.I848L of *UNC13D*; another two patients (oligoarticular and systemic JIA, respectively) were identified as carriers of the p.M170I mutation in the *NFIL3* gene (online supplemental table S8). These mutations are known to be responsible for monogenic forms of systemic JIA and oligoarticular JIA, respectively.[14 15] Interestingly, the findings of these variants in these JIA subtypes are different from those reported in literature and suggest certain shared genetic basis of the JIA subtypes.

We identified six rare missense SNVs and one stop-gain SNV which are predicted to be deleterious by both polyphen and SIFT, and observed in at least two out of three polyarticular subgroups without any carriers in controls (table 1). These recurrent rare coding variants in JIA cases only are located in the genes *SAMD9, CROCC, RABEP2, ZNF639, ATF6B, FGD2* and *SRCAP*, all of which have either been associated with autoimmune diseases in the previous common-variant association studies or have shown at least medium expression level in immune tissues and immune cell types (online supplemental table S9, online supplemental figure S5, S6),[16] suggesting the convergence of common variants and rare variants for autoimmune diseases. The knockout mouse model of *SRCAP* demonstrated defects in the haematopoietic system (online supplemental table S10). The number of recurrent rare variants identified in oligoarticular and systemic JIA subgroups is small due to the very limited sample size, but we observed deleterious functional variants in genes highly related to immunedeficiency or autoimmune disorders, such as *EEF1AKMT2*, *ADCY7* and *CR2* (online supplemental table S9). Knocking out each of these three genes in mice demonstrated severe immunodeficiency phenotypes and that of *ADCY7* showed additional neurological defects (online supplemental table S10). Fisher's exact test by including the RNA-seq data of 242 GTEx samples as healthy controls yielded three deleterious variants of significant association with JIA ($p<0.05$): *SAMD9* p.R459X, *CROCC* p.R1097P in polyarticular subtype and *ODF2L* p.A241V in oligoarticular subtype (online supplemental table S5–S7).

Replication study based on WES data of JIA patients

Furthermore, we conducted the replication study using the WES data of JIA subjects in the database of the Centre for Applied

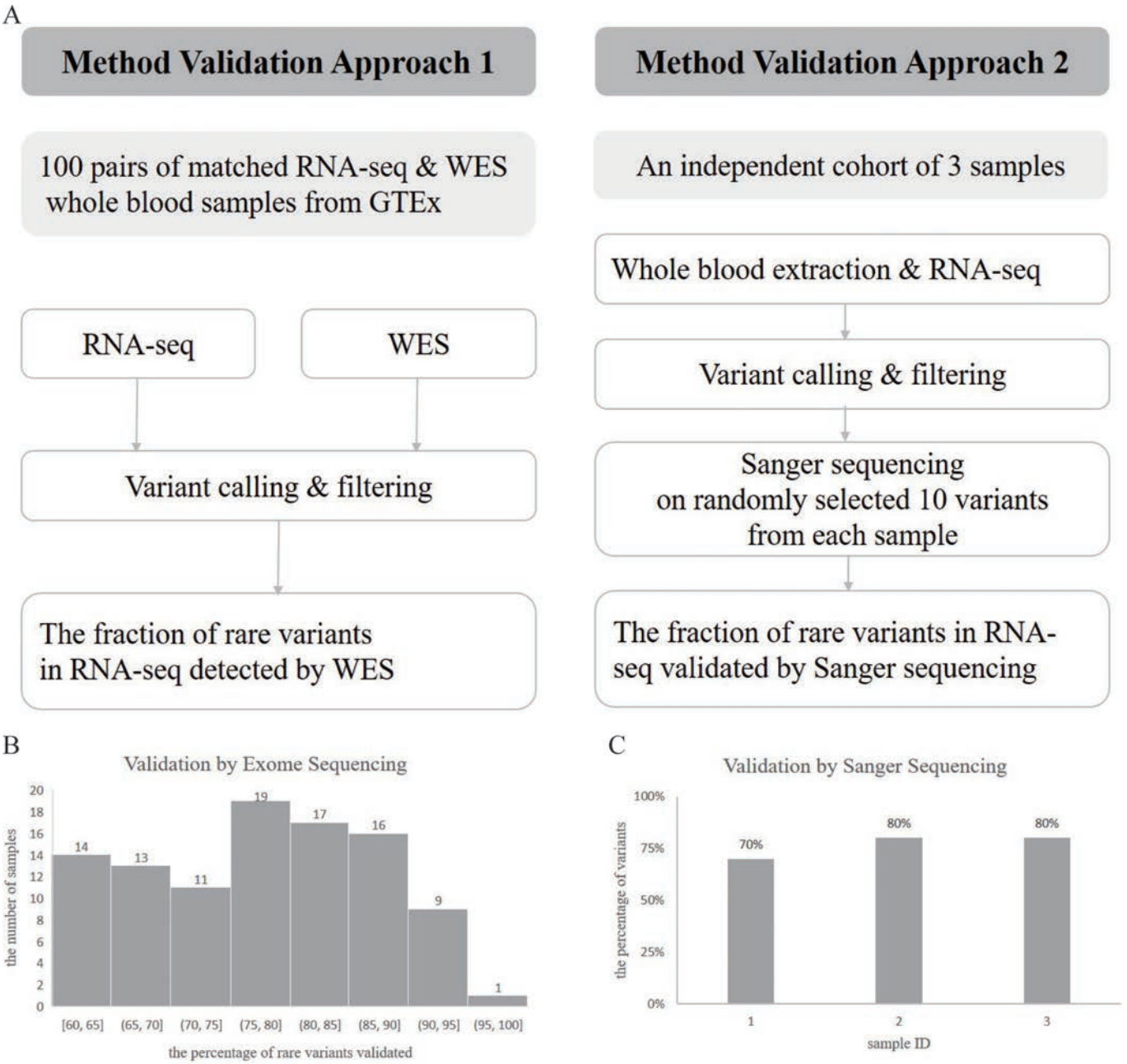

Figure 1 The validation of RNA-seq rare variant calling and filtering pipeline by whole-exome sequencing and Sanger sequencing. (A). The outline of our two approaches for validating functional rare variants called from RNA sequencing data. (B) Summary of the fraction of rare functional variants called from RNA-sequencing data was detected in whole-exome sequencing (WES) data of each of the 100 randomly selected GTEx samples. The fraction ranges were indicated on the x-axis, and the number of GTEx samples in each range was indicated on the y-axis. (C) The fraction of variants valided by Sanger sequencing among the 10 randomly selected rare functional variants called from RNA-sequencing data of each of the three samples. The sample IDs were indicated on the x-axis, and the fraction of variants validated was shown on the y-axis.

Genomics, at The Children's Hospital of Philadelphia (online supplemental table S3). As only the number of polyarticular JIA subjects (online supplemental table S3) is sufficient for a meaningful replication study, we focused on this subtype. We identified 10 of the recurrent rare coding variants in the DNA-seq data of the 38 polyarticular JIA subjects, and one of them is deleterious by SIFT functional prediction (table 1, online supplemental table S5). The deleterious mutation is located in the gene *ATF6B* which encodes a transcription factor, activating target genes of the unfolded protein response (UPR). Seven deleterious variants were identified in the largest polyarticular subgroup in GSE79970, while 4 and 3 variants were found in the other two polyarticular subgroups, respectively, which is proportional to the sample size of each subgroup. It is as expected that only a limited number of variants were replicated due to the extremely low frequency nature of these variants and the small sample size of the our JIA WES dataset, as well as the composition of subjects in this cohort with diverse ancestry (online supplemental table S11). Rare variants are known to be subjected to population specificity. The finding of the previously reported JIA mutations and the shared findings between the RNA-seq and DNA-seq data provide evidence that the recurrent rare coding variants from the RNA-seq data are likely to be *bona fide* JIA variants.

Table 1 Recurrent rare coding variants enriched in the JIA cases of the RNA-seq datasets and the whole-exome sequencing dataset

SNV(hg19)	cytoBand	REF	ALT	Gene	Type	AA change	RNA-seq JIA allele*	P value	WES JIA allele	gnomAD_ genome_ ALL	SIFT and polyphen
1: 17 280 821	1p36.13	G	A	*CROCC*	Missense	p.R1097P	3	0.048	0	9.04E-05	D
3:179 051 234	3q26.33	A	G	*ZNF639*	Missense	p.E161G	2	0.107	0	4.00E-04	D
6:32 083 657	6p21.33	G	C	*ATF6B*	Missense	p.I654M	2	0.107	2	4.06E-05	D
6:36 995 793	6p21.2	C	T	*FGD2*	Missense	p.R608W	2	0.107	0	3.66E-05	D
7:92 734 036	7q21.2	G	A	*SAMD9*	Stopgain	p.R459X	4	0.022	0	2.85E-05	.
16:28 916 281	16p11.2	C	A	*RABEP2*	Missense	p.D565Y	2	0.107	0	7.35E-05	D
16:30 727 465	16p11.2	C	T	*SRCAP*	Missense	p.R858C	2	0.107	0	6.90E-05	D

*The number of alleles among the healthy controls in the RNAseq dataset is 0.
AA Change, amino acid change; ALT, the alternative allele called; Chr pos, the single nucleotide variant shown as chromosome: position on human genome build hg19; cytoBand, human chromosome cytoband; D, deleterious; Gene, gene symbol; gnomAD_ genome_ ALL, The variant allele frequency in the Genome Aggregation Database (gnomAD) genome database; JIA, juvenile idiopathic arthritis; NA, not available; Polyphen, Polyphen annotation; REF, the allele in the reference genome; SIFT, SIFT annotation; Type, the variant type; WES, whole-exome sequencing .

Gene-based collapsing analysis for JIA

We further conducted collapsing analysis of 63 rare variants at the gene level using software RVTEST which included CMC, KBAC and SKAT burden test methods. We found three genes *NPIPB5*, *SAMD9* and *ODF2L* showing consistent significant correlation with JIA in the association testing against the 284 healthy controls by three methods ($p<0.05$) (online supplemental table S12). Most of the 63 recurrent rare variants were located in distinct genes, thus the gene-based results are similar to the Fisher's exact test results at the single-variant level, except for gene *NPIPB5* containing two variants at chr16:22 545 380 and 16:22 546 840.

Effect of recurrent rare coding variants on gene expression

To evaluate if the rare coding variants affected gene expression, we used RNA-seq data to compare gene expression between the three groups composed of JIA patients with JIA-enriched SNVs, JIA patients without SNVs and healthy controls. Four polyarticular JIA patients carrying an early stop codon in the gene *SAMD9* showed the trend of down-regulated expression in comparison with the other polyarticular JIA patients, or healthy controls ($p=0.0575$ and 0.0581, respectively) (figure 2), which suggest nonsense-mediated mRNA decay. Similar trend of down-regulation was also observed for such stratified analysis based on the *ODF2L* p.A241V genotype, though it was not statistically significant. For the other genes in table 1, the number of carriers was too small (≤3) to make a meaningful comparison of the gene expression.

We also looked into the overall DEGs profile of the polyarticular and oligoarticular subtypes of JIA patients after data normalisation, as the altered gene expression levels are the intermediate phenotype between disease clinical phenotypes and the genetic aetiology which is attributable to both common variants and rare variants. With a threshold of $|FC|>1.5$ and $FDR<0.05$ (FC: fold change; FDR: false discovery rate) (online supplemental table S13), 34 genes were significantly downregulated and 104 genes were significantly upregulated, including 4 down-regulated genes and 43 upregulated genes showing more than twofold change (online supplemental figure S7), most of which are known to be involved in immune regulation.

Pathway enrichment analysis

We conducted pathway analysis to understand how the discovered genes collectively contribute to JIA development. We analysed the 63 genes containing the aforementioned recurrent rare coding variants used WebGestalt toolkit[17]; we also ran a separate analysis with it on the 138 DEGs of meta-analysis of polyarticular and oligoarticular subtypes of JIA and 763 DEGs of systemic JIA reported previously. We observed pathways involved in immune regulation, like thyroid hormone synthesis enriched among genes bearing recurrent rare coding variants (online supplemental figure S8, online supplemental table

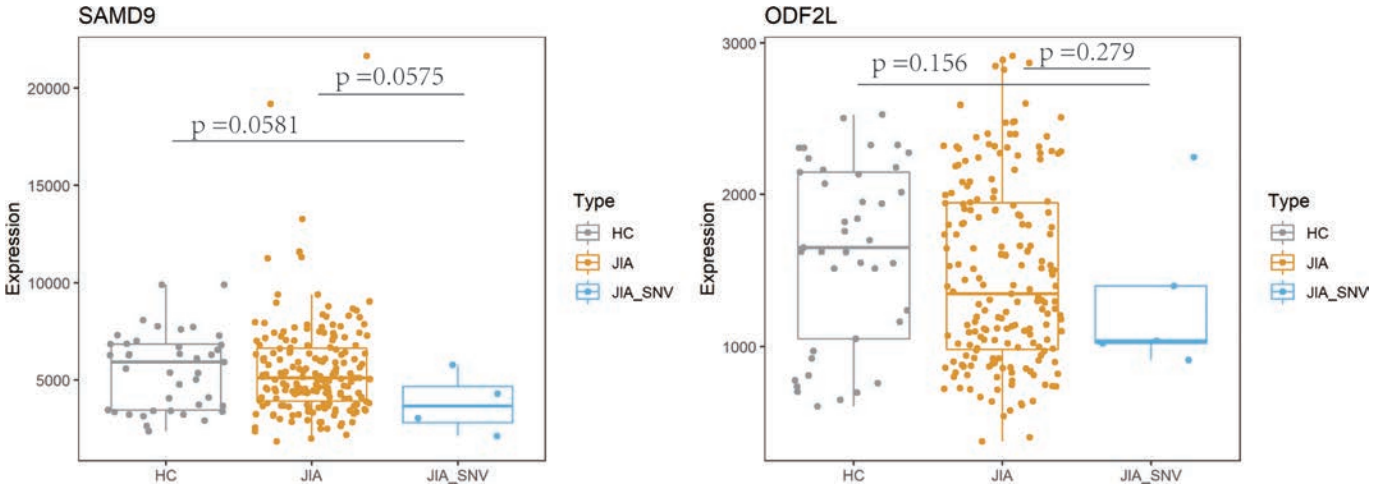

Figure 2 The gene expression of JIA patients carrying rare coding variants in SAMD9 p.R459X and ODF2L p.A241V in the corresponding genes compared with JIA patients or healthy controls not carrying these variants. JIA, juvenile idiopathic arthritis.

S14). Twelve immune-related pathways including TNF signalling pathway, Th17 cell differentiation, NF-kappa B signalling pathway, NOD-like receptor signalling pathway were enriched by both in DEGs of polyarticular/oligoarcular and systemic JIA (online supplemental figure S8,online supplemental table S15, S16). This suggests that it is the altered immune gene expression and dysregulated signalling pathways that may mediate the cumulative molecular effects of the recurrent rare coding variants and different JIA subtypes may share similar immune pathways. We also observed the enrichment of pathways 'Lysosome', 'Protein processing in endoplasmic reticulum', suggesting the potential involvement of UPR and the ER-associated degradation in the pathogenesis of JIA. This is consistent with the important roles of the UPR genes in rheumatoid arthritis (RA) and osteoarthritis.[18–20]

DISCUSSION

Taken together, we have identified recurrent rare coding variants associated with JIA based on RNA-seq data. The approach by the RNA-seq data focuses on expressed sequences. Considering the functional importance of these genomic regions compared with unexpressed regions, this approach represents higher specificity for disease variant discovery. At the same time, the RNA-seq data enables us to investigate the potential effects of genetic variants on gene expression simultaneously. We observed rare coding variants which may affect the expression of the corresponding gene. It has been hypothesised that common and rare genetic variants result in altered gene expression, which further change cellular function and then lead to various symptom domains.[21] The distinct and shared symptom domains together constitute the clinical syndrome. Here, we showed that RNA-seq can be applied to identify rare variants enriched in disease samples based on stringent variant calling pipeline and filtering criteria. The variants in the expressed genes may have direct impact on signalling pathways and cellular functions. By these analyses, we can make more potential use of the rich resource of information in RNA-seq data to establish the genome-transcriptome-phenome relationships.

In our analysis, we found novel mutations in genes which are known to be involved in immune regulation or implicated in immune disorders. Nonsense mutation of *SAMD9* results in a decreased expression of *SAMD9* as expected. SMAD9 mutations have been shown to cause autoimmune disorders. For example, homozygous mutation p.K1495E and compound heterozygous mutations p.K1495E and p.R344X cause normophosphatemic familial tumorous calcinosis, which usually occur to regions associated with frequent trauma and inflammatory manifestations.[22 23] In vitro experiments showed that *SAMD9* is under the regulation of interferon-γ and knockdown *SAMD9* gene expression by shRNA led to the decreased levels of cytokines production in Jurkat cells, including TNF-α, IL-8 and IL-4, all of which have implicated in the pathophysiology of JIA.[24]

Recurrent mutations in gene *ATF6B* have been detected in RNA-seq data and further replicated in WES data. It is a transcription factor which is activated by endoplasmic reticulum stress response (ERSR).[25] In *ATF6B*-deficient mice, the function of CD8 T cell is impaired and insufficient INF-gamma is produced due to decreased level of *ATF6B* in dendritic cells.[26] GWAS found two SNPs of *ATF6B* are significantly associated with asthma.[27] In RA, ER stress drives inflammatory cells to release cytokines, which results in synoviocyte proliferation and proinflammatory cytokine production. ERSR has thus been considered as a therapeutic target in RA.[18] It has also been shown that UPR and ERSR play important roles in the development of osteoarthritis.[19 20] The identification and validation of rare *ATF6B* variant in polyarthritis JIA patients suggests the likely involvement of UPR and ERSR in JIA as well, consistent with the findings from the adult types of arthritis.

In addition, the common variants in the gene *ADCY7, EEF1AKMT2, IRF7 and SCRAP* have been reported to be significantly associated with inflammatory bowel disease, systemic lupus erythematosus, autoimmune thyroid disease and paediatric autoimmune diseases (online supplemental table S9), while several of these genes have also been linked to neuropsychiatric diseases consistent with several studies that reported shared disease loci between immune disease and neuropsychiatric disorders.[28–30] Furthermore, the knock-out mouse model of several gene showed severe defects in the immune or haematopoietic system. These observations provide supportive evidence for the potential involvement of these genes in the pathogenesis of JIA.

Although there is phenotypic difference between JIA subtypes, they share common inflammatory reactions at the joints. In the gene expression analysis, we observed the common upregulation of multiple genes between the polyarticular/oligoarticular and systemic subgroups, such as the genes *TNFAIP3, NFKBIA, PTGS2 and CLEC4D*, which suggest the alteration of common inflammation pathways between these JIA subtypes. Our pathway analysis further confirmed this conclusion by showing the commonly changed pathways like the IL-17/TNF signalling and multiple other pathways (online supplemental table S15, S16). The common alteration in gene expression and cellular process may reflect the shared genetic basis between JIA subtypes. Our recent meta-analysis on common variants of 7 JIA subtypes (Integrative Genetics Analysis of Juvenile Idiopathic Arthritis Identifies Novel Loci)[31] and the finding of rare *UNC13D* mutations in the polyarticular JIA provide further supportive evidence for this notion.

There are two major limitations in this study. First, rare coding variants were called based on RNA-seq data which may be subjected to certain discrepancies from DNA-seq data due to biological and technical reasons.[32] However, the large amount of publicly available RNA-seq data and the improvement of the RNA-seq variant calling algorithm provide us opportunity to identify rare pathogenic variants and analyse its effect on gene expression based on the RNA-seq data.[33] Our method validation studies, the identification of variants in the previously reported genes and the replication by WES data provide supportive evidence for the feasibility of utilising RNA-seq data in the identification of disease-associated rare variants. This study may serve as a pilot study in this regard, especially in the JIA research area where the number of disease sample and sample amount are limited. The second limitation is that the small sample size in our study, which is especially a major hindrance for rare variant study. Rare variants are often population specific which also increase the difficulty for a replication study. The variants identified in our study warrant further investigation in a cohort with larger sample size.

In summary, our results revealed novel rare coding variants and genes associated with JIA, highlighting the convergence of both common and rare variants in JIA (and possibly other autoimmune diseases), together with the pleiotropic genes underlying the development of autoimmune diseases. Meta-analysis of the RNA-seq data reveals that DEGs are highly enriched in immune pathways. The cumulative effects of rare coding variants exhibited on cellular activities may be mediated and amplified by their effects on immune gene expression.

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Contributors XM was mainly involved in the data analysis, processing and summarisation. XH and PW were mainly responsible for drafting manuscript. QX, HH and JL were responsible for conception and design of study and revising the manuscript critically for important intellectual content. Other authors have partially participated in the study. All listed authors have seen and approved the manuscript.XH and PW are co-second authors.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval Ethical approval for this study was obtained from CHOP Institutional Review Board (CHOP IRB#4886) and carried out in accordance with the nationally approved guidelines.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Data are available on reasonable request. Three RNA-seq datasets are from public database the Gene Expression Omnibus (GSE79970, GSE81259 and GSE112057). Whole-exome sequencing data are available upon reasonable request. All data requests should be addressed to HH.

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TRANSLATIONAL SCIENCE

Meta-analysis of 208370 East Asians identifies 113 susceptibility loci for systemic lupus erythematosus

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ABSTRACT

Objective Systemic lupus erythematosus (SLE), an autoimmune disorder, has been associated with nearly 100 susceptibility loci. Nevertheless, these loci only partially explain SLE heritability and their putative causal variants are rarely prioritised, which make challenging to elucidate disease biology. To detect new SLE loci and causal variants, we performed the largest genome-wide meta-analysis for SLE in East Asian populations.

Methods We newly genotyped 10 029 SLE cases and 180 167 controls and subsequently meta-analysed them jointly with 3348 SLE cases and 14 826 controls from published studies in East Asians. We further applied a Bayesian statistical approach to localise the putative causal variants for SLE associations.

Results We identified 113 genetic regions including 46 novel loci at genome-wide significance ($p<5\times10^{-8}$). Conditional analysis detected 233 association signals within these loci, which suggest widespread allelic heterogeneity. We detected genome-wide associations at six new missense variants. Bayesian statistical fine-mapping analysis prioritised the putative causal variants to a small set of variants (95% credible set size ≤10) for 28 association signals. We identified 110 putative

Key messages

What is already known about this subject?
- Genome-wide association studies have identified nearly 100 susceptibility loci for systemic lupus erythematosus (SLE) risk.
- The known SLE loci explain partially the disease heritability.

What does this study add?
- This study identified 113 genomic regions including 46 novel loci for SLE risk.
- The study prioritised 110 putative causal variants including 10 putative causal variants with high confidence (posterior probability ≥0.8).

How might this impact on clinical practice or future developments?
- These findings revealed new genetic basis for SLE and generated molecular mechanisms hypotheses for further investigations.

causal variants with posterior probabilities ≥0.1 for 57 SLE loci, among which we prioritised 10 most likely putative causal variants (posterior probability ≥0.8). Linkage disequilibrium score regression detected genetic correlations for SLE with albumin/globulin ratio (r_g=−0.242) and non-albumin protein (r_g=0.238).

Conclusion This study reiterates the power of large-scale genome-wide meta-analysis for novel genetic discovery. These findings shed light on genetic and biological understandings of SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterised by the production of autoantibodies that damage multiple organs.[1] Considerable genetic predisposition contributes to SLE aetiology.[2] To date, nearly 100 susceptibility loci have been identified for SLE, mainly through genome-wide association studies (GWASs).[3–8] However, these loci collectively only explain ~30% of SLE heritability[9] and their biology, in terms of causal variants, effector genes and cell types and pathological pathways that mediate genetic effects, has not yet been fully characterised.[10]

Genome-wide association meta-analyses have been performed to uncover new genetic associations for SLE in Asians,[11] Europeans[12] and trans-ancestral populations.[9] However, the study sample sizes were relatively modest, which limits their ability for genetic discovery. GWASs have successfully linked genetic variants with human common diseases and traits.[13] Nonetheless, only ~8% of GWAS participants are East Asians.[14] East Asians have a unique population genetic history and may have ethnicity-specific genetic architecture involved in the development of disease and manifestations. For example, SLE has a remarkably higher prevalence and younger age of onset in Asians.[15 16] Genetic heterogeneity may explain, at least partly, the phenotypic diversity of SLE between East Asians and Europeans.[9] Hence, large-scale East Asian investigations may provide an opportunity to identify unique genetic associations even for the same diseases and traits that have already been well studied in Europeans.[17]

METHODS

Study participants

We recruited a total of 10 029 SLE cases and 180 167 healthy controls in three independent case–control cohorts from mainland China, Korea and Japan. We analysed additionally 3348 SLE cases and 14 826 controls that were published in our previous East Asian SLE GWASs[4 6–9] to increase statistical power. All the cases fulfilled the revised American College of Rheumatology SLE classification criteria or were diagnosed by collagen disease physicians (online supplemental table 1). Each participant provided written informed consent.

Genome-wide association analyses

We newly genotyped 10 029 SLE cases and 180 167 controls, and revisited raw genome-wide genotype data in 3348 SLE cases and 14 826 controls from the five published studies.[4 6–9] Quality controls were conducted for each of the eight data sets. Genotype imputation was accomplished using reference panels from the 1000 Genomes Project (1KGP) phase 3 v5[18] and population-specific reference panels[19] in IMPUTE2/4[20 21] or MINIMAC4.[22]

We tested association between SLE risk and genotype dosages in each data set using a logistic regression or linear mixed model in PLINK,[23] SNPTEST[24] or EPACTS (https://genome.sph.umich.edu/wiki/EPACTS) (online supplemental table 1). Within each data set, we filtered out association results based on imputation quality (IMPUTE info or MINIMAC r^2 ≤0.3), minor allele frequency (MAF) ≤0.5% or Hardy-Weinberg equilibrium test $p<1.0\times10^{-6}$ in controls. For each cohort, the association analysis for the X chromosome was conducted separately by sex and then meta-analysed across both men and women. For data sets analysed using a linear mixed model (online supplemental table 1), allelic effects and standard errors were converted to a log-odds scale to correct for case–control imbalance.[25]

FIXED-EFFECTS META-ANALYSIS

We aggregated the association summary statistics from the eight data sets using a fixed-effects inverse-variance meta-analysis in METAL.[26] We applied a genomic control correction to each association summary statistic. Heterogeneity

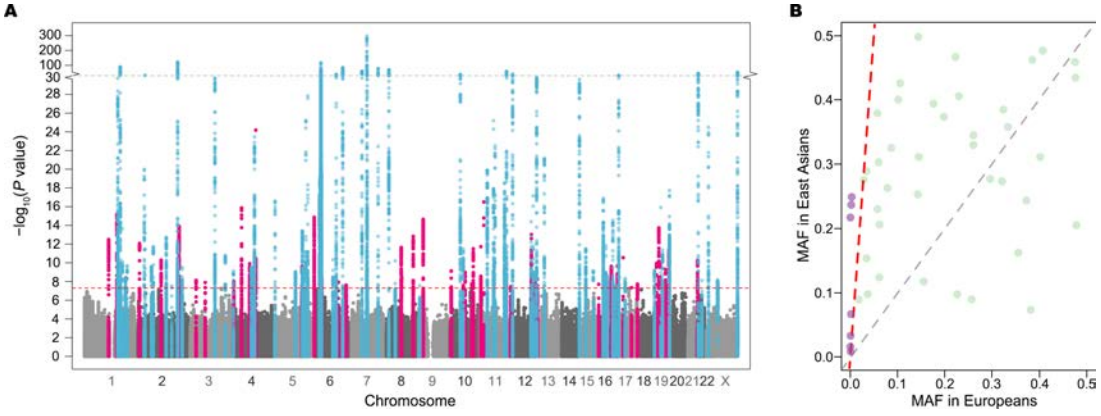

Figure 1 Summary of meta-analysis association results and comparison of MAFs for lead variants within the 46 novel loci between East Asians and Europeans. (A) Manhattan plot of genome-wide association meta-analysis results from 208 370 SLE East Asians including 13 377 SLE cases and 194 993 controls. Minus log10-transformed association p values (y-axis) are plotted along chromosomal positions (x-axis). Known and novel loci are highlighted in light blue and pink, respectively. The red dashed line denotes the genome-wide association significance threshold of $p=5\times10^{-8}$. The grey dashed line represents $p=10^{-30}$, at which the y-axis breaks. (B) Comparison of MAFs of lead variants within the 46 novel loci between East Asians (y-axis) and non-Finnish Europeans (x-axis) in the Genome Aggregation Database (gnomAD) v3. Variants with more than 10 times higher MAFs in East Asians are coloured purple above a red dashed line. MAF, minor allele frequency.

Table 1 Association results for the 46 novel susceptibility loci for systemic lupus erythematosus

Region	CHR	Position	Variant	EA	NEA	EAF	OR	SE	P value	I^2	P_{Het}	N	Nearest gene
1	1	117 043 302	rs9651076	A	G	0.431	1.117	0.015	3.26E−13	10.7	0.347	208 370	*CD58*
2	1	157 108 159	rs116785379	C	G	0.107	1.211	0.024	6.68E−16	43.7	0.114	208 370	*ETV3*
7	1	201 979 455	rs3806357	A	G	0.251	1.106	0.017	4.25E−09	0.0	0.672	208 370	*ELF3*
9	2	7 573 079	rs75362385	T	G	0.321	0.887	0.017	8.40E−13	68.3	0.007	208 370	*LOC100506274*
14	2	111 877 174	rs73954925	C	G	0.878	1.169	0.024	5.11E−11	56.4	0.043	208 370	*BCL2L11*
18	2	198 929 806	rs7572733	T	C	0.260	1.143	0.017	1.25E−14	0.0	0.647	208 370	*PLCL1*
20	3	28 072 086	rs438613	T	C	0.588	0.920	0.014	7.52E−09	69.4	0.006	208 370	*LINC01980*
21	3	72 225 916	rs7637844	A	C	0.871	0.877	0.023	1.28E−08	0.0	0.906	208 370	*LINC00870*
25	4	2 700 844	rs231694	T	C	0.380	1.111	0.018	9.71E−09	23.7	0.269	57 253	*FAM193A*
26	4	40 307 587	rs113284964	G	GCTTC	0.371	1.134	0.015	1.35E−16	67.2	0.009	208 370	*LINC02265*
27	4	79 644 279	rs6533951	A	G	0.350	1.111	0.016	1.25E−10	61.4	0.024	208 370	*LINC01094*
28	4	84 146 996	rs6841907	T	C	0.729	0.906	0.016	1.10E−09	43.5	0.115	208 370	*COQ2*
31	4	109 061 618	rs58107865	C	G	0.227	0.802	0.021	6.57E−25	1.1	0.409	208 370	*LEF1*
34	5	131 120 338	rs370449198	A	AC	0.922	0.721	0.060	4.41E−08	0.0	0.408	187 562	*FNIP1*
35	5	131 829 578	rs2549002	A	C	0.682	0.905	0.016	2.40E−10	20.6	0.279	208 370	*IRF1*
40	6	243 302	rs9503037	A	G	0.693	0.881	0.016	1.36E−15	42.3	0.123	208 370	*LOC285766*
43	6	36 715 031	rs34868004	CA	C	0.225	1.104	0.017	4.46E−09	40.7	0.134	208 370	*CPNE5*
46	6	116 690 849	rs9488914	T	C	0.920	0.862	0.026	1.14E−08	65.3	0.013	208 370	*DSE*
48	6	154 570 651	rs9322454	A	G	0.659	1.090	0.015	2.42E−08	0.0	0.430	208 370	*IPCEF1*
54	8	71 330 166	rs142937720	A	AAGTGGCC	0.383	0.894	0.016	2.27E−12	67.9	0.008	208 370	*NCOA2*
55	8	72 894 959	rs17374162	A	G	0.411	0.917	0.015	3.02E−09	35.7	0.169	208 370	*MSC-AS1*
56	8	129 425 593	rs16902895	A	G	0.678	1.122	0.016	1.48E−13	0.0	0.801	208 370	*LINC00824*
58	9	21 267 087	rs7858766	T	C	0.538	1.139	0.016	2.25E−15	0.0	0.825	208 370	*IFNA22P*
59	10	5 910 746	rs77448389	A	G	0.913	0.855	0.025	7.30E−10	0.0	0.584	208 370	*ANKRD16*
62	10	64 411 288	rs10995261	T	C	0.240	0.909	0.017	2.57E−08	43.9	0.113	208 370	*ZNF365*
63	10	73 466 709	rs10823829	T	C	0.718	0.910	0.016	1.05E−09	0.0	0.771	208 370	*CDH23*
64	10	105 677 911	rs111447985	A	C	0.073	1.172	0.028	1.72E−08	0.0	0.526	208 370	*STN1*
65	10	112 664 114	rs58164562	T	C	0.748	0.892	0.016	3.14E−12	33.3	0.186	208 370	*BBIP1*
66	11	4 113 200	rs3750996	A	G	0.834	1.167	0.022	1.89E−12	0.0	0.522	208 370	*STIM1*
67	11	18 362 382	rs77885959	T	G	0.978	1.694	0.062	3.16E−17	0.0	0.511	204 433	*GTF2H1*
74	12	4 140 876	rs2540119	T	C	0.544	1.086	0.015	3.51E−08	44.9	0.106	208 370	*PARP11*
77	12	103 916 080	rs6539078	T	C	0.591	0.894	0.015	9.49E−14	0.0	0.916	208 370	*LOC105369945*
79	12	121 368 518	rs3999421	A	T	0.506	0.910	0.016	1.29E−09	47.3	0.091	208 370	*XLOC_009911*
81	12	133 040 182	rs200521476	G	GCATCAC	0.812	0.875	0.023	5.66E−09	26.7	0.235	208 370	*FBRSL1*
86	15	101 529 012	rs35985016	A	G	0.930	0.843	0.030	1.95E−08	0.0	0.897	204 433	*LRRK1*
90	16	50 089 207	rs11288784	G	GT	0.365	0.902	0.016	2.38E−10	0.0	0.664	208 370	*HEATR3*
93	16	79 745 672	rs11376510	G	GT	0.737	0.898	0.017	2.23E−10	0.0	0.719	208 370	*MAFTRR*
95	17	7 240 391	rs61759532	T	C	0.076	1.235	0.032	2.79E−11	24.9	0.247	208 370	*ACAP1*
97	17	47 468 020	rs2671655	T	C	0.651	1.087	0.015	4.60E−08	0.0	0.756	208 370	*LOC10272459*
98	17	76 373 179	rs113417153	T	C	0.193	0.893	0.020	1.90E−08	2.1	0.403	208 370	*PGS1*
100	18	77 386 912	rs118075465	A	G	0.147	1.140	0.020	1.16E−10	0.0	0.543	208 370	*LOC284241*
101	19	948 532	rs2238577	T	C	0.455	0.885	0.016	1.83E−14	60.8	0.026	208 370	*ARID3A*
102	19	6 697 088	rs5826945	A	T	0.929	0.836	0.028	9.67E−11	50.0	0.075	208 370	*C3*
105	19	33 072 768	rs12461589	T	C	0.248	0.898	0.017	5.00E−10	0.0	0.510	208 370	*PDCD5*
106	19	49 851 746	rs33974425	CCAGCTGCAT	C	0.702	1.120	0.016	4.40E−12	42.6	0.121	208 370	*TEAD2*
108	22	18 649 356	rs4819670	T	C	0.210	1.151	0.022	5.53E−11	0.0	0.650	208 370	*USP18*

CHR, chromosome; EA, effect allele; EAF, effect allele frequency; I^2, genetic heterogeneity I^2 statistics at scale of 0% to 100%; N, study sample size; NEA, non-effect allele; OR, Odds ratio; P_{Het}, P-values for the χ^2 test of genetic heterogeneity; Region, unique ID for genomic region; ;SE, Standard error of odds ratio.

in allelic effect sizes among data sets was assessed using Cochran's Q statistic. We excluded genetic variants available in only a single data set. We defined SLE susceptibility loci by merging ±250 kilobases (kb) windows around genome-wide associated variants to ensure that lead single nucleotide polymorphisms (SNPs) were at least 500 kb apart. We defined lead variants as the most significant SLE-associated variant within each locus. A locus was considered novel if the lead SNP was at least 500 kb away from any previously reported SLE-associated variants.

Approximate conditional association analysis

To dissect distinct association signals at each SLE locus, we performed an approximate conditional analysis using GCTA COJO[27] with genome-wide meta-analysis summary statistics based on linkage disequilibrium (LD) estimated from 7021 unrelated Chinese controls. The Chinese reference individuals for LD calculation were retrieved from the Chinese study using the Illumina Infinium Global Screening Array data (online supplemental table 1), excluding first-degree and second-degree relatives.

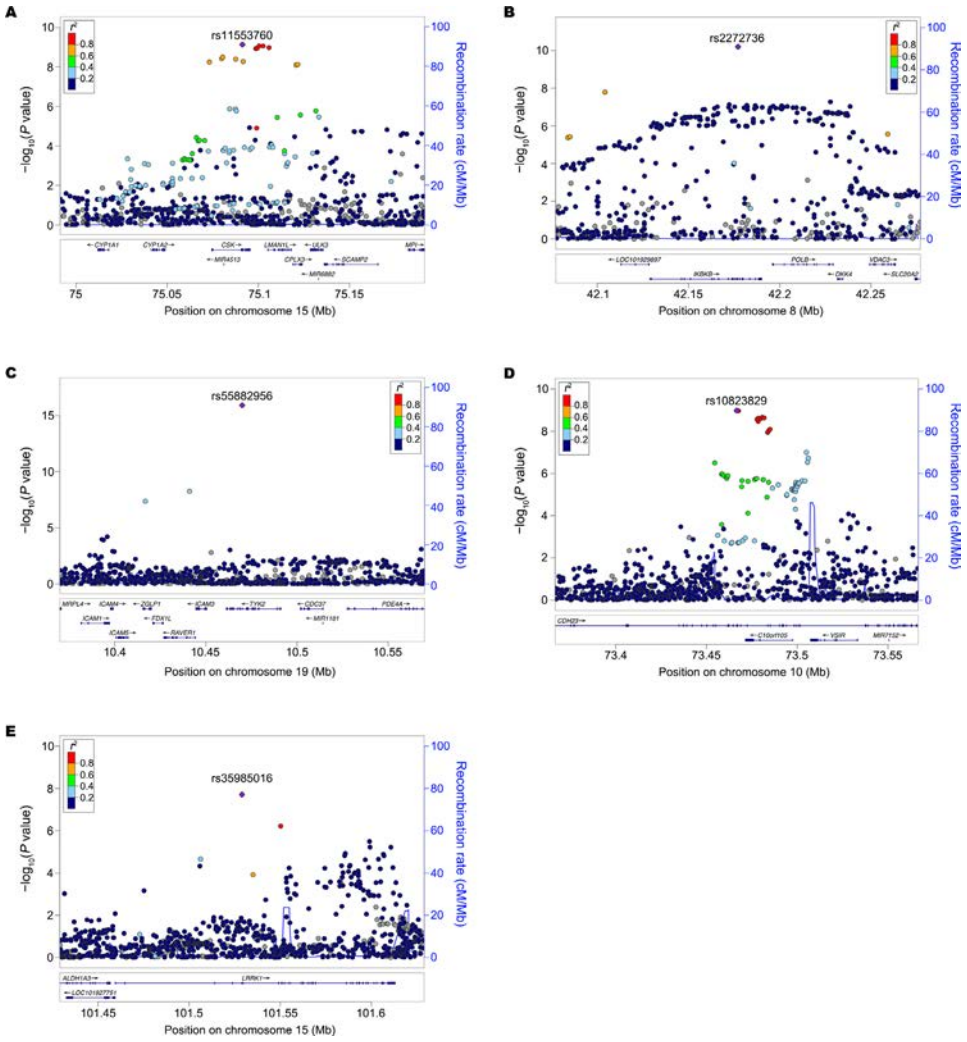

Figure 2 New lead exonic variants identified at three known (*CSK*, *IKBKB* and *TYK2*) and two novel (*CHD23* and *LRRK1*) loci. (A) rs11553760 (synonymous variant) at *CSK*. (B) rs2272736 (p.Arg303Gln, missense variant) at *IKBKB*. (C) rs55882956 (p.Arg703Trp, missense variant) at *TYK2*. (D) rs10823829 (synonymous variant) at *CHD23*. (E) rs35985016 (p.Lys203Glu, missense variant) at *LRRK1*. The lead SNP is labelled as purple diamond. The LD is estimated from 7021 Chinese samples. LD, linkage disequilibrium; Mb, megabases; SNP, single nucleotide polymorphism.

Bayesian statistical fine-mapping analysis

To prioritise causal variants in SLE susceptibility loci, a statistical fine-mapping analysis was performed using FINEMAP v1.4 software,[28] with meta-analysis z-scores and LD matrices estimated from the 7021 Chinese reference individuals. We used default priors and parameters in FINEMAP, assuming at most five causal signals in the ±250 kb region around a lead variant at each SLE locus. FINEMAP computed a posterior probability (PP) for each genetic variant being the true putative causal variant. For each association signal, we ranked the candidate putative causal variants in a descending order of their PPs, and then built a 95% credible set of causal variants by including the ordered variants until their cumulative PP reached 0.95.

Heritability estimation by LD score regression

Overall SLE heritability h^2 explained by genome-wide variants was estimated using the LD score regression model[29] with LD scores[18] from the 1KGP East Asian descendants, based on an SLE population prevalence of 0.03% in East Asian populations.[1] SLE heritability estimate was further partitioned according to known and novel SLE loci using stratified LD score regression.[30] The boundary of each SLE locus was arbitrarily defined as ±500 kb flanking the lead SLE-risk variant.

Genetic correlation between SLE and other traits by LD score regression

We calculated genetic correlations between 98 traits (39 diseases[17] and 59 quantitative traits[31] and SLE by using bivariate LD score regression.[32] We used the LD scores[18] from the 1KGP East Asian descendants, limited the genetic variants to the HapMap3 SNPs and removed the variants with extended human leucocyte antigen (*HLA*) region (chromosome 6: 25 to 34 megabases (Mb)).

Patient and public involvement

Patients and the public were not involved in the design or analysis of this study.

RESULTS

Identification of 46 novel SLE susceptibility loci

We performed a large genome-wide association meta-analysis in 13 377 SLE cases and 194 993 controls of East Asians (online supplemental table 1). To the best of our knowledge, this is the largest genetic association study of SLE to date. The effective sample size (N_{eff}=50 072) is three-fold and four-fold larger than

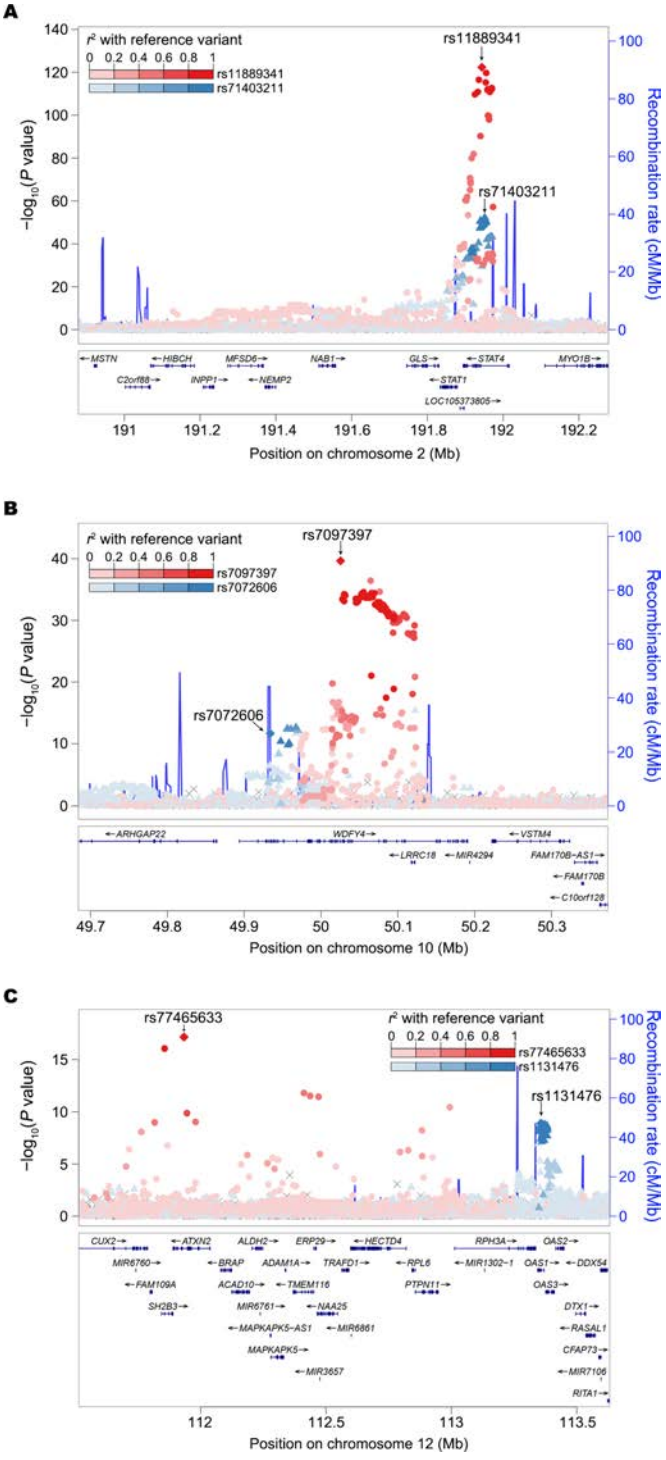

Figure 3 Two independent association signals identified. (A) At two intronic variants within known *STAT4* locus. (B) At known (rs7097397, p.Arg1816Gln) and new (rs7072606, p.Ser214Pro) missense variants within *WDFY4* locus. (C) A known intronic variant within *ATXN2* gene and a new (rs1131476, p.Ala352Thr) missense variant within *OAS1* gene. The lead and secondary index variants are labelled in diamond. The lead variant and its LD proxies are in red while the secondary signal index variant and its LD proxies are in blue. The LD is estimated from 7021 Chinese samples. LD, linkage disequilibrium; Mb, megabases.

that of the largest published trans-ancestry[9] and East Asian[11] meta-analyses, respectively.

We tested associations for 11 270 530 genetic variants in a fixed-effects meta-analysis. A quantile–quantile plot showed that test statistics were well-calibrated, with a genomic-control inflation factor $\lambda_{GC}=1.06$ (indicating that ancestry effects had been well controlled; online supplemental figure 1). LD score regression[29] showed that polygenic effects (89.4%), rather than biasses, primarily caused the inflation residual (estimated mean $\chi^2=1.32$ and LD-score intercept=1.03).

We detected 26 379 genetic variants associated with SLE at $p<5\times10^{-8}$ within 113 loci (figure 1A and online supplemental table 2), of which 46 were novel (table 1). The pairwise LD between lead variants was low (LD r^2 <0.002). For seven novel loci, MAFs of the lead SNPs were 10-fold higher in East Asians than in Europeans (figure 1B). Two of them and their LD neighbours (r^2 ≥0.2 in either East Asians or Europeans) would be undetectable in Europeans with the same effective sample size and risk magnitude due to low statistical power (<10%; online supplemental table 3).

Associations at exonic variants

The meta-analysis identified lead missense variants in two novel loci (*CHD23* and *LRRK1*; figure 2A,B and online supplemental table 2). In addition, we detected three new exonic variants (including two missense variants) within the reported SLE loci including *CSK* (rs11553760), *IKBKB* (rs2272736) and *TYK2* (rs55882956) genes (figure 2C–E and online supplemental table 2). They were not correlated with previously reported exonic variants within the same genes (LD r^2 <0.02 in East Asians or Europeans; online supplemental table 4), suggesting possible allelic heterogeneity of these genes. We replicated four known associations for missense variants at *AHNAK2* (rs2819426),[33] *IRAK1* (rs1059702),[34] *NCF2* (rs13306575) and *WDFY4* (rs7097397; online supplemental table 2).[35 36]

Secondary association signals within SLE loci

To dissect the source of association signals at each locus, we conducted an approximate conditional analysis using GCTA[27] with meta-analysis summary statistics and LD estimates from 7021 unrelated Chinese controls. We acknowledge the limitations of using LD estimation from a single population for a meta-analysis of diverse East Asians. We identified a total of 233 independent association signals with conditional $p<5\times10^{-8}$, 169 of which arose from non-*HLA* regions (online supplemental table 5). We observed from two to four signals at each of 28 non-*HLA* loci (including seven novel loci). For example, we discovered two distinct association signals within the known *STAT4* locus, including the previously reported SNP rs11889341[12] and the new insert-deletion variant (indel) rs71403211 (figure 3A). For the 46 novel loci, we discovered 55 distinct signals (online supplemental table 5 and figure 2). We noticed that most of the signal index variants (n=190, 82%) are common (MAF ≥5%) with modest effects (online supplemental table 5).

Approximate conditional analysis detected two novel missense variants at *WDFY4* and *OAS1* genes. We detected two distinct signals within *WDFY4*, including the known (rs7097397)[37] and a new (rs7072606) missense variant (LD r^2=0.02 between two variants in East Asians), which suggests allelic heterogeneity at this locus (figure 3B). We provided for the first time genome-wide association evidence at a missense variant within *OAS1* (rs1131476, LD r^2=0.78 with rs1051042, which is a known missense variant but only exhibited suggestive significance with SLE in previous study,[33] figure 3C and online supplemental table 5).

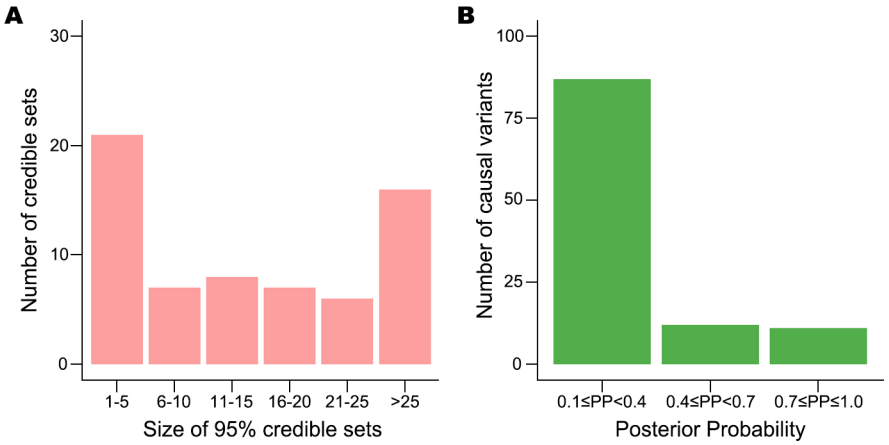

Figure 4 Results of statistical fine-mapping analysis. (A) Number of 95% credible sets of putative causal variants, binned by their sizes. (B) Number of potential causal variants with posterior probabilities (PP) ≥0.1, which are considered to be the true causal variants.

Prioritisation of causal variants

To prioritise putative causal variants, we conducted a Bayesian statistical fine-mapping analysis for 111 loci using FINEMAP[28] after excluding complex associations involving the *HLA* and 7q11.23. We found exactly the same number of association signals in 57 loci between FINEMAP causal configuration with the highest posterior probability and the GCTA approximate conditional test. To be conservative, we only summarised the statistical fine-mapping results for these 57 regions, which contained 65 association signals (online supplemental table 6).

For each signal, we built a credible set of putative causal variants with a 95% probability of including the true causal variants. The size of 28 credible sets was small (size ≤10; figure 4A). Among the 110 putative causal variants with posterior probability ≥0.1 (figure 4B), we found four coding variants (3.6%), which implies that most of these associations are probably induced by non-coding causal variants. The prioritised variants are available to be tested as potential targets in perturbation experiments. For example, the allele-specific regulatory activity of the intronic variant (rs10036748) with the highest posterior probability (0.387) in the *TNIP1* locus was recently experimentally characterised in SLE.[38]

We pinpointed a single most likely causal variant with high confidence (posterior probability ≥0.8) for four known (*ATXN2*, *BACH2*, *DRAM1*/*WASHC3* and *NCF2*) and six novel (17p13.1, *ELF3*, *GTF2H1*, *LRRK1*, *LOC102724596*/*PHB* and *STIM1*) loci (online supplemental table 6). For example, we prioritised rs61759532 as a putative causal variant at the novel 17p13.1 locus (PP=0.999). This variant is located in an intron of *ACAP1*, which encodes a key regulator of integrin traffic for cell adhesion and migration.[39]

SNP-BASED HERITABILITY

To assess the proportion of phenotypic variance explained by common variants, we applied LD score regression[29] to the meta-analysis results. Assuming a population prevalence of 0.03% for SLE,[1] we estimated the liability-scale SNP-based heritability from all non-*HLA* variants as h^2_{SNP} = 7.24% (SE=0.78%). The 66 known and 46 novel non-*HLA* loci explained 62.6% (SE=4.9%) and 22.1% (SE=2.6%) of this overall SNP-based heritability, respectively.

Genetic correlation with other diseases/traits

To explore shared genetics between SLE and various traits, we calculated genetic correlations of SLE with 39 complex diseases and 59 quantitative traits in Biobank Japan participants using bivariate LD score regression[32] (online supplemental table 7). As expected, we detected significant positive genetic correlations between SLE and two other autoimmune diseases: rheumatoid arthritis (r_g=0.437) and Graves' disease (r_g=0.318). In addition, we found unreported genetic correlations (FDR<0.05) with albumin/globulin ratio (r_g=−0.242) and non-albumin protein (r_g=0.238).

DISCUSSION

Here, we carried out the largest-ever genome-wide association meta-analysis for SLE and identified 113 risk loci including 46 novel regions for SLE in 208 370 East Asians including 13 377 SLE cases and 194 993 controls. This study revealed new genetic predispositions for SLE and generated hypotheses for further studies to investigate diseases functional mechanisms.

Epidemiological studies have found the higher prevalence of SLE in East Asians and heterogeneous disease manifestations across ethnicities.[15 16] Previous investigations suggested genetics might explain the phenotypic heterogeneity.[9] We observed that the MAFs of the index variants for several novel genetic associations were much higher in East Asians than in Europeans. Specifically, we suggested two novel loci were more likely specific to East Asians. These findings might help explain the genetic basis of SLE phenotypic heterogeneity between East Asians and Europeans. The results reinforce the power of large-scale genetic association for genetic discovery of SLE in relatively less studied populations.

We identified 11 exonic variants including two missense variants within novel loci *CHD23* and *LRRK1*, four novel missense variants within known SLE loci *IKBKB*,[9] *TYK2*,[9] *WDFY4*[37] and *OAS1*,[33] and three known missense variants within known *AHNAK2*,[33] *IRAK1*[34] and *NCF2*.[35 36] These findings suggested allelic heterogeneity within several of these loci and highlighted the disease-risk effects of genes *AHNAK2*, *CSK*, *IKBKB*, *IRAK1*, *NCF2*, *OAS1*, *TYK2* and *WDFY4* within eight known loci, and *CDH23* and *LRRK1* within two novel loci which potentially alter gene product activity in an allele-specific manner. The novel gene *CHD23* plays a role in cell migration[40] while *LRRK1* encodes a multiple-domain leucine-rich repeat kinase. A previous study observed that *LRRK1*-deficient mice exhibited a profound defect in B-cell proliferation and survival and impaired B-cell receptor-mediated NF-κB activation,[41] which suggested that the association within this region might confer the risk of SLE through modulating the NF-κB pathway and the activities of B cells. We noted that the Bayesian statistical fine-mapping analysis prioritised the lead missense variant rs35985016 as the most likely putative

causal variant for this association. This variant is highly frequent in our study individuals but is rare in Europeans. The molecular mechanisms in SLE risk worthy further investigations.

In the present study, we localised the putative causal variants for SLE genetic association in high resolution. Our findings indicated that the putative causal variants for the majority of SLE associations were non-coding variants. We provided targets of candidate putative causal variants with high confidence for several SLE loci. These findings are worthy for further exploration in functional experiments. We showed the regulatory effect of one of the putative causal variants in an accompanied paper. We acknowledged the limitation of a small LD reference panel from single population in the Bayesian statistical fine-mapping analysis.

We found for the first time the significant genetic correlations between SLE, albumin/globulin ratio and non-albumin protein. These findings might reflect the renal complications commonly developed in SLE patients who have been reported to have significantly lower albumin/globulin ratio and higher serum globulin than healthy controls in epidemiological studies.[42] These shared genetic basis findings might suggest a common pathway underlying the SLE risk and kidney function in addition to the direct damage of SLE autoantibodies on kidney.

In summary, we detected 46 novel loci for SLE risk in the largest meta-analysis and prioritised putative causal variants for 65 causal signals. This study highlights the power of large-scale genetic association study in East Asian populations. The findings reveal the genetic predispositions for SLE and provide clues for further the investigation of disease mechanisms.

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CLINICAL SCIENCE

New composite endpoint in early diffuse cutaneous systemic sclerosis: revisiting the provisional American College of Rheumatology Composite Response Index in Systemic Sclerosis

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ABSTRACT

Objectives American College of Rheumatology Composite Response Index in Systemic Sclerosis (ACR-CRISS) is a composite endpoint to assess the likelihood of improvement in diffuse systemic sclerosis. ACR-CRISS is a weighted score and includes five core set measures: modified Rodnan skin score, FVC% predicted, health assessment questionnaire–disability index, and patient and clinician global assessments.

Methods We analysed core set measures from 354 participants who participated in three placebo-controlled trials. We generated 10 development datasets, randomly selected from 2/3 of the participants, stratified by study and treatment group. The remaining participants (1/3 of the participants) formed the validation sets. Risk differences (RDs) between active and placebo treatments were calculated by averaging over the replicate datasets; bootstrap 95% CIs for the RDs to estimate the magnitude of treatment effects.

Results In the development sets (n=237), the proportion of participants in the active group had statistically higher improvement in >1 of 5 core set measures versus the placebo group. For example, the proportion who improved by ≥20% in ≥3 core set measures was 49.4% in the active versus338.9% in the placebo; RD: 10.5%, 95% CI4.9 % to 16.1%. In the validation sets (n=117), the proportion who improved by ≥20% in ≥3 core set measures was 50.3% in the active versus35.63% in the placebo (RD:114.8%, 95% CI 3.1% to225.7%). Similar trends were seen with larger percentage cut-offs.

Conclusion Revised CRISS, as assessed by the proportion of participants who improved by a certain percentage in ≥3 of 5 core set measures, is a potential new composite outcome measure.

Key messages

What is already known about this subject?

- Systemic sclerosis is a multisystem heterogeneous disease. American College of Rheumatology Composite Response Index in Systemic Sclerosis (ACR-CRISS) is a composite endpoint used to assess the likelihood of improvement in diffuse cutaneous systemic sclerosis (dcSSc).
- ACR-CRISS includes five core set measures: modified Rodnan skin score, FVC% predicted, health assessment questionnaire–disability index, and patient and clinician global assessments.
- ACR-CRISS is a weighted score (making it difficult to interpret) and has high floor and ceiling effects.
- We analysed core set measures from 347 patients who participated in three placebo-controlled trials and assessed for proportion of participants who improve above a certain threshold, similar to ACR 20% response criteria for rheumatoid arthritis.

What does this study add?

- Participants who were on active medication had statistically higher improvement in >1 of 5 core set measures versus the placebo group.
- The proportion who improved by ≥20% in ≥3 core set measures was 49.4% in the active versus 38.9% in the placebo; risk difference: 10.5%, 95% CI4.9 % to 16.1%.
- The same trends were seen for different cutpoints for ≥3 core set measures favouring the active medication group.

How might this impact clinical practice or future developments?

- The proposed new composite endpoint (revised CRISS) may provide easy interpretation and reduce floor and ceiling effects in clinical trials of dcSSc.

INTRODUCTION

Systemic sclerosis (SSc, scleroderma) is an immune-mediated rheumatic disease characterised by autoimmunity, vasculopathy, and fibrosis in the skin and internal organs.[1–3] It has the highest case fatality of any rheumatic disease. One subclassification of this disease, diffuse cutaneous SSc (dcSSc), has a 10-year mortality rate of 50% and disease management is focused on organ-specific complications.

Provisional American College of Rheumatology Composite Response Index in Systemic Sclerosis (ACR-CRISS) is a composite endpoint and was designed to capture the global or holistic evaluation of the likelihood of improvement in early SSc.[4] It integrates worsening or incident cases of cardiopulmonary-renal involvement and incorporates changes in five core set measures— modified Rodnan skin score (mRSS), per cent predicted

FVC (FVC%, health assessment questionnaire–disability index (HAQ-DI), and patient (PGA) and clinician (CGA) global assessments. ACR-CRISS was able to differentiate active therapy from placebo in recent trials,[3 5–8] which showed statistically different and clinically important changes in the ACR CRISS, highlighting the importance of global assessment in a multisystem heterogeneous disease. However, ACR-CRISS is derived from a two-step algorithm with probabilities of improvement based on core set measures that are weighted differently in each study. Thus, the endpoint has the potential for a single core set measure to drive overall response without clearly demonstrating a treatment benefit on one or more of the other core set measures. In addition, recent top line data (not published in medical journal) from lenabasum and autotaxin inhibitor trials showed ceiling effect of ACR-CRISS in the placebo and active therapy groups.[9]

To address these concerns, we explored the performance of the five core set measures in ACR-CRISS that were collected in three recent placebo-controlled randomised controlled trials (RCTs). We used the concept that was first proposed by Paulus *et al*,[10] who developed a composite score based on statistical analysis to assess the activity of disease-modifying anti-rheumatic drugs (DMARDs) in rheumatoid arthritis (RA). These criteria, known as the Paulus criteria, required the improvement of 20% in at least four of six core set measures in RA and the criteria were able to differentiate the DMARD therapies from placebo in available RCTs. This later led to the development of ACR 20% response criteria that has been adopted as an acceptable endpoint for regulatory approval for RA.[11]

The objective of the current analysis is to assess if a certain percentage of improvement in five core set measures, as incorporated in the ACR-CRISS, can differentiate the active medication group from placebo. Our hypotheses were that a greater proportion of participants on active medication will show statistically significant improvement compared with placebo for each of the five core set measures, and a statistically significant proportion of participants will improve by a predefined percentage in >1 core set measures (eg, three of five core set measures with 20% improvement) favouring active medication group. We tested our hypotheses in three RCTs that assessed abatacept versus placebo (in a phase II trial) and tocilizumab versus placebo (in phase II and phase III trials) in early dcSSc using 95% CIs for the risk difference between treatments.

PATIENTS AND METHODS

Description of three trials

The three RCTs recruited patients with early dcSSc and mRSS was the primary outcome measure for all trials. The RCTs were double-blind, placebo-controlled where escape therapy was allowed with immunosuppressive therapy, if there was worsening of dcSSc.

Abatacept phase II trial (ASSET) randomly assigned 88 participants 1:1 to receive abatacept 125 mg subcutaneously (SC) or matching placebo, stratified by duration of dcSSc (ClinicalTrials.gov NCT02161406) in a 52-week trial.[3 12] The primary endpoint was the change from baseline to week 52 in mRSS. Key inclusion criteria were the disease duration of ≤36 months (defined as time from the first non−Raynaud phenomenon manifestation). For disease duration of ≤18 months, an mRSS ≥10 and ≤35 was required at the screening visit. For disease duration of >18 to ≤36 months, an mRSS of ≥15 and ≤45 was required.

Tocilizumab (TCZ) phase II trial randomly assigned 87 participants 1:1 to receive weekly tocilizumab 162 mg or placebo SC for 48 weeks (ClinicalTrials.gov NCT01532869).[5 8] The primary endpoint was the change from baseline to week 48 in mRSS. The key inclusion criteria included of ≤60 months' disease duration (from first non–Raynaud phenomenon manifestation) and a mRSS of 15–40 units at screening.

Tocilizumab phase III trial randomly assigned 212 participants 1:1 to receive double-blind weekly tocilizumab 162 mg or placebo SC for 48 weeks (ClinicalTrials.gov NCT02453256).[6] The primary endpoint was the change from baseline to week 48 in mRSS. The key inclusion criteria included ≤60 months' disease duration (from first non–Raynaud phenomenon manifestation) and a mRSS of 10–35 units at screening.

Methods

We pooled participants from the three RCTs to estimate the magnitude of treatment effects and the impact of the varying thresholds for improvement in various sets of the core set measures (ie, revised CRISS outcomes) to differentiate active and placebo treatment groups.

Statistical analysis

We defined improvement for four core set measures (mRSS, HAQ-DI, PGA, CGA) as the relative improvement from baseline to 1 year, varying the threshold of improvement in 5% increments from 10%, 15%, 20%, …, 50%, 55% and 60% (ie, at 11 different cut points). We also defined improvement for the pulmonary core set measure—per cent predicted FVC (FVC%)—as 5% and 10% relative improvement from baseline to 1 year (ie, at two cut points). We assessed whether improvement was seen in at least one, two, three, four or all five core set measures (ie, five levels of improvement).

We summarised the proportion of participants who demonstrated improvements for the five core set measures by treatment group, based on their relative improvement in four of the five core set measures and FVC% (ie, 11×2 or 22 combinations of cut points for the five core set measures). In addition, for each cut point (eg, 10% improvement in mRSS, HAQ-DI, patient GA and clinician GA and 5% improvement in FVC%), we calculated the proportion of participants with at least one, two, three, four or all five improvements by treatment group, resulting in an additional five summaries per cut point. We calculated the risk difference (RD; proportion of participants who improved in active medication group—proportion of participants who improved in placebo group) as a measure of the sensitivity of each of the core set measures and each revised CRISS outcome to treatment differences.

Development and validation sets

To assess the validity of our estimates, we divided the pooled data from the three RCTs into development and validation sets, using random split-sample validation.[13] Because of potential differences among the three trials with respect to treatments, demographic and baseline characteristics, we generated 10 development sets, randomly selected from two-thirds of the pooled patients, stratified by study and treatment group. The remaining participants (one-third of the pooled patients) formed the validation sets. For each set (either development or validation), we did the following: (1) we calculated the proportion of participants who improved in each treatment group and the associated RD (resulting in 10 estimates for each set); (2) we averaged the proportion of participants who demonstrated improvements by treatment group (over the 10 sets); (3) we used bootstrapping methods to estimate RD and its 95% CI (based on 100 bootstraps).[13]

Table 1 Baseline demographics of participants in three randomised controlled trials

	Overall n=387	Placebo n=195	Active n=192	P value	ASSET n=88	TCZ II n=87	TCZ III n=212	P value
Age, years								
N	386	195	191	0.68*	88	87	211	0.50†
Mean (SD)	48.7 (12.4)	48.9 (12.7)	48.5 (12.2)		49.4 (12.6)	49.6 (12.3)	48.1 (12.4)	
Female sex, n (%)	305 (79.81)	161 (82.56)	144 (75.00)	0.07‡	66 (75.00)	67 (77.01)	172 (81.13)	0.44‡
Race, n (%)								
White	327 (84.50)	168 (86.15)	159 (82.81)	0.68‡	72 (81.82)	78 (89.66)	177 (83.49)	0.01§
African American	17 (4.39)	8 (4.10)	9 (4.69)		6 (6.82)	6 (6.90)	5 (2.36)	
Asian	32 (8.27)	13 (6.67)	19 (9.90)		5 (5.68)	2 (2.30)	25 (11.79)	
Other	11 (2.84)	6 (3.08)	5 (2.60)		5 (5.68)	1 (1.15)	5 (2.36)	
Baseline core set measures								
mRSS (0–51)								
n	386	195	191	0.67*	88	87	211	<0.0001†
Mean (SD)	22.1 (7.3)	21.9 (7.1)	22.4 (7.5)		22.5 (7.7)	26.0 (6.5)	20.4 (6.9)	
HAQ-DI (0–3)								
n	382	192	190	0.25*	88	86	208	0.057†
Mean (SD)	1.2 (0.7)	1.2 (0.7)	1.1 (0.7)		1.1 (0.7)	1.3 (0.7)	1.2 (0.7)	
Patient Global Assessment (0–10 VAS)								
N	381	192	189	0.046*	85	87	209	<0.0001†
Mean (SD)	5.4 (2.4)	5.7 (2.3)	5.2 (2.4)		4.1 (2.4)	6.1 (2.0)	5.7 (2.3)	
Clinician Global Assessment (0–10 VAS)								
n	372	187	185	0.65*	86	87	199	<0.0001†
Mean (SD)	5.8 (1.8)	5.7 (1.7)	5.8 (1.9)		4.8 (1.7)	6.2 (1.5)	6.0 (1.8)	
FVC% predicted								
n	384	193	191	0.0487¶	88	86	210	0.0918**
Mean (SD)	82.5 (14.6)	84.0 (15.1)	81.1 (14.1)		85.3 (15.1)	80.7 (13.6)	82.1 (14.8)	

*Wilcoxon rank sum test.
†Kruskal-Wallis test.
‡Chi-square test.
§Fisher exact test.
¶2-sample t-test.
**ANOVA test and Fisher exact test.
HAQ-DI, health assessment questionnaire–disability index; mRSS, modified Rodnan skin score; VAS, visual analogue scale.

We calculated the floor (defined as the proportion of patients achieving ACR-CRISS of <0.005) and ceiling effects (defined as the proportion of patients achieving ACR-CRISS of >0.995) of ACR-CRISS. We also assessed the relationship between the proportion of participants who achieved at least 10%, 20%, 30%, 40%, 50% and 60% improvement in at least three of five core set measures in revised CRISS and ACR-CRISS by displaying box plots of ACR-CRISS for the improved and not improved groups based on revised CRISS. We summarised those associations using point-biserial correlations.

All analyses were conducted in SAS V.9.4 (SAS Institute).

RESULTS

There were 387 participants in the three RCTs. Eighty per cent of the pooled participants were women, with mean (SD) baseline age of 48.7 (12.4) years, mRSS of 22.1 (7.3), HAQ-DI of 1.2 (0.7), PGA (on 0–10 scale) of 5.4 (2.4), CGA (on 0–10 scale) of 5.8 (1.8) and FVC% of 82.5 (14.6)%; (table 1). Of the 387 participants, 33 (8.5%) met step 1 (cardio-pulmonary-renal worsening) and were considered not improved and not included in the subsequent analysis as our goal was to analyse the performance of five core set measures. These included 4 in TCZ II (4 in placebo and 0 in active medication groups), 19 in TCZ III (13 in placebo and 6 in active medication groups) and 10 in ASSET (5 in each treatment group; table 2). We observed statistically significant higher PGA (p=0.047) and higher FVC% predicted (p=0.049) in placebo group at baseline (table 1). We also observed statistically significant heterogeneity among studies in race (p=0.01), and baseline mRSS (p<0.0001), PGA (p<0.0001) and CGA (p<0.0001).

Performance of five core set measures: development data sets

The proportion of participants (n=237, validation sets) who improved by ≥10% to ≥60% (in 5% increments) were numerically higher in the active therapy versus placebo group for all four core set measures mRSS, HAQ-DI, PGA and CGA and for FVC% at 5% and 10% relative improvement the majority of the time (table 3 and figure 1). PGA was not numerically higher in the active group, compared with the placebo group, for 10% and 20% thresholds, and mRSS was not numerically higher for the 60% threshold. When we assessed the proportion of participants who improved by ≥1 in 5 core set measures, these were numerically higher favouring active therapy versus placebo group—except the proportion of participants with all five improvements was not numerically higher in active, compared with placebo, for the 60% threshold. As an example, the proportion of participants who improved by ≥20% in ≥1 core set measure was 90.1% in active therapy versus 81.2% in the placebo group, in ≥2 core set measures was 74.4% in active therapy versus 61.6% in the placebo group, in ≥3 core set measures was 49.4% in active therapy versus 38.9% in the placebo group and in ≥4 core set measures was 24.9% in active therapy versus 17.1% in placebo group (table 3 and figure 1).

Table 2 Performance of the ACR-CRISS in the three randomised controlled trials, including floor and ceiling effects

Trials	Active, n=192					Placebo, n=195				
	Meeting Step 1	Median score at 12 months*	ACR-CRISS ≥0.6 at 12 months*	% with ceiling effect*†	% with floor effect*†	Meeting Step 1	Median score at 12 months*	ACR-CRISS ≥0.6 at 12 months*	% with ceiling effect*†	% with floor effect*†
Overall, n=387	11	0.86	61.9%	32.1%	15.7%	22	0.19	46.5%	17.8%	32.6%
ASSET, n=88	5	0.84	64.3%	35.7%	21.4%	5	0.04	41.9%	6.5%	41.9%
TCZ phase II, n=87	0	0.25	44.4%	25.9%	29.6%	4	0.03	27.6%	10.3%	41.4%
TCZ phase III, n=212	6	0.90	88.6%	32.9%	8.9%	13	0.74	56.5%	26.1%	24.6%

ACR-CRISS <0.005.
*Step 1 participants not included.
†Ceiling effect: ACR-CRISS >0.995 and floor effect.

Performance of five core set measures: validation data sets

Similar to the development sets, we saw analogous trends in the validation sets (n=117) where the proportion of participants who improved by ≥10% to ≥60% (in 5% increments) were numerically higher in the active therapy versus placebo group for all five core set measures mRSS, HAQ-DI, PGA, CGA and FVC% the majority of the time. In addition, the patterns were similar for the proportion of participants with at least one to all five core set measures on active therapy numerically larger than those in the placebo group. The magnitude of the effects was comparable between the development and validation sets; for example, the proportion of participants who improved by ≥20% in ≥1 core set measure was 92.7% in active therapy versus 80.1% in the placebo group, in ≥2 core set measures was 75.8% in active therapy versus 57.7% in the placebo group, in ≥3 core set measures was 50.3% in active therapy versus 35.6% in the placebo group and in ≥4 core set measures was 27.7% in active therapy versus 13.6% in the placebo group (table 4 and online supplemental figure 1). We used the same method as described for development sets to calculate RD and 95% CI.

Development and validation sets using FVC cut-off 10% improvement

The data were similar when we used an FVC% improvement of ≥10% instead of ≥5% (online supplemental tables 1 and 2).

Performance of ACR-CRISS versus revised CRISS

ACR-CRISS showed a ceiling effect (defined as proportion of patients achieving ACR-CRISS of >0.995) of 17.8% in the placebo group and 32.1% in the active therapy group (table 2). In addition, there was a high floor effect (defined as proportion of patients achieving ACR-CRISS of <0.005) of 15.7% and 32.6% in active and placebo groups, respectively.

Figure 2 displays boxplots of ACR-CRISS between the improved and non-improved groups based on revised CRISS with thresholds of ≥10%, ≥20%, …, ≥60%. At improvement thresholds of ≥10% and ≥20%, the median ACR-CRISS was 0.99 among those with improvement versus 0.01 among those without (correlation coefficient of 0.63 and 0.62, respectively, p<0.001 for each comparison). The magnitude of difference is attenuated as the threshold increases (correlation coefficients from 0.38 to 0.59, p<0.001 for each comparison), but differences remain statistically significant.

DISCUSSION

SSc is a multisystem heterogeneous disease with variable disease course. Traditionally, clinical trials have focused on using mRSS as the primary outcome measure in dcSSc due to its relationship to internal organ involvement in early disease and meeting the OMERACT filters. However, recent trials have shown marked heterogeneity in early disease despite enriching the trial population of disease duration, biomarkers and/or mRSS cut-off.[3 6 8 14] As an example, post hoc analyses from the abatacept phase II trial show that skin gene expression predicted differential responses in mRSS, FVC% and HAQ-DI.

ACR-CRISS was developed to address the limitations of mRSS and other outcome measures using well-established consensus and evidence-based input. ACR-CRISS, a global measure, is based on a probability score of 0.0 (no improvement) to 1.0 (marked improvement) and includes two steps. The first step assesses for worsening or incident cases of cardio-pulmonary-renal involvement and gives a score of 0.0. For those who do not meet step 1, a weighted probability score is calculated that

Table 3 Proportion of participants who achieved a predefined percentage of improvement for each core set measure and ≥1 core set measures in development data set

Improvement	Measures	PBO n=116	Active n=121	Risk difference (95% CI)	Improvement	Measures	PBO n=116	Active n=121	Risk difference (95% CI)
10%	mRSS	68.9	78.2	9.3 (5.2 to 14)	15%	mRSS	60.2	76.2	16 (11.6 to 21.1)
	HAQ-DI	38.3	52.1	13.7 (8.6 to 18.5)		HAQ-DI	35.7	46.0	10.2 (4.7 to 17.5)
	Patient GA	56.8	54.6	−2 (−10.2 to 2.8)		Patient GA	49.6	50.6	1.1 (−7.1 to 5.6)
	Clinician GA	64.5	78.1	13.7 (9 to 20.1)		Clinician GA	61.8	73.6	11.8 (5.8 to 19.3)
	FVC%	11.8	19.2	7.3 (4 to 11.6)		FVC%	11.8	19.2	7.3 (4 to 11.6)
	At least 1 improvement	87.7	96.5	8.9 (4 to 11.7)		At least 1 improvement	83.2	93.1	10.1 (5.9 to 12.8)
	At least 2 improvements	73.7	80.7	6.9 (2 to 13.2)		At least 2 improvements	64.4	78.0	13.5 (8.9 to 19.1)
	At least 3 improvements	46.6	56.4	9.7 (4.5 to 15.9)		At least 3 improvements	41.9	53.1	11.1 (5.7 to 17)
	At least 4 improvements	19.0	33.0	13.9 (9.9 to 23.4)		At least 4 improvements	17.8	28.3	10.6 (5 to 20.6)
	All 5 improvements	2.1	6.0	3.9 (2 to 5.7)		All 5 improvements	1.4	3.8	2.3 (0.9 to 3.8)
20%	mRSS	54.9	72.7	17.8 (11.9 to 21.4)	25%	mRSS	50.5	65.3	14.8 (9.3 to 18.8)
	HAQ-DI	35.2	40.5	5.3 (−0.2 to 10.5)		HAQ-DI	30.8	37.1	6.2 (1 to 10.7)
	Patient GA	47.4	46.9	−0.4 (−8.2 to 3.6)		Patient GA	42.3	43.6	1.3 (−5.1 to 6.8)
	Clinician GA	60.9	72.2	11.3 (4.7 to 18.3)		Clinician GA	54.0	68.3	14.3 (5.8 to 23.1)
	FVC%	11.8	19.2	7.3 (4 to 11.6)		FVC%	11.8	19.2	7.3 (4 to 11.6)
	At least 1 improvement	81.2	90.1	9 (4 to 12.9)		At least 1 improvement	75.1	87.2	12.2 (7.9 to 15.9)
	At least 2 improvements	61.6	74.4	12.8 (7.9 to 19.1)		At least 2 improvements	54.6	67.9	13.2 (7.9 to 21.3)
	At least 3 improvements	38.9	49.4	10.5 (4.9 to 16.1)		At least 3 improvements	34.2	45.1	10.8 (4.1 to 15.9)
	At least 4 improvements	17.1	24.9	7.8 (3 to 14.9)		At least 4 improvements	15.0	21.1	6 (0.2 to 10.9)
	All 5 improvements	1.4	3.8	2.3 (0.9 to 3.8)		All 5 improvements	1.4	3.8	2.3 (0.9 to 3.8)
30%	mRSS	45.2	53.7	8.5 (4 to 13.1)	35%	mRSS	39.7	47.8	8.1 (6.2 to 11.2)
	HAQ-DI	25.5	31.6	6.1 (2.4 to 11.6)		HAQ-DI	22.7	30.9	8.2 (3.5 to 13.7)
	Patient GA	35.5	39.8	4.4 (−0.6 to 9.1)		Patient GA	31.4	34.9	3.4 (−0.9 to 7.3)
	Clinician GA	50.8	61.4	10.6 (2.6 to 19)		Clinician GA	47.8	54.1	6.2 (0.9 to 15.1)
	FVC%	11.8	19.2	7.3 (4 to 11.6)		FVC%	11.8	19.2	7.3 (4 to 11.6)
	At least 1 improvement	70.3	85.6	15.4 (11.2 to 20.7)		At least 1 improvement	65.7	80.7	14.9 (10.9 to 21.9)
	At least 2 improvements	49.3	60.5	11.1 (5.2 to 17.6)		At least 2 improvements	46.4	54.4	7.9 (3.5 to 14)
	At least 3 improvements	29.2	34.5	5.2 (−1.4 to 8.8)		At least 3 improvements	23.5	29.5	5.9 (−1.2 to 10.7)
	At least 4 improvements	10.4	15.3	4.9 (2 to 9.1)		At least 4 improvements	8.7	13.3	4.5 (2.5 to 8.2)
	All 5 improvements	1.4	2.2	0.8 (−1.1 to 2)		All 5 improvements	1.4	2.2	0.8 (−1.1 to 2)
40%	mRSS	33.5	41.6	8.1 (4.6 to 12.1)	45%	mRSS	29.3	37.1	7.9 (4.2 to 12.1)
	HAQ-DI	22.1	26.6	4.5 (0.6 to 9.9)		HAQ-DI	18.8	23.1	4.3 (0.8 to 6.7)
	Patient GA	27.4	32.6	5.1 (−0.7 to 10.3)		Patient GA	26.5	29.9	3.4 (−3.8 to 6.6)
	Clinician GA	45.5	50.2	4.7 (−1 to 14.2)		Clinician GA	41.5	43.5	1.9 (−2.2 to 8.1)
	FVC%	11.8	19.2	7.3 (4 to 11.6)		FVC%	11.8	19.2	7.3 (4 to 11.6)
	At least 1 improvement	62.8	73.4	10.6 (6.5 to 16.2)		At least 1 improvement	60.2	70.9	10.7 (6.9 to 16.3)
	At least 2 improvements	41.9	48.9	6.9 (1.6 to 11.7)		At least 2 improvements	36.8	42.1	5.2 (0.2 to 9.1)
	At least 3 improvements	19.0	26.4	7.3 (2 to 12)		At least 3 improvements	15.9	21.8	5.8 (2.4 to 9.2)
	At least 4 improvements	7.9	12.9	5 (2.9 to 7.3)		At least 4 improvements	7.1	10.8	3.7 (1.9 to 5.6)
	All 5 improvements	1.4	2.2	0.8 (−1.1 to 2)		All 5 improvement	1.4	1.5	0.1 (−1.1 to 1)

Continued

Table 3 Continued

Improvement	Measures	PBO n=116	Active n=121	Risk difference (95% CI)	Improvement	Measures	PBO n=116	Active n=121	Risk difference (95% CI)
50%	mRSS	26.5	30.8	4.3 (1.7 to 8.3)	55%	mRSS	21.5	24.4	2.8 (–1.9 to 7)
	HAQ-DI	18.1	22.5	4.5 (1.2 to 7.3)		HAQ-DI	16.4	18.5	2.1 (–1.8 to 4.7)
	Patient GA	24.0	27.0	2.9 (–3.8 to 6.5)		Patient GA	17.4	26.2	8.8 (3.3 to 11.4)
	Clinician GA	34.2	39.8	5.6 (2.5 to 10.6)		Clinician GA	29.1	32.9	3.9 (1.6 to 7.7)
	FVC%	11.8	19.2	7.3 (4 to 11.6)		FVC%	11.8	19.2	7.3 (4 to 11.6)
	At least 1 improvement	56.2	65.4	9.3 (5 to 12.7)		At least 1 improvement	49.0	60.9	12.1 (7.5 to 16.4)
	At least 2 improvements	32.1	37.6	5.4 (0.4 to 10.3)		At least 2 improvements	26.4	30.6	4.1 (–0.1 to 9.5)
	At least 3 improvements	12.7	21.1	8.4 (6.2 to 11.9)		At least 3 improvements	9.8	17.6	7.7 (3.4 to 12)
	At least 4 improvements	7.1	8.3	1.2 (–1.1 to 3.7)		At least 4 improvements	5.5	6.5	1 (–0.9 to 2.8)
	All 5 improvements	0.9	1.5	0.6 (–0.1 to 1)		All 5 improvements	0.9	1.0	0.1 (–1 to 1)
60%	mRSS	19.4	17.8	–1.7 (–6.7 to 5.2)					
	HAQ-DI	15.6	17.8	2.3 (–1.9 to 5.8)					
	Patient GA	15.2	20.2	5.1 (0.2 to 8.6)					
	Clinician GA	27.3	29.4	2.1 (–1.9 to 5.8)					
	FVC%	11.8	19.2	7.3 (4 to 11.6)					
	At least 1 improvement	47.1	56.4	9.4 (5 to 14.4)					
	At least 2 improvements	23.1	23.9	0.7 (–3.7 to 7.8)					
	At least 3 improvements	8.4	13.6	5.1 (1.5 to 10.2)					
	At least 4 improvements	5.5	5.8	0.4 (–1.9 to 2)					
	All 5 improvements	0.9	0.5	–0.4 (–1 to 0)					

5% improvement is calculated for FVC% in all improvement level; risk difference=proportion of participants who improved in active medication group–proportion of participants who improved in placebo group.
GA, global assessment; HAQ-DI, health assessment questionnaire–disability index; mRSS, modified Rodnan skin score; PBO, placebo.

incorporates changes in five physical or functional areas—mRSS (assessment of skin), FVC% predicted (assessment of lungs), HAQ-DI (measure of patient function), PGA and CGA. The weights are derived from physician consensus of ranking real patient profiles. The ACR-CRISS has worked well in four recent prospective trials including lenabasum, abatacept and tocilizumab phase II and III, statistically favouring active therapy from placebo ($p<0.05$ for all analyses). Apart from lenabasum phase II trial, all thetrials incorporated mRSS as the primary outcome measure and showed non-significant trends favouring the active therapy in mRSS.[14] Despite the non-significant results in the mRSS, tocilizumab had robust influence on FVC% predicted with preservation of lung function in two separate trials and abatacept had clinically meaningful impact on HAQ-DI.

The positive results with the ACR-CRISS has been discussed in the scleroderma community where the researchers have queried the interpretation of the probability score. A cut-off of ≥0.60 has been proposed as a clinically meaningful cut point for the ACR-CRISS.[4] However, there is concern that the ACR-CRISS score can be driven by one core set measure, especially mRSS, since it has the highest weight in the probability score. In addition, recent topline data from lenabasum phase III trial had the ACR-CRISS score of 0.887 in the placebo group at week 52, suggesting a ceiling effect, and a similar trend was seen in a 6-month double-blind phase II trial of autotaxin inhibitor versus placebo[9]; both trials allowed background immunosuppressive therapy as part of the trial design.

To address this, we followed the principles laid by Paulus *et al* that were later modified to develop the ACR 20% response criteria for ACR20,[10] the gold standard for approval of drugs by regulatory agencies for RA, and was later adopted for juvenile arthritis and psoriatic arthritis. Paulus *et al* analysed four RCTs in RA,[10] which were conducted as part of a consortium with an agreed set of core set measures, but no pre-specified primary outcome measures. One of the rationales of the composite index in RA was a lack of interpretation and comparison between different trials where similar outcome measures were incorporated, with certain measures showing statistically significant differences while others did not. In addition, the data were presented as mean and median changes over time, and it was difficult for a clinician to assign clinical importance to the data. Trial design has improved over the last 30 years where SSc trials are pre-specifying the appropriate primary outcome measure and statistical testing. From a purely statistical interpretation of the three RCTs analysed for this report, the trials are negative. However, these trials provide a platform to explore and develop new composite endpoints as all three RCTs included five core set measures and had one or more core set measures that favoured active medication over placebo, similar to the process proposed by Paulus *et al*. Using pooled data from three clinical trials (and reinforced by analyses to support internal validity using development and validation sets), we showed that the active therapies had a higher proportion of patients who improved in ≥1 core set measure compared with the placebo. The effect was consistent from 10% to 60% improvement in ≥1 core set measure.

We found that the active therapy group had consistent results, but there were some differences in the placebo response between the three RCTs (data not shown). This can be explained by different inclusion and exclusion criteria and geographical locations where the trials were conducted. The abatacept and tocilizumab phase II trials were conducted in North America and Europe whereas tocilizumab phase III trial was conducted in multiple countries throughout the world. The higher placebo

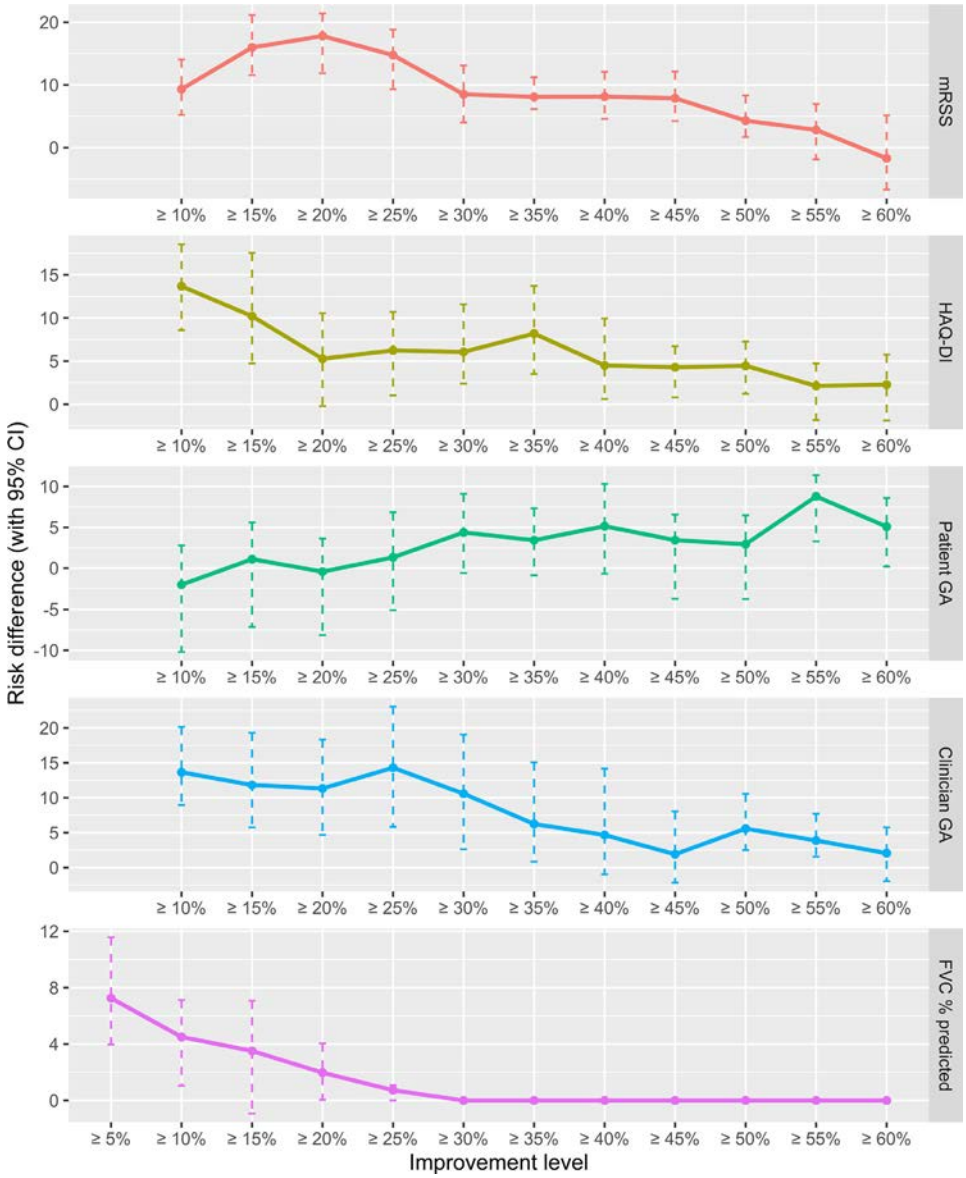

Figure 1 Risk difference (proportion of participants who improved in active medication group—proportion of participants who improved in placebo group) for improvement ≥10% in 5% increments in the three randomised controlled trials in the development data set . GA, global assessment; HAQ-DI, Health Assessment Questionnaire–Disability Index; mRSS, modified Rodnan skin score.

response in the tocilizumab phase III may reflect expectations of the patients in different countries or other unexplained variables.

In our current analysis, the ACR CRISS showed both ceiling and floor effect that may impact responsiveness to change.[15] We believe that recent trials on background immunosuppressives may increase the ceiling effect (although this needs to be analysed). We also acknowledge that the proposed revised CRISS measures are a dichotomous index. Although it is well known that dichotomising a continuous outcome variable reduces power and precision, the current analysis of ACR-CRISS indicates that there is bimodal distribution with discontinuity in values, and we believe that the impact of this loss of power and precision in revised CRISS may be balanced by the improvement in clinical interpretation.

Although the different cut-offs showed trends favouring active therapy, we propose two ways to consider incorporating the revised CRISS as a starting point for future trials (online supplemental figure 2). First, we can consider ACR-CRISS 20 or 25% which translates into at least 20% or 25% improvement in mRSS, HAQ-DI, PGA and CGA (with 5% or 10% improvement in FVC). This is based on the minimal clinically important differences (MCIDs) that are published in different rheumatic diseases, including SSc, for five core set measures. For mRSS, an improvement of 24% is considered as the MCID.[16] For HAQ-DI, the published MCID estimate is 0.22 and the mean HAQ-DI scores in the three RCTs was 1.2—a relative change of 19%. A change of one unit (on a 0–10 scale) is considered as the MCID estimate for global assessments and the mean baseline scores for PGA and CGA in the three RCTs were 5.4 and 5.8, a 18%–19% relative change. For the FVC%, we only evaluated 5% and 10% relative improvement as an improvement above this level is unreasonable in fibrotic progressive lung fibrosis.[17 18] In the three RCTs presented here, there were 15.6% of participants who improved by ≥5% and 7.8% who improved by ≥10%. In the SENCIS trial with established interstitial lung disease, the percentage who improved by 5% and 10% were approximately 7.0%–12.9%, respectively.[19] In addition, the intra-observer variability of FVC% was 5% in the SLS I and II and improvement

Table 4 Proportion of participants who achieved a predefined percentage of improvement for each core set measure and ≥1 core set measures in the validation data set

Improvement	Measures	PBO n=57	Active n=60	Rate difference (95% CI)	Improvement	Measures	PBO n=57	Active n=60	Rate difference (95% CI)
10%	mRSS	65.6	81.3	15.8 (6.1 to 24.4)	15%	mRSS	58.9	79.5	20.7 (10.2 to 30.1)
	HAQ-DI	33.7	54.5	21 (11.4 to 30.8)		HAQ-DI	30.9	45.3	14.6 (0.8 to 25)
	Patient GA	57.0	57.7	0.5 (–8.3 to 16.2)		Patient GA	48.8	53.9	4.9 (–3.7 to 20.8)
	Clinician GA	61.4	80.4	18.9 (5.7 to 28.9)		Clinician GA	57.8	76.8	18.9 (3.7 to 31.3)
	FVC%	11.1	20.4	9.5 (0.3 to 15.7)		FVC%	11.1	20.4	9.5 (0.3 to 15.7)
	At least 1 improvement	86.8	97.3	10.4 (4.3 to 20)		At least 1 improvement	82.1	94.4	12.1 (6.4 to 20)
	At least 2 improvements	69.1	80.7	11.8 (-1.7 to 20.4)		At least 2 improvements	59.9	78.4	18.6 (6.8 to 27.3)
	At least 3 improvements	44.0	59.5	15.7 (3 to 25.8)		At least 3 improvements	39.4	54.5	15.3 (3.1 to 25.7)
	At least 4 improvements	15.6	36.6	21 (1.5 to 28.3)		At least 4 improvements	14.2	32.4	18.1 (–2.5 to 29.5)
	All 5 improvements	1.8	9.3	7.5 (3.8 to 11.1)		All 5 improvements	1.2	6.0	4.9 (1.9 to 7.7)
20%	mRSS	53.4	76.8	23.5 (15.9 to 35.7)	25%	mRSS	48.1	70.2	22.3 (14.2 to 33.3)
	HAQ-DI	29.8	40.6	10.8 (0.9 to 21.2)		HAQ-DI	26.3	37.6	11.4 (2.9 to 21.2)
	Patient GA	46.9	49.3	2.2 (–5.5 to 17)		Patient GA	40.8	46.0	5.2 (–5.3 to 17.6)
	Clinician GA	57.3	75.3	17.9 (3.7 to 31.6)		Clinician GA	50.9	72.5	21.6 (3.8 to 38.8)
	FVC%	11.1	20.4	9.5 (0.3 to 15.7)		FVC%	11.1	20.4	9.5 (0.3 to 15.7)
	At least 1 improvement	80.1	92.7	12.4 (4.4 to 22.1)		At least 1 improvement	74.3	90.7	16.2 (8.6 to 24.1)
	At least 2 improvements	57.7	75.8	18.1 (4.8 to 27.5)		At least 2 improvements	49.8	71.4	21.8 (4.8 to 31.9)
	At least 3 improvements	35.6	50.3	14.8 (3.1 to 25.7)		At least 3 improvements	31.0	45.4	14.6 (4 to 27.5)
	At least 4 improvements	13.6	27.7	14.1 (–0.4 to 23)		At least 4 improvements	11.8	23.7	12.0 (2.0 to 23.3)
	All 5 improvements	1.2	6.0	4.9 (1.9 to 7.7)		All 5 improvements	1.2	6.0	4.9 (1.9 to 7.7)
30%	mRSS	42.6	60.3	17.7 (8.7 to 26.8)	35%	mRSS	37.5	52.6	15.2 (8.9 to 19.3)
	HAQ-DI	22.4	32.9	10.4 (–1.7 to 17.3)		HAQ-DI	20.0	32.3	12.3 (0.3 to 21.2)
	Patient GA	33.7	41.5	7.7 (–1.3 to 17.9)		Patient GA	27.6	37.6	10.1 (2.0 to 18.5)
	Clinician GA	48.4	65.3	16.9 (–0.4 to 33.0)		Clinician GA	45.5	58.7	13.3 (–4.6 to 23.9)
	FVC%	11.1	20.4	9.5 (0.3 to 15.7)		FVC%	11.1	20.4	9.5 (0.3 to 15.7)
	At least 1 improvement	70.1	88.1	17.9 (6.6 to 25.9)		At least 1 improvement	65.3	84.5	19.3 (4.6 to 27.4)
	At least 2 improvements	44.5	65.0	20.5 (7.1 to 31.5)		At least 2 improvements	40.4	57.8	17.5 (5.0 to 25.8)
	At least 3 improvements	27.0	37.5	10.7 (3.3 to 23.6)		At least 3 improvements	20.6	34.0	13.7 (4.4 to 27.3)
	At least 4 improvements	7.0	17.8	10.8 (2 to 16.2)		At least 4 improvements	6.4	14.2	7.7 (0 to 12.1)
	All 5 improvements	1.2	3.3	2.2 (–0.2 to 5.8)		All 5 improvements	1.2	3.3	2.2 (–0.2 to 5.8)
40%	mRSS	31.6	45.3	13.7 (5.1 to 20.6)	45%	mRSS	27.9	40.6	12.5 (3.6 to 19.8)
	HAQ-DI	19.2	29.2	10.0 (–1.7 to 17.3)		HAQ-DI	15.5	24.4	8.9 (3.8 to 16.7)
	Patient GA	23.3	34.2	11.0 (0 to 23.1)		Patient GA	23.1	29.6	6.5 (0 to 21.2)
	Clinician GA	41.2	55.9	14.8 (–4.4 to 26)		Clinician GA	38.0	50.5	12.6 (0.2 to 19.7)
	FVC%	11.1	20.4	9.5 (0.3 to 15.7)		FVC%	11.1	20.4	9.5 (0.3 to 15.7)
	At least 1 improvement	61.1	79.7	18.5 (6.8 to 27.2)		At least 1 improvement	58.5	76.9	18.4 (6.8 to 25.3)
	At least 2 improvements	35.4	53.1	18.0 (8 to 29.5)		At least 2 improvements	31.8	45.6	14.0 (6.0 to 23.7)
	At least 3 improvements	15.6	28.5	13.1 (3 to 23.4)		At least 3 improvements	12.0	24.3	12.4 (5.3 to 19.3)
	At least 4 improvements	6.0	13.0	6.9 (2.0 to 10.9)		At least 4 improvements	5.6	9.3	3.6 (–0.5 to 7.2)
	All 5 improvements	1.2	3.3	2.2 (–0.2 to 5.8)		All 5 improvements	1.2	2.7	1.6 (–0.2 to 3.8)

Continued

Table 4 Continued

Improvement	Measures	PBO n=57	Active n=60	Rate difference (95% CI)	Improvement	Measures	PBO n=57	Active n=60	Rate difference (95% CI)
50%	mRSS	25.5	35.8	10.3 (1.7 to 15.7)	55%	mRSS	21.5	27.2	5.9 (–2.5 to 15.2)
	HAQ-DI	14.9	23.6	8.7 (2.2 to 15.3)		HAQ-DI	14.0	19.9	5.7 (0.2 to 13.4)
	Patient GA	21.8	27.6	5.7 (–1.9 to 19.3)		Patient GA	14.6	27.2	12.6 (7.4 to 23.6)
	Clinician GA	32.3	45.1	12.8 (2.7 to 19.1)		Clinician GA	26.8	37.7	10.8 (3.0 to 15.5)
	FVC%	11.1	20.4	9.5 (0.3 to 15.7)		FVC%	11.1	20.4	9.5 (0.3 to 15.7)
	At least 1 improvement	54.5	70.6	15.9 (8.8 to 23.6)		At least one improvement	47.1	66.0	18.8 (10.1 to 28.8)
	At least two improvement	29.2	41.0	12 (2 to 22.7)		At least two improvement	22.8	33.8	11.2 (0 to 20.2)
	At least three improvement	10.4	23.7	13.4 (6 to 18.2)		At least three improvement	8.2	19.0	10.9 (2 to 19.4)
	At least four improvement	5.6	8.5	2.8 (-2.6 to 7.2)		At least four improvement	5.0	6.4	1.3 (-2.6 to 5)
	All five improvement	0.2	2.7	2.5 (1.9 to 4.1)		All five improvement	0.2	2.0	1.8 (0 to 3.6)
60%	mRSS	19.6	21.0	1.5 (-12.5 to 12.2)					
	HAQ-DI	13.6	19.3	5.5 (-1.5 to 14.2)					
	Patient GA	12.7	19.2	6.3 (-1.2 to 16.3)					
	Clinician GA	23.7	34.3	10.6 (3.1 to 18.7)					
	FVC%	11.1	20.4	9.5 (0.3 to 15.7)					
	At least one improvement	44.9	61.5	16.5 (6.5 to 25.1)					
	At least two improvement	19.4	25.9	6.7 (-8.0 to 15.7)					
	At least three improvement	7.0	15.3	8.4 (-2.0 to 15.5)					
	At least four improvement	5.0	5.8	0.7 (-2.6 to 5.0)					
	All five improvement	0.2	1.0	0.8 (-0.1 to 2.0)					

5% improvement is calculated for FVC% in all improvement level; Risk Difference=proportion of participants who improved in active medication group – proportion of participants who improved in placebo group.

beyond this can be considered clinically important.[18] A second option is to limit the proportion of patients who improve in the placebo group to <20% in the composite endpoint, similar to the Paulus criteria. Analysing the development and validation sets, a cut-off of 40% for at least three of five core set measures achieved a placebo effect of <20%.

Based on our current analyses and review of the published and unpublished data from recent trials (such as lenabasum phase III data), we believe that revised CRISS may provide an anchor for clinical meaningfulness and provide assurance to the clinicians and regulators that the improvement in three of five core set measures (as an example) with improvement of ≥20% is driven by more than one core set measure (and not driven by mRSS, which has the greatest weight in the ACR-CRISS). This, in turn, will improve the interpretation of the data (similar to RA RCTs). Finally, we show a high floor and ceiling effect of ACR-CRISS, and the revised CRISS has an advantage to limit the ceiling effect as different cut points can be chosen, as done in RA (such as ACR 20%, 50% and 70% response criteria).

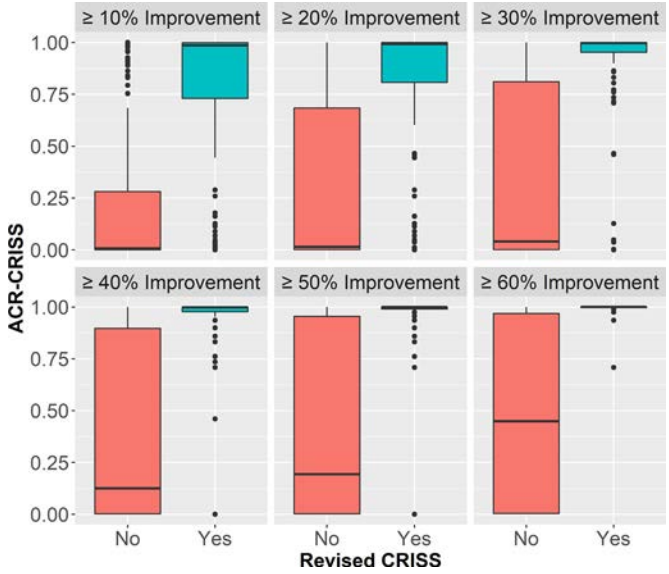

Figure 2 Concordance between American College of Rheumatology Composite Response Index in Systemic Sclerosis (ACR-CRISS) vs revised CRISS from 10% to 60%, in 10% increments.

For incorporation in an RCT, we propose that the researchers continue to include step 1 score in the assessment of revised CRISS (online supplemental figure 2). Step 1 consists of cardiopulmonary-renal involvement and consideration should be given to add significant gastrointestinal dysmotility requiring parenteral or enteral nutrition and significant digital ischaemia requiring hospitalisation, gangrene or amputation (as they are important to end-organ damage in early SSc). If a patient meets step 1, they are considered not improved and given a percentage change of 0 for each core set item and included in step 2. For the remaining patients who do not meet step 1, an appropriate cut-off should be proposed in step 2 that may range from 20% to 40% for at least three of five core set measures (as discussed previously), but should be driven by future trials, with and without background immunosuppressive therapies.

The strengths of the current analysis include careful evaluation of three RCTs with individual-level data. Second, we carefully estimated treatment differences for various definitions using separate development and validation sets using patient-level data.

The limitations of this study include the analysis of trials with negative primary endpoint of mRSS. Tocilizumab clinical trials

showed a large favourable benefit on FVC and abatacept showed statistical improvements in HAQ-DI and CGA. In addition, all three RCTS showed trends favouring other core set measures and we considered it as an appropriate database for this exercise. In addition, there was apparent heterogeneity in the RD between the development and validation sets (tables 3 and 4) that stratified sampling did not completely address; thus, our results should be validated in an independent cohort. Finally, all three RCTs were performed with no background immunosuppressive therapies, and the response may be different in those with background immunosuppressive therapies.

In conclusion, we show that the proportion of patients who achieved a predefined percentage of improvement in ≥1 core set measures was higher in active therapy versus placebo group and propose a new composite outcome measure for early dcSSc, which addresses certain limitations of ACR-CRISS score. This composite measure should be considered preliminary and rigorously tested in recently completed and ongoing clinical trials oallowing background immunosuppressive therapy to assess its performance versus ACR-CRISS.

Contributors All listed authors provided substantial contributions to the conception or design of the work; or the acquisition, analysis or interpretation of data for the work; drafted the work or revised it critically for important intellectual content; had final approval of the version to be published; and have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Competing interests DK reports grants from NIH/NIAMS, grants from Immune Tolerance Network, grants and personal fees from Bayer, grants from Bristol Myers Squibb, grants from Horizon, grants from Pfizer, personal fees from Acceleron, personal fees from Acetlion, personal fees from Amgen, personal fees from Blade Therapeutics, personal fees from Boehringer Ingelheim, personal fees from CSL Behring, personal fees from Corbus, personal fees from Cytori, personal fees from Galapagos, personal fees from Genentech/Roche, personal fees from GSK, personal fees from Horizon, personal fees from Merck, personal fees from Mitsubishi Tanabe Pharma, personal fees from Regeneron, personal fees from Sanofi-Aventis, personal fees from United Therapeutics, other from Impact PH, personal fees from Eicos Sciences, and personal fees and other from CiviBioPharma/Eicos Sciences outside the submitted work. SH has nothing to report. CJFL reports other from Genentech during the conduct of the study; other from Genentech outside the submitted work and owns stock in Roche. CS reports statistical consulting from Eicos Sciences outside the submitted work.

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TRANSLATIONAL SCIENCE

Anti-centromere antibodies target centromere–kinetochore macrocomplex: a comprehensive autoantigen profiling

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ABSTRACT

Objectives Anti-centromere antibodies (ACAs) are detected in patients with various autoimmune diseases such as Sjögren's syndrome (SS), systemic sclerosis (SSc) and primary biliary cholangitis (PBC). However, the targeted antigens of ACAs are not fully elucidated despite the accumulating understanding of the molecular structure of the centromere. The aim of this study was to comprehensively reveal the autoantigenicity of centromere proteins.

Methods A centromere antigen library including 16 principal subcomplexes composed of 41 centromere proteins was constructed. Centromere protein/complex binding beads were used to detect serum ACAs in patients with SS, SSc and PBC. ACA-secreting cells in salivary glands obtained from patients with SS were detected with green fluorescent protein-fusion centromere antigens and semiquantified with confocal microscopy.

Results A total of 241 individuals with SS, SSc or PBC and healthy controls were recruited for serum ACA profiling. A broad spectrum of serum autoantibodies was observed, and some of them had comparative frequency as anti-CENP-B antibody, which is the known major ACA. The prevalence of each antibody was shared across the three diseases. Immunostaining of SS salivary glands showed the accumulation of antibody-secreting cells (ASCs) specific for kinetochore, which is a part of the centromere, whereas little reactivity against CENP-B was seen.

Conclusions We demonstrated that serum autoantibodies target the centromere–kinetochore macrocomplex in patients with SS, SSc and PBC. The specificity of ASCs in SS salivary glands suggests kinetochore complex-driven autoantibody selection, providing insight into the underlying mechanism of ACA acquisition.

Key messages

What is already known about this subject?

- Anti-centromere antibodies (ACAs) are detected in various autoimmune diseases such as Sjögren's syndrome (SS), systemic sclerosis (SSc) and primary biliary cholangitis (PBC) and correlate with characteristic symptoms such as Raynaud's phenomenon and sclerodactyly.

What does this study add?

- Comprehensive serum ACA profiling revealed broad specificity for the centromere–kinetochore macrocomplex, and the specificity of autoantibodies was not different in patients with SS, SSc and PBC.
- Antibody-secreting cells in the salivary glands of ACA-positive SS patients were specific to the part of the centromeric structure, termed the 'kinetochore' rather than CENP-B, which is known as the major autoantigen corresponding to ACA.

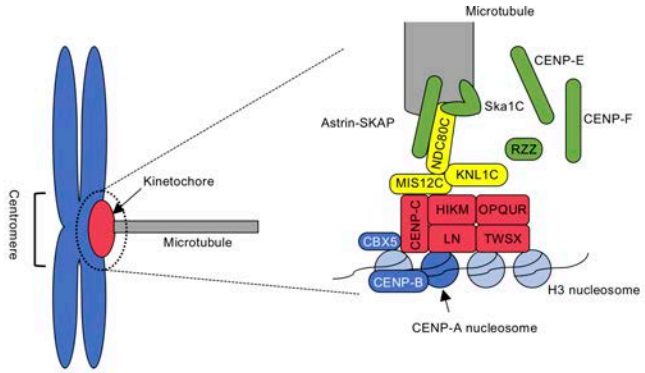
How might this impact on clinical practice or future developments?

- This study conducted a detailed analysis of the specificity of ACAs, providing further insights into pathognomonic autoantibodies common to multiple autoimmune diseases.
- The combination of multiple conformational centromere antigens could detect serum ACAs with higher sensitivity than conventional ACA detection methods.

INTRODUCTION

Anti-centromere antibodies (ACAs) are well-known autoantibodies detected in various autoimmune diseases. Although serum ACAs are frequently detected in patients with systemic sclerosis (SSc), they are also detected in other autoimmune diseases such as Sjögren's syndrome (SS) and primary biliary cholangitis (PBC), and the presence of ACAs is associated with the overlap of these three diseases.[1–3]

In the anti-nuclear antibody (ANA) test, ACAs show a characteristic staining pattern called the discrete-speckled pattern, which reflects the localisation of the centromere.[4] Recently, the molecular structure of the centromere has been rapidly clarified. Its framework structure is understood as a combination of specific centromeric chromatin, characterised by the replacement of histone H3 by CENP-A and the macromolecular complex 'kinetochore', which is assembled on the centromere-specific nucleosome.[5] The centromere binds to microtubules via inner and outer kinetochore structure during cell division. Schematic illustration of



Location	Antigen name	Component protein	Presumed function
Centromeric chromatin	CBX5*	CBX5* (also known as HP1α)	Constitute heterochromatin and may interact with MIS12.
	CENP-A*	CENP-A*	Replace histone H3 and form centromere-specific nucleosome.
	CENP-B*	CENP-B*	Bind to α satellite DNA at centromere and may function through CENP-C assembling.
Inner kinetochore; Constitutive Centromere-Associated Network (CCAN)	CENP-C*	CENP-C*	Assembled on the centromeric chromatin and serve as a platform for the outer kinetochore binding.
	CENP-HIKM	CENP-H*, -I*, -K, -M*	
	CENP-TWSX	CENP-T*, -W, -S, -X	
	CENP-OPQUR	CENP-O*, -P*, -Q*, -U, -R	
	CENP-LN	CENP-L, -N	
Outer kinetochore; KMN-network	KNL1 complex (KNL1C)	KNL1, ZWINT	Serve as the key link between the CCAN and microtubules. NDC80C bind to microtubules, MIS12C connect with CCAN, and KNL1C play an important role in mitotic checkpoint control.
	MIS12 complex (MIS12C) *	MIS12, PMF1, DSN1, NSL1	
	NDC80 complex (NDC80C)	NDC80, NUF2, SPC24, SPC25	
Outer kinetochore; other proteins	Astrin-SKAP complex	Astrin, SKAP, LC8, MYCBP	Interact with NDC80C and stabilize the kinetochore-microtubule binding.
	CENP-E*	CENP-E*	Bind to microtubules, kinetochores, and transport chromosomes along the microtubules.
	CENP-F	CENP-F	Associated in kinetochore-microtubule binding and dynein regulation.
	Rod-Zw10-Zwilch (RZZ) complex	Rod, Zw10, Zwilch	Interact with KNL1C, recruit dynein to kinetochore.
	Ska1 complex (Ska1C)	SKA1, SKA2, SKA3	Recruited by NDC80C and promote the kinetochore-microtubule binding.

Figure 1 Schematic illustration of the centromere-kinetochore-microtubule interface. CENP-A replaces histone H3 and forms centromere-specific nucleosome. CBX5 and CENP-B bind to H3 nucleosome and centromeric DNA, respectively. The kinetochore complex is constructed on the CENP-A nucleosome and interacts with microtubules. The key kinetochore subcomplexes are the constitutive centromere-associated network (CCAN; divided into CENP-C, CENP-HIKM, CENP-TWSX, CENP-LN, and CENP-OPQUR) and the KMN-network (divided into the KNL1 complex, the MIS12 complex, and the NDC80 complex). The Astrin-SKAP complex and the Ska1 complex stabilise the kinetochore-microtubule binding. CENP-E, CENP-F, and the RZZ complex associate in kinetochore-microtubule binding and chromosome transportation. *Known autoantigens in autoimmune diseases.[12 15 17]

centromere–kinetochore–microtubule interface is described in figure 1 based on the cited references.[6–11]

Among a number of component molecules, CENP-A, CENP-B, CENP-C and CBX5 are known targets of ACAs.[12 13] In particular, CENP-B is thought to be the main autoantigen because the presence of anti-CENP-B antibody is highly consistent with the ACAs detected by the ANA test.[14] Some other centromere proteins were also identified as autoantigens in the sera of ACA-positive patients: CENP-D, -E, -G, -H, -I, -J, -M, -T, -O, -P and -Q[12 15]; however, the spatial relationship of these antigens has not been taken into account, and the autoantigenicity of newly identified centromere proteins remains unclear. In addition, although

Table 1 Clinical characteristics of the patients who underwent serum analysis

Disease type	HC n=68	SS n=86	SSc n=35	PBC n=10	Overlap n=42
Female %	82	97	86	100	100
Age (y), median (IQR)	44 (31–50)	61 (47–72)	59 (50–71)	60 (56–72)	64 (55–74)
ANA discrete-speckled %	NA	10	34	30	69
Anti-CENP-B antibody positive %	1.5	10	37	30	71
Disease specific antibody positive %		Anti-SSA 83 Anti-SSB 42	Anti-Topo 1 31 Anti-RNAPIII 6	AMA 80	
Disease type/ complicated disease %		Primary SS 85 Secondary SS 15	lcSSc 71 dcSSc 29		SS+SSc 27 SS+PBC 17 SSc+PBC 29 SS +SSc+ PBC 29

ACA, anti-centromere antibody; AMA, anti-mitochondrial antibody; dcSSc, diffuse cutaneous systemic sclerosis; HC, healthy controls; lcSSc, limited cutaneous systemic sclerosis; NA, not assessed; PBC, primary biliary cholangitis; RNAPIII, RNA polymerase III; SS, Sjögren's syndrome; SSc, systemic sclerosis; Topo 1, topoisomerase 1.

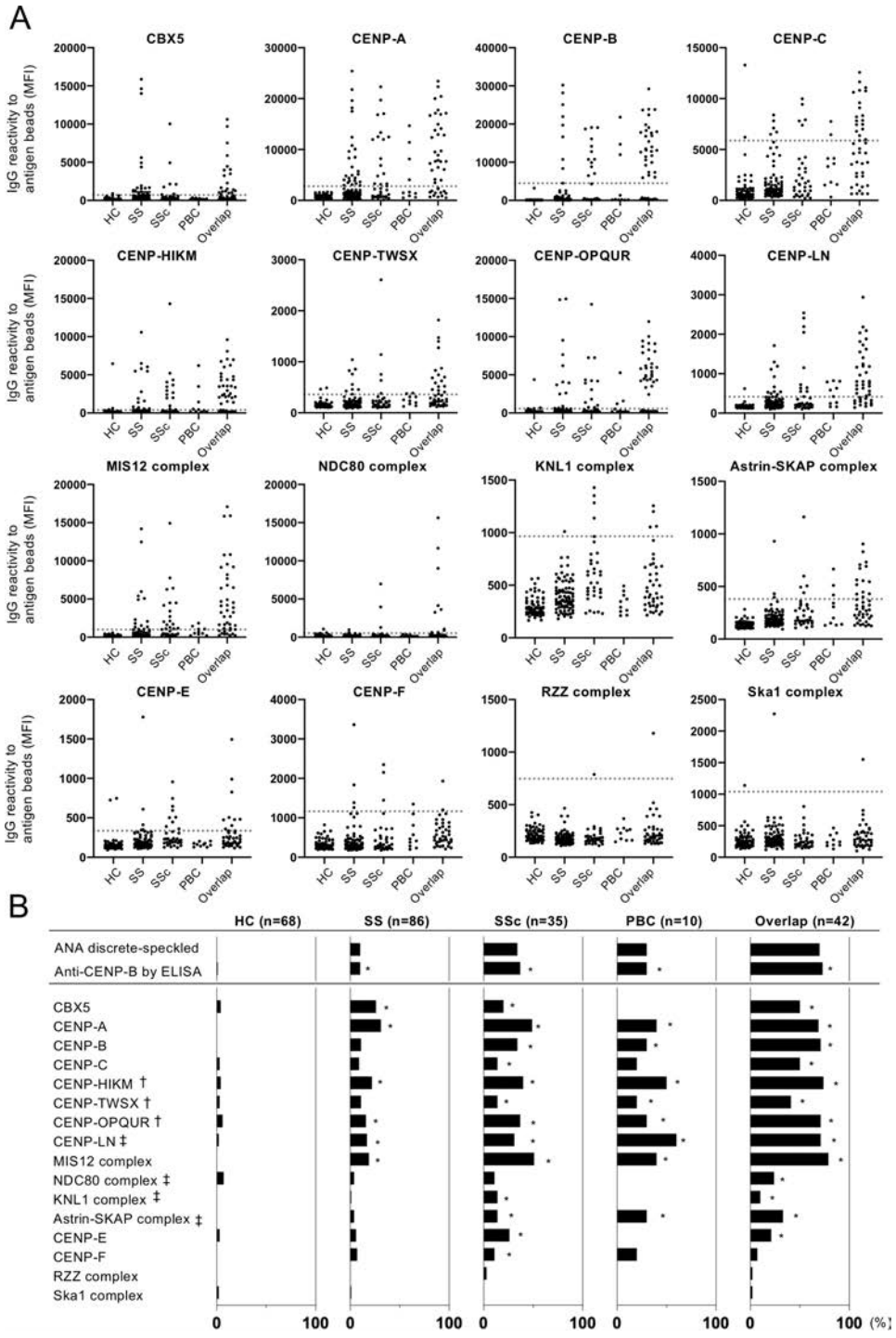


Figure 2 Profiling of serum IgG ACAs. The serum IgG autoantibody titres against each centromere antigen were analysed by the antigen-binding bead assay with the sera of patients with SS (n=86), SSc (n=35), PBC (n=10), overlap disease (n=42), and healthy controls (HC; n=68). (A) Each symbol represents the antibody level in an individual's serum and the dotted line indicates the cut-off value, which was determined by the median plus 5IQR of MFI in HC. (B) Bar graphs show the prevalence of autoantibodies against centromere antigens measured as MFI in each disease group. The prevalence of the discrete-speckled pattern by the ANA test and anti-CENP-B antibody by ELISA are shown in the top. *p<0.05 between each disease group and the HC group; †novel autoantigen as a form of complex, at least one component molecule is known as an autoantigen; ‡novel autoantigen identified in this assay. The data of CBX5, CENP-A, CENP-B, CENP-C, and the MIS12 complex were obtained from our previous study.[17] ANA, anti-nuclear antibody; ELISA, enzyme-linked immunosorbent assay; MFI, mean fluorescence intensity.

several studies have focused on the distinct epitope specificity of major antigens (ie, CENP-A, -B and -C), comparing patients with SS and SSc,[13 16] few studies have been performed on the prevalence of antibodies against other centromere proteins.

Our recent study of autoantibodies produced in salivary glands demonstrated that many autoantibodies recognise native conformational epitopes. We developed an antigen-binding bead assay by using mammalian cell line-derived proteins as antigens,

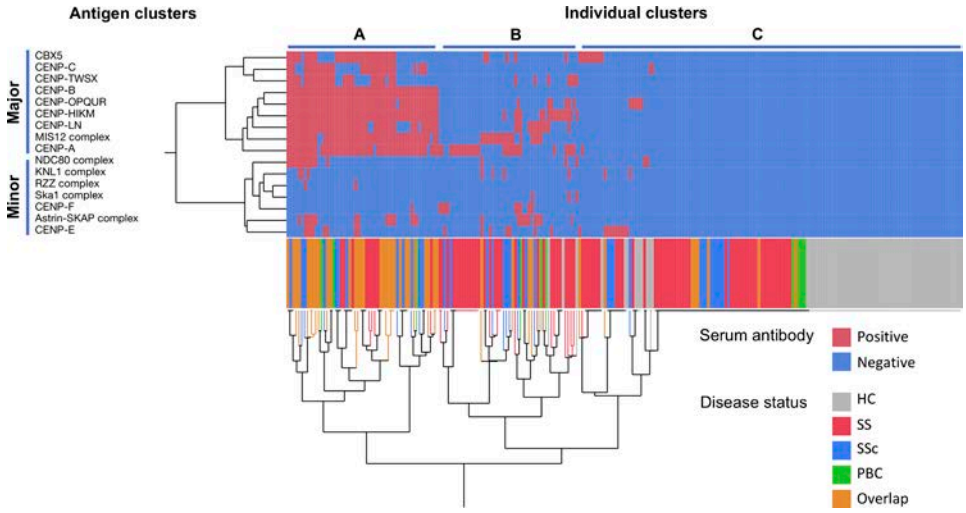


Figure 3 Clustering analysis of the centromere antigens and individuals. The sera of patients with SS (n=86), SSc (n=35), PBC (n=10), or overlap disease (n=42), and HC (n=68) were analysed by the antigen-binding bead assay. The serum antibody positivity for each target antigen (rows) in each individual (columns) is indicated by the appropriate colour. Hierarchical clustering produced a dendrogram among target antigens (left) and individuals (bottom). Coloured lines below the matrix indicate the disease status of the individuals. HC,healthy controls; PBC, primary biliary cholangitis; SS, Sjögren's syndrome;SSc, systemic sclerosis.

which provided higher sensitivity for the detection of autoantibodies than conventional ELISA.[17]

In this study, we constructed a centromere antigen library including 41 centromere proteins, which were selected based on the latest information about the centromere structure. To clarify the true target of ACAs and to identify differences by disease, we examined serum autoantibodies against this library using an antigen-binding bead assay and investigated the spatial relationship of antibody-secreting cells (ASCs) in salivary gland tissue.

METHODS

Clinical samples

Serum samples were obtained from patients with SS, SSc or PBC, and salivary gland samples were collected from patients with clinically suspected SS who underwent a lip biopsy at Keio University Hospital. The diagnosis was made according to the 2016 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for primary SS,[18] the 2013 ACR/EULAR classification criteria for SSc[19] and the clinical practice guidelines for PBC established in 2017.[20] Sera of healthy controls (HCs) were used as controls.

Preparation of the centromere antigen library

A total of 41 centromere proteins were cloned into pEFs vector or pcDNA3.4 vector as a centromere protein library, combined with the streptavidin-binding peptide tag and green fluorescent protein (GFP) at the N-terminus and expressed by 293T cells. Most of them were cotransfected to construct subcomplexes according to the molecular structure of the centromere.[8 9 21–23] Antigens were purified by streptavidin beads and electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by silver staining (Aproscience, Tokushima, Japan) and western blotting with anti-human GFP antibody (unconjugated, 1GFP63, BioLegend, California, USA) and horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (GE Healthcare, Buckinghamshire, UK) (see online supplemental figure S1). Nucleic acid of CENP-E was purchased from Kazusa DNA Research Institute (Chiba, Japan). Other detailed methods were described previously.[17]

Serum autoantibody detection by antigen-binding bead assay

The protocols for bead coupling and measurements of serum antibody titres were described previously.[17] In short, antigens expressed by 293T cells were attached to Dynabeads M-280 Streptavidin (Thermo Fisher Scientific, Massachusetts, USA). Antigen-binding beads were incubated with sera of subjects, washed and then stained with anti-human IgG-Fc antibody (APC, goat-F(ab')$_2$ fragment, Jackson ImmunoResearch, Pennsylvania, USA) and anti-human IgA-Fc antibody (DL405, goat-F(ab')$_2$ fragment, Jackson ImmunoResearch). The titres of antibodies were analysed by FACSVerse and FlowJo software (BD Biosciences, California, USA). Anti-CENP-B antibody was measured by anti-CENP-B ELISA (ORGENTEC, Mainz, Germany) according to the manufacturer's instructions.

Monoclonal antibodies against newly identified centromere autoantigens

We previously examined lesion antibody specificity by cloning antibodies from human salivary glands and analysed their reactivity to recombinant centromere antigens.[17] In this study, these cloned antibodies were comprehensively analysed with a newly developed centromere antigen library. The detailed method is described in online supplemental information.

Direct detection of antibody-producing cells in salivary glands

Fresh-frozen sections of labial salivary gland samples were incubated with GFP fusion antigens and anti-CD138 antibody and anti-mouse IgG1 antibody. ASCs were semiquantified using confocal microscopy. The detailed method is provided in online supplemental information.

Statistics

The cut-off value of a serum antibody against a specific antigen was determined by the median plus 5IQR of the mean fluorescent intensity in 68 HCs. A doubling of the difference between the third quartile and the median (Q3−Q2) was substituted for IQR when the skewness of a distribution in HCs was over

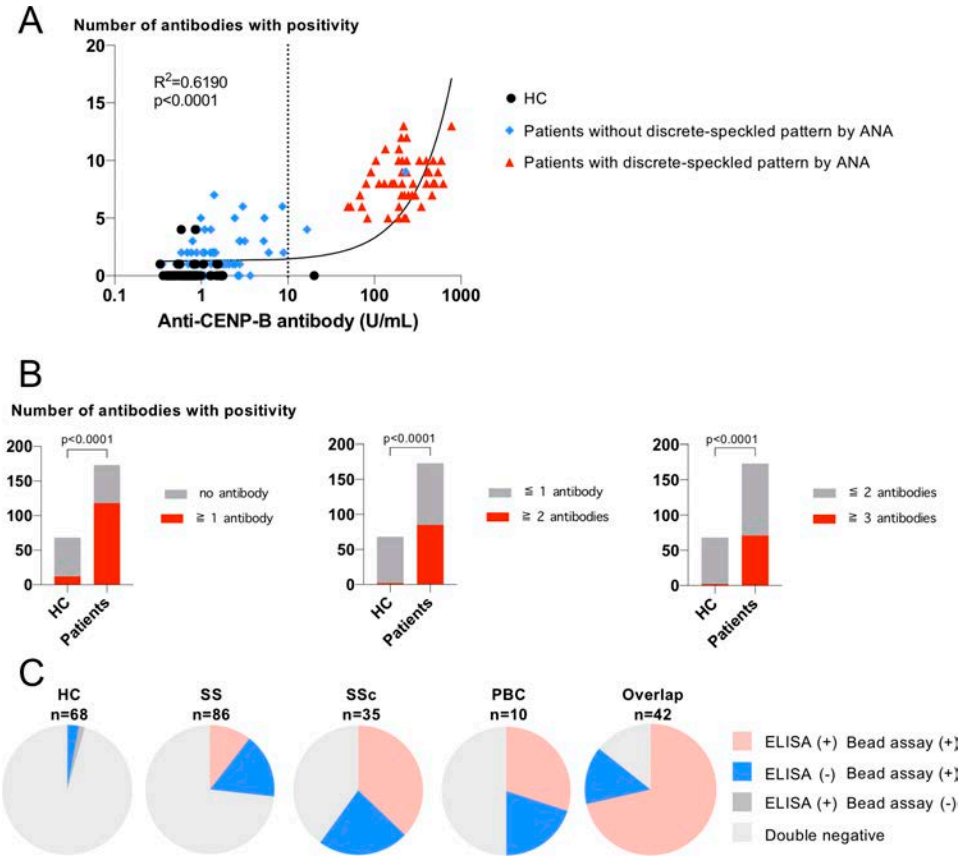


Figure 4 Comparison between the antigen-binding bead assay and conventional methods; ANA test and anti-CENP-B antibody by ELISA. (A) The numbers of antibodies with positivity in sera were plotted against the titres of anti-CENP-B antibody. Linear regression lines, the correlation coefficient, and p value of the regression line are shown. The dotted line indicates cut-off value by ELISA. (B) Number of individuals with and without bead assay positivity depending on various cut-off values. (C) Pie charts show positive rates for anti-CENP-B antibody by ELISA and the centromere antigen-binding bead assay. Having two or more antibodies against centromere antigens was considered as positive. Individuals who were additionally identified as ACA positive by the bead assay are shown by blue. ANA, anti-nuclear antibody; ELISA, enzyme-linked immunosorbent assay; HC, healthy controls.

1.[24] Exceptionally, the cut-off value of anti-CENP-B IgG antibody was determined by receiver operating characteristic analysis, in which patients with positive anti-CENP-B antibody by ELISA were defined as positive. The Wilcoxon rank sum test was applied to compare continuous variables, and two-sided Fisher's exact test was applied to compare categorical variables. P values <0.05 were considered statistically significant. Unsupervised hierarchical clustering by Ward's method and principal component analysis (PCA) were performed to analyse the serum autoantibody profile. GraphPad Prism 8 (GraphPad Software, California, USA) and JMP V.13 (SAS Institute, North Carolina, USA) were used to perform the analyses.

RESULTS

Serum anti-centromere antibody analysis

We recruited a total of 241 individuals with SS (n=86), SSc (n=35), PBC (n=10) or two or more diseases above (overlap; n=42) and HC (n=68) for serum antibody analysis. The clinical characteristics of the subjects are shown in table 1.

The IgG reactivity of each individual sera against centromere antigens is shown in figure 2A. The reactivity against CENP-HIKM, CENP-TWSX, CENP-OPQUR and CENP-LN was observed with a similar tendency to those against previously known autoantigens: CBX5, CENP-A, CENP-B, CENP-C and the MIS12 complex (MIS12C). Reactivity against the NDC80 complex (NDC80C), the KNL1 complex (KNL1C), the Astrin–SKAP complex, CENP-E and CENP-F was also seen with relatively low frequency. The Rod-Zw10-Zwilch (RZZ) complex and the Ska1 complex showed negligibly low reactivity in the sera. Although we also performed the same analysis with IgA antibodies, the titres and antibody positivity were low, and the differences between the HC group and each disease group were less apparent than those of IgG antibodies (online supplemental figure S2).

As shown in figure 2B, the positive rates of antibodies against CENP-HIKM, CENP-TWSX, CENP-OPQUR, CENP-LN, NDC80C, KNL1C and the Astrin–SKAP complex were significantly higher in at least one disease group than in the HC group. CENP-LN, NDC80C, KNL1C, and the Astrin-SKAP complex were newly identified as centromere autoantigens. In CENP-HIKM, CENP-TWSX, and CENP-OPQUR, at least one component molecule was previously reported as an autoantigen; however, this is the first study to examine in complex form.

Clustering of autoantigens and individuals

Next, we performed clustering analysis to visualise the serum autoantibody profile of each individual and analysed the correlations among antigens. Unsupervised hierarchical clustering identified two antigen clusters (figure 3). We refer to the first cluster, including CBX5, CENP-A, CENP-B, CENP-C, CENP-HIKM, CENP-TWSX, CENP-OPQUR, CENP-LN, and MIS12C, as the 'major antigen' cluster and the second cluster, including the

Table 2 Clinical characteristics and antibody specificity of lesion antibody-secreting cells in salivary glands

Patient ID	S3	S10	LB32	LB73	LB90	LB117	LB101	LB93	LB17	LB25	LB46	LB19	LB23	LB47	LB48
Age, sex	51, F	71, F	31, F	29, F	70, F	70, F	65, F	65, F	47, F	86, F	50, F	54, F	60, F	38, F	47, F
Disease	sSS, PBC, MCTD	pSS	pSS	sSS, SSc	pSS	pSS	sSS, PMR	pSS	pSS	pSS	pSS	pSS	pSS	nonSS	nonSS
Anti-CENP-B antibody	+	+	+	+	+	+	+	+	NA	NA	NA	NA	–	–	NA
ANA discrete-speckled	- *	+	+	+	+	+	+	+	–	–	–	–	–	–	–
ANA	>2560 Sp+C	640 H+D	640 H+D+N	2560 D	320 H+D	160 D	320 D	160 D	40 Sp	80 H+Sp	80 Sp	<40	<40	40 H+Sp	80 H+Sp
Anti-SSA antibody	+	+	–	–	–	+	–	–	+	+	+	–	–	–	–
Anti-SSB antibody	+	–	–	–	–	–	–	–	+	+	–	–	–	–	–
Rheumatoid factor	+	+	–	+	NA	+	–	–	–	–	+	+	–	–	–
Greenspan grade	3	3	4	4	4	4	4	1	3	4	4	3	4	2	1
Medication	+ †	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Extraglandular symptom	PH	–	Erythema	–	–	–	–	–	–	–	–	–	–	–	–
Antibody-secreting cell															
CBX5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CENP-A	±	±	–	–	±	±	–	–	–	–	–	–	–	–	–
CENP-B	–	±	–	±	–	±	–	–	–	–	–	–	–	–	–
CENP-C	+++	+++	++	+++	++	+	–	–	–	–	–	–	–	–	–
CENP-HIKM	+++	–	++	±	+++	+++	–	–	–	–	–	–	–	–	–
CENP-TWSX	–	–	–	–	–	±	–	–	±	±	–	–	–	–	–
CENP-OPQUR	++	–	–	–	–	+	–	–	–	–	–	–	–	–	–
CENP-LN	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
MIS12 complex	+++	++	+++	+++	+++	+++	–	–	–	–	–	–	–	–	–
NDC80 complex	+	+	–	++	–	–	–	–	–	–	–	–	–	–	–
KNL1 complex	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Astrin-SKAP complex	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CENP-E	–	–	–	–	–	±	–	–	–	–	–	–	–	–	–
CENP-F	–	–	–	±	–	–	–	–	–	–	–	–	–	–	–
RZZ complex	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ska1 complex	–	–	–	±	–	–	–	–	–	–	–	–	–	–	–

Slides were examined at a magnification of ×200. –, undetectable; ±, one cell in multiple fields; +, 1–3 cells in one field; ++, 4–8 cells in one field; +++,>8 cells in one field
The patient IDs correspond to those in our previous study.[17]
*ACA converted to positive after 1 year of immunosuppressive therapy.
†Treated with prednisolone 10 mg/day.
C, cytosol; D, discrete-speckled; F, female; H, homogeneous; LB, lip biopsy; MCTD, mixed connective tissue disease; N, nucleolar; NA, not assessed; PH, pulmonary hypertension; PMR, polymyalgia rheumatica; pSS, primary Sjögren's syndrome; RZZ, Rod-Zw10-Zwilch; S, salivary gland; Sp, speckled; SS, Sjögren's syndrome; ; sSS, secondary Sjögren's syndrome.

others, as the 'minor antigen' cluster. In the major autoantigen cluster, the titre of each antibody was mutually correlated in addition to the prevalence (online supplemental figure S3A).

When we focused on the disease status, the participants seemed to be classified into three groups. A total of 54 individuals in cluster A showed a broad spectrum of autoantibodies against the major antigens. For the remaining participants, cluster B, with 50 individuals, had 1–7 antibodies against major or minor antigens, whereas cluster C, with 137 individuals, had few or none. Although patients with overlapping diseases tended to be classified as cluster A and most HCs were in cluster C, the result of clustering based on the antibody profile was not consistent with disease specificity. We further performed PCA of serum IgG antibody reactivity and revealed overlapping 95% confidence ellipses across patients in the SS, SSc, PBC and overlap groups, suggesting similar patterns of antibody specificity in these diseases (online supplemental figure S3B). These results indicated that the specificity of ACAs is generally shared across disease phenotypes.

Antigen bead assay identified potential ACA-positive patients

Next, we analysed the clinical significance of comprehensive bead-based autoantibody detection compared with standard ACA detection methods, ANA tests and anti-CENP-B antibodies by ELISA. As shown in figure 4A, the numbers of autoantibodies against centromere proteins were well correlated with their titres of anti-CENP-B antibody by ELISA (r^2=0.6190, p<0.0001). The presence of a discrete-speckled pattern by the ANA test was remarkably consistent with the positivity of the anti-CENP-B antibody by ELISA.

Although most HCs have no more than one antibody, some patients without ACA by standard ACA detection methods have two or more antibodies. Defining the bead assay positivity as having two or more antibodies, the bead assay clearly distinguished patients with SS, SSc or PBC with high specificity (figure 4B, online supplemental table S1). As shown in figure 4C, the bead assay identified 14%–23% of additional patients as ACA positive compared with standard methods in each disease group (15% in SS, 23% in SSc, 20% in PBC and 14% in overlap).

To confirm the clinical significance of additionally identified ACAs, we compared the characteristics of patients with or without anti-CENP-B antibody. The clinical characteristics of bead assay-positive SS patients with or without anti-CENP-B antibody by ELISA were comparable between the two groups (online supplemental table S2). For patients with SSc, the clinical characteristics differed in the two groups (online supplemental table S3). Although all anti-CENP-B antibody-positive patients presented limited cutaneous SSc, half of the bead assay-positive and ELISA-negative patients showed diffuse cutaneous SSc with anti-topoisomerase 1 antibody (ATA). The number of patients with PBC was too small to compare clinical characteristics (online supplemental table S4).

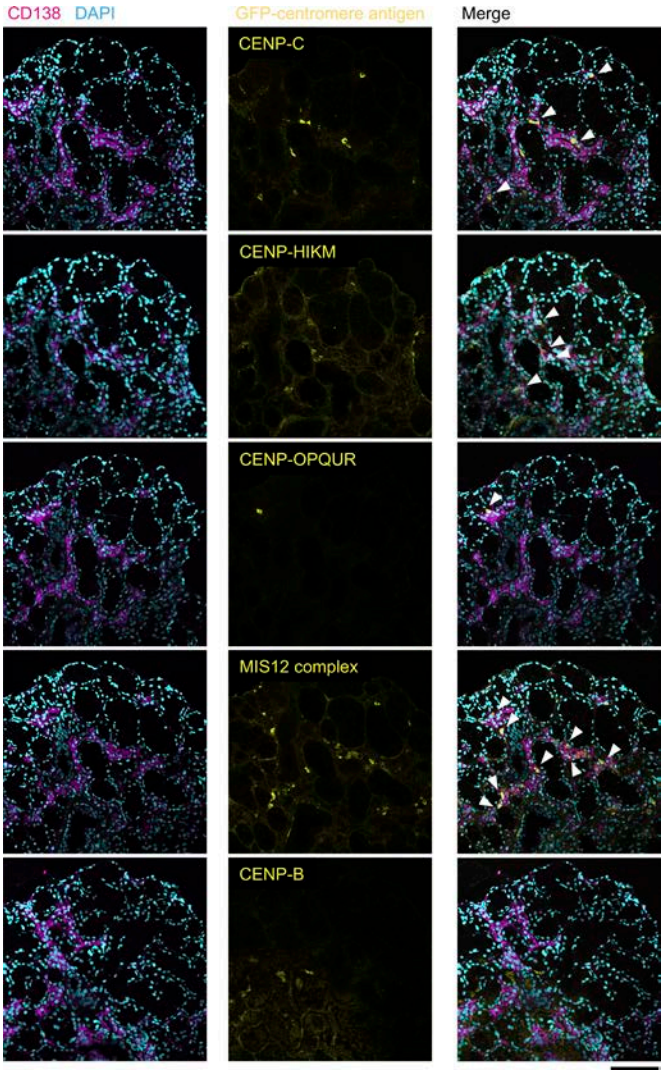


Figure 5 The distribution of ASCs against various kinetochore antigens observed in serial salivary gland sections despite a lack of ASCs against CENP-B (magnification, ×200). Serial sections of salivary gland samples obtained from a serum ACA-positive patient (LB117) were stained with GFP-centromere antigens (yellow), CD138 (magenta), and DAPI (cyan). Arrowheads indicate ASCs against centromere antigens. Antibodies against CENP-C, CENP-HIKM, CENP-OPQUR, and the MIS12 complex were secreted in the same area of salivary glands from distinct ASCs. Scale bar: 100 µm. ASCs, antibody-secreting cells; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein.

Monoclonal antibodies from SS salivary glands recognised the newly identified centromere antigens

In a previous study, we produced 256 antibodies from the ASCs of SS salivary glands, some of which were reacted against known centromere autoantigens.[17] In this study, we searched for antibodies that recognise new centromere autoantigens. The results are shown in online supplemental table S5. We identified two antibodies against CENP-HIKM, one against CENP-OPQUR and one against NDC80C in addition to the previously identified ACAs.

Antigen specificity of ASCs in salivary glands

Next, we comprehensively analysed the specificity of ASCs in target organs using a centromere antigen library. Due to limitations in sample collection, the study was limited to salivary glands in patients with SS. Fresh-frozen sections of labial salivary glands were stained with GFP-fusion centromere antigens and CD138 as a cell surface marker of ASCs. Representative images are shown in online supplemental figure S4.

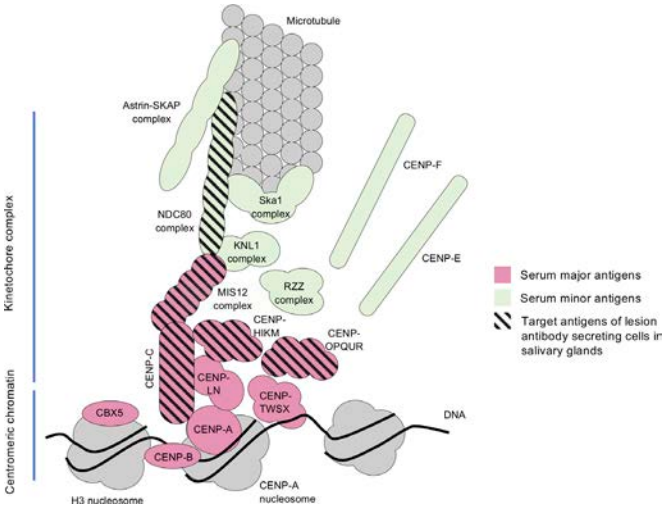


Figure 6 Schematic summary of autoantigenicity of centromere-kinetochore macrocomplex. Centromeric antigens, CCAN (CENP-C, CENP-HIKM, CENP-TWSX, CENP-LN, and CENP-OPQUR), and the MIS12 complex were the main targets of serum antibodies in patients with SS, SSc, and PBC. By contrast, antibody-secreting cells in salivary glands in patients with SS were specific to kinetochore antigens. CCAN, constitutive centromere-associated network; PBC, primary biliary cholangitis; SS, Sjögren's syndrome; SSc, systemic sclerosis.

The results are summarised in table 2. Among ACA-positive patients (n=8), 6 patients had lesion ASCs targeting centromere antigens. The most frequent target antigens were CENP-C (n=6) and MIS12C (n=6), followed by CENP-HIKM (n=4), NDC80C (n=3) and CENP-OPQUR (n=2). ASCs targeting other major antigens were scarce or not identified in salivary glands. ASCs in patients with ACA-negative SS (n=5) and patients with sicca symptoms without fulfilling SS diagnosis (n=2) showed negative results. These results were consistent with the results of the specificity of monoclonal antibodies from ACA-positive SS salivary glands.

In addition, an analysis of serial sections demonstrated the distribution of ASCs against various antigens (CENP-C, CENP-HIKM, CENP-OPQUR and MIS12C) in the same area (figure 5). This result suggested that the centromere complex, particularly the kinetochore protein complex, was processed and presented to ASCs in SS salivary glands.

Finally, we illustrated a schematic figure of the centromere–kinetochore complex and its autoantigenicity in the sera of patients with various autoimmune diseases and in SS salivary glands (figure 6). Serum antibodies target centromeric chromatin (CENP-A, CENP-B and CBX5), the constitutive centromere-associated network (CCAN; consisting of CENP-C, CENP-HIKM, CENP-TWSX, CENP-LN and CENP-OPQUR) and MIS12C, whereas target antigens of lesion ASCs were dominant in the kinetochore complex.

DISCUSSION

We provided the comprehensive mapping of ACAs targets within autoimmune diseases. We demonstrated that the CCAN was the major target of serum autoantibodies as well as the previously known autoantigens CBX5, CENP-A, CENP-B, CENP-C and MIS12C in patients with SS, SSc and PBC. These results

indicated that the centromere–kinetochore macrocomplex is the main target of serum ACAs. In addition, the autoantigenicity of centromere antigens was shared among patients with SS, SSc and PBC. With regards to the ASCs in SS salivary glands, kinetochore antigens, rather than centromeric proteins such as CENP-B, were the dominant targets of ASCs as opposed to serum ACAs.

Several studies indicated that ACA may cross-react with some non-centromere proteins.[25 26] Although we could not rule out cross-reactivity with non-centromere proteins, however, we found that when an autoantibody is acquired against at least one major centromere antigen, it is likely to be accompanied by multiple ACAs as shown in figure 3. These results could not be explained by molecular mimicry alone and suggest that ACAs would recognise the structure of the centromere complex, rather than a single epitope.

Although a previous study showed that serum ACAs had relatively low or no reactivity against centromere proteins other than CENP-A, CENP-B and CENP-C,[12] their reactivity might be underestimated due to the usage of non-mammalian cell-derived individual proteins regardless of their intermolecular association. We previously reported that using human cell-derived antigens and coexpression of the complexed proteins enables highly sensitive detection of autoantibody. Accumulating evidence about the molecular structure of the centromere[6 21] enabled us to build a centromere antigen library in which the intermolecular conformation was taken into account. In this study, we identified multiple novel targets of ACAs, such as CENP-HIKM, CENP-TWSX, CENP-OPQUR, CENP-LN, NDC80C, KNL1C and the Astrin–SKAP complex. We believe that this concept, using conformational antigens for antibody detection, could be applicable to identify novel autoantibodies in other autoimmune conditions.

Although several studies have focused on the distinct epitope specificity comparing patients with SS and SSc, demonstrating that antibodies against CBX5 and CENP-C are frequently seen in SS compared with SSc,[13 16] our present study clarified the similarity in serum autoantibodies against nine major autoantigens. Several reports demonstrated the frequent concurrence of SS/SSc, SS/PBC and SSc/PBC in the presence of ACAs.[27 28] Moreover, the presence of ACAs is associated with characteristic symptoms such as Raynaud's phenomenon, sclerodactyly and sicca syndrome regardless of whether classification criteria are fulfilled.[29 30] Taken together, these results indicate that patients with ACA-positive SS, SSc and PBC have common clinical and immunological characteristics, strengthening our idea that novel disease classification, 'ACA-related disease', could be added to the disease category.

We further demonstrated the potential for the clinical application of the assay with multiple centromere antigens. Previous studies showed that the ELISA results of the anti-CENP-B antibody highly corresponded with the discrete-speckled pattern by the ANA test[31]; hence, these principal 2 methods could only detect the common population. Our data demonstrated that 14%–23% of the patients with SS, SSc or PBC had autoantibodies against multiple centromere antigens but not CENP-B. Furthermore, these antibodies were highly specific to autoimmune diseases. Additionally identified ACA positivity might have comparable clinical significance to anti-CENP-B antibody in patients with SS because the clinical characteristics were similar regardless of the presence of anti-CENP-B antibody. In SSc patients, although the clinical characteristics of ACA-positive patients with or without anti-CENP-B antibody were different in accordance with the prevalence of ATA, this result was consistent with that of previous reports that ATA and ACA were not mutually exclusive, and the clinical manifestations of ATA and ACA double-positive patients were similar to those of ATA single-positive patients.[32 33]

ASCs in the salivary glands of ACA-positive patients with SS showed reactivity to various centromere antigens and characterised by specificity to kinetochore antigens. There is accumulating evidence of local antibody production in target organs of systemic autoimmune diseases.[34] In SS, several studies described the antigen-driven immune response and autoantibody production from B cells within salivary glands.[35 36] In our study, the observed diversity of ASCs against kinetochore proteins corroborated the presence of serum various ACAs in autoimmune diseases. Although serum autoantibodies showed similar reactivity against major autoantigens, including both centromeric chromatin and kinetochore proteins, ASCs in the salivary glands showed specificity to kinetochore proteins. In addition, immunostaining of serial sections with various kinetochore antigens showed the local accumulation of distinct anti-kinetochore ASCs. These results suggested that the kinetochore complex is presented to B cells and causes kinetochore-driven antibody selection within sialadenitis in SS.

We note that this study has several limitations. The diagnostic potential of our method should be verified in a large cohort of seronegative autoimmune disease patients because many of the patients in our cohort were already diagnosed with conventional disease-specific autoantibodies. In addition, our result might not reflect the serology in early stage of each disease because the patients, especially in SSc group, had relatively long disease duration. Moreover, the result from ASCs of SS salivary glands could show the indirect evidence of kinetochore-driven antibody selection, however, further research is needed to clarify the precise mechanism of ACA acquisition. The specificity of ASCs in affected organs other than SS salivary glands remains a challenge due to the limitation of sample collection.

In conclusion, our study presented the precise mapping of ACA targets, indicating that the centromere–kinetochore macrocomplex is the main target of serum ACAs and that patients with ACA-positive SS, SSc and PBC form distinct subgroups in terms of the similarity of antibody specificity. The acquisition of ACAs might be the result of kinetochore complex-driven antibody selection in affected organs.

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Contributors Study design: NK, MT, KS, KT and TT. Data acquisition: NK, MT, YK, HY, KI, HS, SK, HS, and KT. Data analysis and interpretation: NK, MT and KS. Manuscript drafting: NK, MT, KS and TT. All authors approved the final version of the manuscript.

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Competing interests MT, KS, and TT have applied for a patent of anti-MIS12C antibody as diagnostic marker.

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EPIDEMIOLOGICAL SCIENCE

Coronavirus disease 2019 outcomes among patients with rheumatic diseases 6 months into the pandemic

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ABSTRACT

Objective In earlier studies, patients with rheumatic and musculoskeletal disease (RMD) who got infected with COVID-19 had a higher risk of mechanical ventilation than comparators. We sought to determine COVID-19 outcomes among patients with RMD 6 months into the pandemic.

Methods We conducted a cohort study at Mass General Brigham in Boston, Massachusetts, of patients with RMD matched to up to five comparators by age, sex and COVID-19 diagnosis date (between 30 January 2020 and 16 July 2020) and followed until last encounter or 18 August 2020. COVID-19 outcomes were compared using Cox regression. Risk of mechanical ventilation was compared in an early versus a recent cohort of patients with RMD.

Results We identified 143 patients with RMD and with COVID-19 (mean age 60 years; 76% female individuals) and 688 comparators (mean age 59 years; 76% female individuals). There were no significantly higher adjusted risks of hospitalisation (HR: 0.87, 95% CI: 0.68–1.11), intensive care unit admission (HR: 1.27, 95% CI: 0.86–1.86), or mortality (HR: 1.02, 95% CI: 0.53–1.95) in patients with RMD versus comparators. There was a trend towards a higher risk of mechanical ventilation in the RMD cohort versus comparators, although not statistically significant (adjusted HR: 1.51, 95% CI: 0.93–2.44). There was a trend towards improvement in mechanical ventilation risk in the recent versus early RMD cohort (10% vs 19%, adjusted HR: 0.44, 95% CI: 0.17–1.12).

Conclusions Patients with RMD and comparators had similar risks of poor COVID-19 outcomes after adjusting for race, smoking and comorbidities. The higher risk of mechanical ventilation in the early RMD cohort was no longer detected in a recent cohort, suggesting improved management over time.

Key messages

What is already known about this subject?

- Patients with rheumatic disease and providers continue to be concerned about the risks of poor outcomes from COVID-19.
- Earlier studies observed a higher risk of mechanical ventilation in patients with rheumatic disease versus comparators early in the pandemic.

What does this study add?

- Six months into the ongoing COVID-19 pandemic, we found that patients with rheumatic disease had no significantly higher risk of hospitalisation, intensive care unit admission or death compared with those without rheumatic disease after adjusting for race, smoking and comorbidities.
- There was an association between rheumatic disease and risk of mechanical ventilation, but this was attenuated after adjusting for comorbidities.
- There was a temporal trend towards a reduction in risk of mechanical ventilation in the recent versus earlier rheumatic disease cohort.

How might this impact on clinical practice or future developments?

- These findings provide reassurance that rheumatic disease may not place patients at a higher risk of severe COVID-19 respiratory complications or death compared with the general population.
- COVID-19 outcomes may have improved over time, possibly due to improvement in management.

INTRODUCTION

COVID-19, caused by the novel SARS-CoV-2, has become an unprecedented global health crisis, with over 36 million confirmed cases and over 1 million deaths worldwide as of October 2020.[1] Especially as workplaces and schools reopen, whether patients with rheumatic disease and those on immunosuppressive medications are at a higher risk of complications of COVID-19 infection continue to be a concern to both patients and providers.[2] Several case series have suggested that patients with rheumatic disease may not be at a higher risk of severe COVID-19 outcomes,[3–5] although a comparative cohort study from Wuhan, China, reported higher rates of mechanical ventilation among patients with rheumatic disease versus comparators (38% vs 10%, p<0.001).[6] A comparative cohort study from Spain found that having a connective tissue disease was independently associated with a trend towards higher odds of severe COVID-19 (OR: 1.82, 95% CI: 1.00–3.30).[7] Lastly, disease-specific registry studies from the rheumatology and inflammatory bowel disease communities have shown a higher risk of severe COVID-19 with glucocorticoid

use, although not with biologic or targeted synthetic disease-modifying antirheumatic drugs (DMARDs).[8–10]

During the initial crisis phase of the pandemic in Boston, Massachusetts (March and April 2020), we performed a comparative cohort study that demonstrated similar odds of hospitalisation and death but threefold higher odds of mechanical ventilation among 52 patients with rheumatic disease versus 104 matched comparators without the rheumatic disease.[11] In this follow-up study, we examine COVID-19 outcomes and temporal trends in an expanded number of systematically identified patients with rheumatic disease and matched comparators 6 months into the ongoing COVID-19 pandemic.

METHODS

Study population

Mass General Brigham (MGB) is a large, multi-centre healthcare system that includes tertiary care hospitals (Massachusetts General Hospital and Brigham and Women's Hospital), community hospitals, and primary and specialty outpatient centres in the greater Boston, Massachusetts, area. We identified patients seen at MGB who were ≥18 years of age and had a positive test result for SARS-CoV-2 by PCR clinical assay between 30 January 2020 and 16 July 2020, using the MGB centralised data warehouse, Research Patient Data Registry.[12] Patients diagnosed in the outpatient setting were required to have at least one follow-up encounter following the positive SARS-CoV-2 test. This study was approved by the MGB Institutional Review Board (2020P000833). Patients were not involved in the design, conduct or reporting of this study.

Rheumatic disease case identification

From this group of patients with confirmed COVID-19, we searched the electronic health record (EHR) for International Classification of Diseases (ICD)-9 and ICD-10 codes to identify patients with a possible rheumatic disease (online supplemental table 1). Rheumatic disease diagnosis was confirmed by manual review of the EHR. Patients with only crystalline arthropathy, fibromyalgia or osteoarthritis were excluded, as these are not typically considered systemic autoimmune rheumatic diseases (or treated with systemic immunomodulators).[13] The following patients were also excluded: remote polymyalgia rheumatica (last prednisone use ≥5 years prior), antiphospholipid antibody syndrome with no prior immunosuppression, and sarcoidosis with no prior immunosuppression or with prior immunosuppression ≥5 years ago. For reference, our first study regarding COVID-19 outcomes in patients with rheumatic disease at MGB included patients with rheumatic disease identified in a similar fashion with a COVID-19 diagnosis date between 30 January 2020 and 8 April 2020.[11] Patients from the first study were also included in the current study.

Non-rheumatic disease comparator identification

Each patient with a rheumatic disease was matched to up to five comparators without a rheumatic disease ICD code from the same COVID-19-positive MGB population, based on age, sex and the index date (the date of collection of initial positive SARS-CoV-2 test ±5 days, since testing criteria changed over time). For comparators with multiple test dates, the date of the first positive result was used.

Data collection

For patients with rheumatic disease, clinical variables of interest regarding the rheumatic disease diagnosis were extracted from the EHR by manual chart review. These included the rheumatic disease diagnosis, immunomodulatory medications (including the specific dose of any glucocorticoid when applicable), rheumatic disease duration and disease activity level (based on the global assessment from the last rheumatology provider note documented in the EHR) as determined by the reviewer.

For both patients with rheumatic disease and comparators, additional variables were extracted from the COVID-19 Data Mart,[14] an EHR-based data enclave established by MGB that includes all patients who have had a lab test for SARS-CoV-2 performed. Variables extracted from the COVID-19 Data Mart included demographics (age, sex and self-identified race/ethnicity), smoking status, medical comorbidities and COVID-19 clinical outcomes (including dates of hospitalisation, intensive care admission, mechanical ventilation and death). Baseline characteristics including demographics, comorbidities, smoking history and body mass index (BMI) were assessed in the 1 year prior to the index date, and the Charlson Comorbidity Index (CCI)[15] was calculated prior to the index date.

Statistical analysis

Categorical variables are presented as number (percentage), and continuous variables are reported as mean±SD or median±IQR, as appropriate. Continuous variables were compared using a two-sample t-test for continuous normally distributed variables or Mann-Whitney U test for continuous non-normally distributed variables. Categorical variables were compared using χ^2 tests.

Baseline was the index date that the initial positive PCR for SARS-CoV-2 was obtained. Person-days (PD) of follow-up were determined for each subject from the index date to the first of any of the following events: occurrence of the outcome of interest, date of the last encounter at MGB or end of the study period (18 August 2020). We calculated incidence rates per 1000 days by dividing the number of events by the number of PD. Multivariable Cox proportional hazard regression models were used to estimate HRs and 95% CIs for the following outcomes in separate models: hospitalisation, intensive care unit admission, mechanical ventilation and death, comparing patients with rheumatic diseases to matched comparators. Covariates in the multivariable models were chosen due to known risk factors for COVID-19 or imbalance between patients with and without rheumatic disease at baseline, in addition to the matching factors of age, sex and date of the test. The first multivariable model adjusted for race and smoking. The second multivariable model adjusted for cardiovascular disease (coronary artery disease, hypertension, heart failure), chronic lung disease (obstructive sleep apnea, chronic obstructive pulmonary disease, asthma and interstitial lung disease) and body mass index. The third and final multivariable models adjusted for race, smoking and CCI (dichotomised as <2 or ≥2). For hospitalisation, intensive care unit admission and mechanical ventilation, death was treated as a competing risk using a cause-specific model yielding subdistribution HRs.[16]

To expand on our previous observations and evaluate time trends in mechanical ventilation in patients with rheumatic disease we divided our rheumatic disease cohort into early and recent cohorts (prior to and after 12 April 2020, respectively, which was the calendar midpoint of all COVID-19 diagnosis dates in the rheumatic disease cohort) and compared the risk of mechanical ventilation between the early and recent cohorts using multivariable Cox proportional hazards regression. To determine whether temporal trends in risk of mechanical

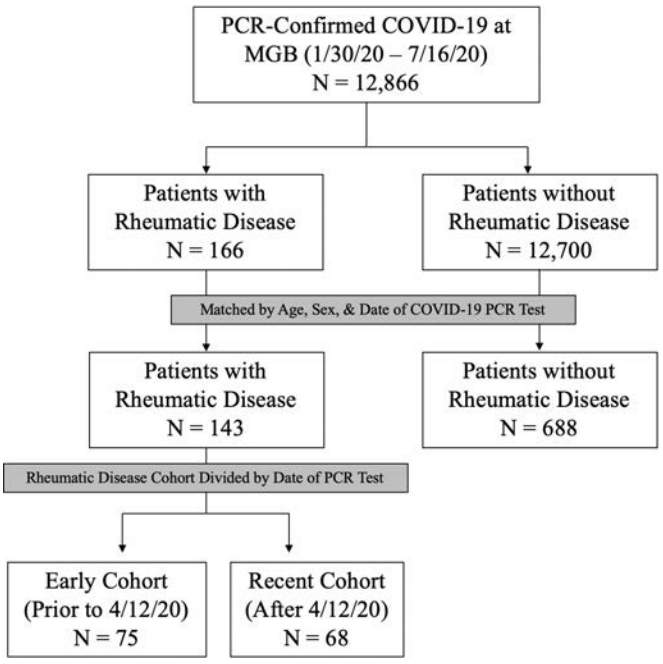

Figure 1 Flow diagram of rheumatic disease. Patients and comparators with COVID-19 infection at Mass General Brigham (MGB). MGB, Mass General Brigham.

ventilation might be related to more mild cases being diagnosed in the recent cohort, we also compared the risk of hospitalisation in the early versus recent cohorts. A similar analysis of temporal trends in mechanical ventilation was performed in the comparator cohort. The level of significance was set as a two-tailed p<0.05, and statistical analyses were completed using SAS statistical software (V.9.4; SAS Institute).

RESULTS

Study population

As of 16 July 2020, there were 12 866 patients with a positive test result for SARS-CoV-2 at MGB. Of these, 733 (6%) had a positive rheumatic disease screen by ICD code, and 143 (1%) had confirmed rheumatic disease on EHR review and were matched to 688 comparators (figure 1).

Patients with rheumatic disease and those without rheumatic disease were well matched; the mean age was 60 years in the rheumatic disease group and 59 years in the comparator group, and 76% were female individuals in each group (table 1). The distribution of race was similar between those with and without the rheumatic disease. The percent with Hispanic ethnicity was similar between groups (8% vs 12%, p=0.16). A higher proportion of patients with rheumatic disease were either former (33% vs 21%) or current (4% vs 3%) smokers (p<0.0003).

There was a higher proportion of patients in the rheumatic disease group with comorbidities including hypertension (54% vs 35%, p<0.0001), coronary artery disease (17% vs 6%, p<0.0001), interstitial lung disease (7% vs 1%, p<0.0001), heart failure (11% vs 6%, p=0.03), asthma (14% vs 8%, p=0.01), chronic obstructive pulmonary disease (COPD) (8% vs 4%, p=0.08), obstructive sleep apnoea (12% vs 5%, p=0.003) and chronic kidney disease (18% vs 8%, p=0.0001). There was a similar proportion of patients in each group with diabetes and malignancy.

Among patients with rheumatic disease, the disease distribution was broad and included rheumatoid arthritis (44; 31%), systemic lupus erythematosus (27; 19%), psoriatic arthritis (10; 7%), other inflammatory arthritis (10; 7%), polymyalgia rheumatica (8; 6%), antineutrophil cytoplasmic antibody-associated vasculitis (6; 4%) and others (table 2). The disease duration was less than 1 year in 1 (1%), 1–4 years in 27 (19%), 5–10 years in 27 (19%) and greater than 10 years in 87 patients (61%). Fifty-three patients (37%) were in remission, whereas 90 (63%) had active disease at the time of COVID-19 diagnosis. Patients with rheumatic disease were on a variety of immunomodulatory medications: 30 (21%) were on hydroxychloroquine, 41 (29%) were on biologic DMARDs, 44 (31%) were on conventional synthetic DMARDs and 4 (3%) were on targeted synthetic DMARDs. Of those on oral glucocorticoids (51; 36%), the median prednisone-equivalent dose was 5 mg/day.

Table 1 Clinical characteristics of patients with rheumatic disease with COVID-19 (N=143) and matched comparators (N=688) at the time of COVID-19 diagnosis

Characteristic	Rheumatic disease (N=143)	No rheumatic disease (N=688)	P value
Age, years (mean±SD)	60±16	59±16	0.75
Female, n (%)	108 (76)	520 (76)	1.00
Race, n (%)			0.19
White	68 (48)	342 (50)	
Black	35 (25)	117 (17)	
Asian	5 (4)	26 (4)	
Other	35 (25)	203 (30)	
Hispanic ethnicity, n (%)	11 (8)	81 (12)	0.16
Body mass index, kg/m^2 (mean±SD)	30.2±6.7	29.5±7.0	0.33
Smoking status, n (%)			0.0003
Never	75 (52)	341 (50)	
Former	47 (33)	146 (21)	
Current	5 (4)	20 (3)	
Unknown	16 (11)	181 (26)	
Comorbidities, n (%)			
Hypertension	77 (54)	241 (35)	<0.0001
Diabetes	30 (21)	123 (18)	0.38
Coronary artery disease	25 (17)	40 (6)	<0.0001
Heart failure	16 (11)	42 (6)	0.03
Asthma	20 (14)	52 (8)	0.01
Chronic obstructive pulmonary disease	11 (8)	29 (4)	0.08
Obstructive sleep apnoea	17 (12)	36 (5)	0.003
Interstitial lung disease	10 (7)	7 (1)	<0.0001
Chronic kidney disease	26 (18)	53 (8)	0.0001
Any neoplasm	41 (29)	162 (24)	0.19
Charlson comorbidity index (median, IQR)	2.0 (1.0–4.0)	0.0 (0.0–2.0)	<0.0001

COVID-19: coronavirus disease 2019.

Outcomes of COVID-19 infection in patients with rheumatic disease

In unadjusted and multivariable models, the risk of hospitalisation was similar in patients with rheumatic disease and comparators (58 (41%) vs 295 (43%), adjusted model 3, HR: 0.87, 95% CI: 0.68–1.11). The risks of intensive care unit admission (28 (20%) vs 96 (14%), adjusted model 3, HR: 1.27, 95% CI: 0.86–1.86) and death (12 (8%) vs 48 (7%), adjusted model 3, HR: 1.02, 95% CI: 0.53–1.95) were also similar in those with rheumatic diseases and comparators, respectively (table 3). The

Table 2 Details of rheumatic disease diagnosis and management at the time of COVID-19 diagnosis (N=143)

Characteristic	n (%)
Rheumatic disease diagnosis	
Rheumatoid arthritis	44 (31)
Systemic lupus erythematosus	27 (19)
Psoriatic arthritis	10 (7)
Other inflammatory arthritis	10 (7)
Polymyalgia rheumatica	8 (6)
ANCA-associated vasculitis	6 (4)
Other vasculitis	6 (4)
Axial spondyloarthritis	5 (4)
Inflammatory myositis	4 (3)
Systemic sclerosis	3 (2)
Undifferentiated connective tissue disease	3 (2)
Sarcoidosis	2 (1)
Mixed connective tissue disease	2 (1)
Juvenile idiopathic arthritis	2 (1)
Kikuchi disease	2 (1)
Giant cell arteritis	2 (1)
Antiphospholipid syndrome	1 (1)
Sjögren's syndrome	1 (1)
Multiple diagnoses*	5 (4)
Rheumatic disease duration (years)	
<1	1 (1)
1–4	27 (19)
5–10	27 (19)
>10	87 (61)
Unknown	1 (1)
Disease activity	
Active	90 (63)
Remission	53 (37)
Baseline rheumatic disease medications	
Biologic DMARDs†	41 (29)
TNF inhibitor	17 (12)
IL-6 receptor inhibitor	3 (2)
B-cell activating factor inhibitor	2 (1)
CD20 inhibitor	11 (8)
IL-17 inhibitor	3 (2)
IL-12/IL-23 inhibitor	1 (1)
CTLA-4 immunoglobulin	4 (3)
C5 inhibitor	1 (1)
Targeted synthetic DMARDs (JAK inhibitors)	4 (3)
Conventional synthetic DMARDs‡	44 (31)
Leflunomide	9 (7)
Azathioprine	6 (4)
Methotrexate	18 (13)
Mycophenolate	10 (7)
Tacrolimus	2 (1)
Sulfasalazine	1 (1)
Cyclophosphamide	1 (1)
Hydroxychloroquine	30 (21)
Oral glucocorticoid	51 (36)
Prednisone-equivalent daily dose (median, IQR, mg)	5 (5 to 10)

*'Multiple diagnoses' category includes patients with overlap features of multiple primary rheumatic diseases.
†One patient was on two biologic DMARDs (rituximab and eculizumab).
‡Three patients were on multiple conventional synthetic DMARDs.
ANCA, antineutrophil cytoplasmic antibody; C5, complement component 5; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DMARD, disease-modifying antirheumatic drug; IL, interleukin; JAK, Janus kinase; TNF, tumour necrosis factor.

Table 3 COVID-19 outcomes in patients with rheumatic disease (N=143) vs matched comparators (N=688)

	Rheumatic disease (N=143)	No rheumatic disease (N=688)
Hospitalisation, n (%)	58 (41)	295 (43)
Total follow-up time (person-days)	5847	21 671
Incidence rate/1000 days (95% CI)	9.90 (7.40–12.50)	13.60 (12.10–15.20)
Unadjusted HR (95% CI)	0.95 (0.75–1.21)	1.0 (Ref)
Adjusted model 1, HR (95% CI)*	0.89 (0.70–1.13)	1.0 (Ref)
Adjusted model 2, HR (95% CI)	0.86 (0.68–1.09)	1.0 (Ref)
Adjusted model 3, HR (95% CI)	0.87 (0.68–1.11)	1.0 (Ref)
Intensive care unit admission, n (%)	28 (20)	96 (14)
Total follow-up time (person-days)	7502	29 746
Incidence rate/1000 days (95% CI)	3.70 (2.30–5.10)	3.20 (2.60–3.90)
Unadjusted HR (95% CI)	1.38 (0.95–2.00)	1.0 (Ref)
Adjusted model 1, HR (95% CI)	1.33 (0.91–1.94)	1.0 (Ref)
Adjusted model 2, HR (95% CI)	1.22 (0.83–1.79)	1.0 (Ref)
Adjusted model 3, HR (95% CI)	1.27 (0.86–1.86)	1.0 (Ref)
Mechanical ventilation, n (%)	22 (15)	63 (9)
Total follow-up time (person-days)	7812	31 042
Incidence rate/1000 days (95% CI)	2.80 (1.60–4.00)	2.00 (1.50–2.50)
Unadjusted HR (95% CI)	1.75 (1.12–2.74)	1.0 (Ref)
Adjusted model 1, HR (95% CI)	1.72 (1.07–2.76)	1.0 (Ref)
Adjusted model 2, HR (95% CI)	1.56 (0.97–2.50)	1.0 (Ref)
Adjusted model 3, HR (95% CI)	1.51 (0.93–2.44)	1.0 (Ref)
Death, n (%)	12 (8)	48 (7)
Total follow-up time (person-days)	8790	33 428
Incidence rate/1000 days (95% CI)	1.40 (0.60–2.10)	1.40 (1.00–1.80)
Unadjusted HR (95% CI)	1.16 (0.63–2.13)	1.0 (Ref)
Adjusted model 1, HR (95% CI)	1.20 (0.62–2.33)	1.0 (Ref)
Adjusted model 2, HR (95% CI)	1.03 (0.54–1.97)	1.0 (Ref)
Adjusted model 3, HR (95% CI)	1.02 (0.53 –1.95)	1.0 (Ref)

*Model 1 adjusted for race and smoking. Model 2 adjusted for cardiovascular disease (coronary artery disease, hypertension, heart failure), chronic lung disease (obstructive sleep apnoea, chronic obstructive pulmonary disease, asthma and interstitial lung disease) and body mass index. Model 3 adjusted for race, smoking and Charlson Comorbidity Index (dichotomised as ≤2 or >2). Matching factors were age, sex and date of initial positive PCR for SARS-CoV-2.

first (adjusted for race and smoking) and second (adjusted for cardiovascular disease, chronic lung disease and BMI) models yielded similar results to the third model (adjusted for race, smoking and CCI) for the outcomes of hospitalisation, intensive care unit admission and death. In contrast, there was a higher risk of mechanical ventilation in patients with rheumatic disease versus comparators in the unadjusted model (22 (15%) vs 63 (9%), HR: 1.75, 95% CI: 1.12–2.74) and the first model, which adjusted for race and smoking (HR: 1.72, 95% CI: 1.07–2.76). However, after adjusting for comorbidities, this difference was attenuated and no longer statistically significant (adjusted model 2, HR: 1.56, 95% CI: 0.97–2.50; adjusted model 3, HR: 1.51, 95% CI: 0.93–2.44).

Among patients with rheumatic disease, there was a trend towards a lower risk of mechanical ventilation in the recent cohort compared with the early cohort (7 (10%) vs 14 (19%), unadjusted HR: 0.45, 95% CI: 0.19–1.10) (figure 2). This trend was similar after adjusting for age, sex and CCI (adjusted HR: 0.44, 95% CI: 0.17–1.12). Indeed, the risk of mechanical ventilation among patients with rheumatic disease versus comparators was significantly elevated in the early cohort (adjusted HR: 1.88, 95% CI: 1.00–3.51) but similar in the recent cohort (adjusted HR: 0.99, 95% CI: 0.40–2.46). In contrast, the risk of hospitalisation was stable in the recent and early cohorts (27 (40%) vs 28 (37%); unadjusted HR: 0.99, 95% CI: 0.63–1.59; adjusted HR: 0.94, 95% CI: 0.59–1.49) (figure 3). In the matched comparators, there was lower unadjusted risk of mechanical ventilation

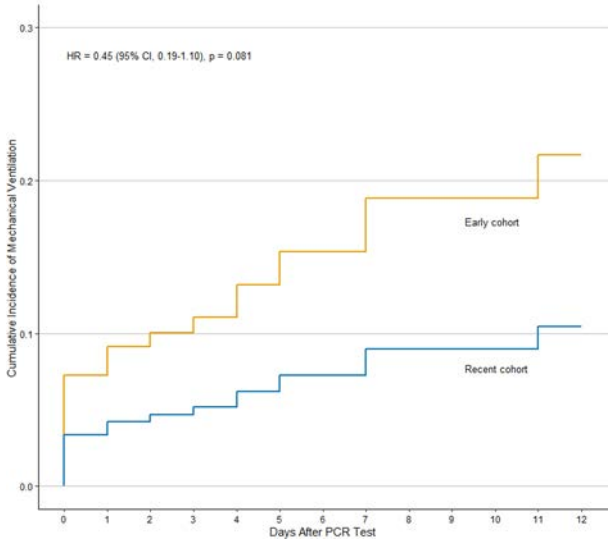

Figure 2 Cumulative incidence of mechanical ventilation in patients with COVID-19 and rheumatic disease in the recent (n=68) vs early (n=75) cohorts.

in the recent cohort compared with the early cohort (HR: 0.58, 95% CI: 0.34–0.97). After adjusting for age, sex and CCI, there was a trend towards a lower risk of mechanical ventilation although not statistically significant (adjusted HR: 0.63, 95% CI: 0.38–1.07).

DISCUSSION

In this large cohort study from a multicentre healthcare system in Boston, Massachusetts, patients with COVID-19 infection and rheumatic disease had similar risks of hospitalisation, intensive care unit admission and mortality versus matched comparators. Patients with rheumatic disease had a higher unadjusted risk of mechanical ventilation versus comparators, although after adjusting for race, smoking and comorbidities, the risk of mechanical ventilation was attenuated and no longer statistically significant. There was a trend towards a lower risk of mechanical ventilation in patients with rheumatic disease diagnosed later in the pandemic versus earlier in the pandemic. Outcomes of COVID-19 infection in patients with rheumatic disease may have improved over time due to improved COVID-19 management, less stress on the healthcare system due to capacity issues during the early surge or increased testing capacity allowing detection of milder cases. Larger cohort studies are needed to fully understand the temporal trends in COVID-19 outcomes in this population.

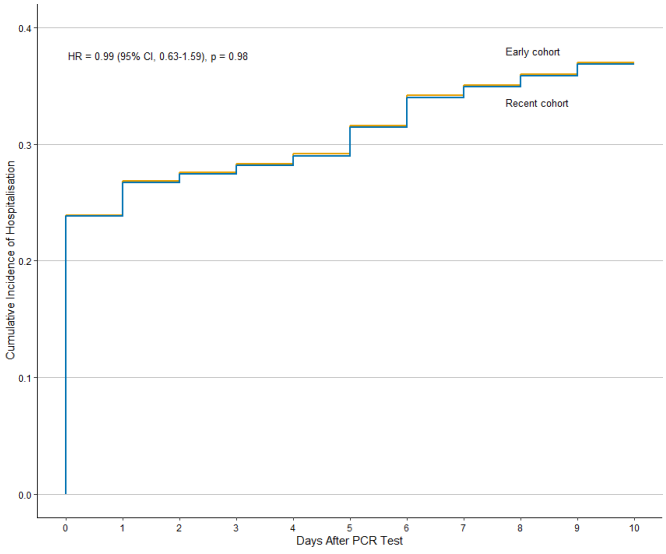

Figure 3 Cumulative incidence of hospitalisation in patients with COVID-19 and rheumatic disease in the recent (n=68) vs early (n=75) cohorts.

Prior comparative cohort studies of patients with rheumatic disease from early in the pandemic reported higher odds of mechanical ventilation in patients with rheumatic disease versus comparators,[6 11] and that having a connective tissue disease was associated with a trend towards higher odds of severe COVID-19 (intensive care unit admission, mechanical ventilation and/or death).[7] Additionally, a cohort study using an EHR database including >52 million patients across 35 healthcare organisations showed that patients with SARS had a higher risk of hospitalisation, intensive care unit admission and mechanical ventilation versus comparators matched on age, sex and race, but this study did not adjust for comorbidities and relied on different definitions for the exposure and outcome.[17] Our study extends through the first 6 months of the COVID-19 pandemic in the greater Boston area and shows no higher risks of hospitalisation, intensive care unit admission, mechanical ventilation or death in patients with rheumatic disease versus comparators after adjusting for comorbidities. Overall, the hospitalisation rate among patients with rheumatic disease in our cohort is similar to that reported in the Global Rheumatology Alliance (GRA) Physician-Reported Registry (46% in the GRA[8] vs 41% in our study), and the case fatality rate of 8% in each group is similar to the overall reported case fatality rate in Massachusetts of 7.4%.[18]

In unadjusted analyses, we observed similar results as in our prior comparative cohort study, which showed threefold higher odds of mechanical ventilation among patients with rheumatic disease versus comparators during the first 2 months of the COVID-19 pandemic in Boston.[11] Ye *et al* also found higher rates of mechanical ventilation among patients with rheumatic disease (n=21) compared with those without, but were unable to adjust for comorbidities.[6] After extending the study period to 6 months and adjusting for comorbidities, we observed no statistically significant higher risk of severe COVID-19 outcomes including mechanical ventilation, in contrast to prior studies. Our current analyses show a trend towards a lower risk of mechanical ventilation among patients with rheumatic disease in the recent cohort as opposed to the early cohort, suggesting possible improvement in COVID-19 outcomes over calendar time.

The trend in improvement in outcomes at MGB mirrors the trends in the USA, where the COVID-19 case fatality rate has improved over time.[19] This improvement in COVID-19 outcomes is likely multifactorial, including potential detection of milder cases with increased testing availability, lower volume of seriously ill patients for hospitals and providers after the initial surge of cases, or improvements in COVID-19 management over time.[20–22] Of note, the risk of hospitalisation remained stable in our rheumatic disease population during the early and recent cohorts, suggesting that the improvement in mechanical ventilation risk is not related to increased testing alone.

Our study has several strengths. As Boston became a hot spot of COVID-19 infection early in the pandemic, there were a relatively large number of confirmed cases within our multicentre healthcare system. We identified patients with

confirmed COVID-19 infection based on positive COVID-19 PCR testing, we confirmed the diagnosis of rheumatic disease by manual chart review, and we selected comparators who had never received a diagnostic code for rheumatic disease, thus reducing the risk of misclassification. The limitations of our study deserve comment. Some of the included covariates in the CCI, such as chronic kidney disease, may be causal intermediates. Collider bias may exist as the outcomes are conditioned on the diagnosis of COVID-19 and this may bias our results towards the null.[23] We were unable to capture outcomes that may have occurred outside of MGB. However, we required patients with rheumatic disease and comparators to have at least one follow-up encounter within our health-care system to reduce the risk of missed outcomes due to loss to follow-up. Our cohort was assembled from MGB, which includes two tertiary care facilities, in Boston, Massachusetts, and may not be generalisable to the entire USA. However, patients from primary care clinics and community hospitals affiliated with MGB were also included. We were unable to perform subgroup analyses by specific rheumatic diseases or medication classes such as oral glucocorticoids given small sample sizes and low event rates. It remains possible that patients with specific diseases or on specific medications may be at a higher risk of poor outcomes of COVID-19 infection. Last, given that MGB was a major site for many randomised placebo-controlled trials evaluating COVID-19 therapies, we are unable to assess the impact of study drugs such as remdesivir.

In conclusion, we found that patients with rheumatic disease had a similar risk of hospitalisation; intensive care unit admission; and death after adjusting for race, smoking and comorbidities. Although prior studies have shown a higher risk of mechanical ventilation in patients with rheumatic disease with COVID-19 versus comparators, our results show a temporal trend towards improvement in risk of mechanical ventilation in patients with rheumatic disease. These results may provide reassurance to patients with rheumatic disease and their providers during the ongoing COVID-19 pandemic. As in the general population, close monitoring of patients with rheumatic disease with risk factors such as pulmonary and cardiovascular comorbidities is warranted, as these patients may be at a higher risk of poor outcomes from COVID-19 infection.

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Contributors NS-B, KMD, JAS and ZSW designed the study, were responsible for the acquisition, analysis and interpretation of data, and drafted and revised the article. TH and RW were involved in data acquisition and revision of the manuscript. XF was involved in data analysis and interpretation and revision of the manuscript. EMG, AMJ, YZ and HC were involved in data analysis and interpretation and revision of the manuscript. All authors approved the final version of the article.

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Realising early recognition of arthritis in times of increased telemedicine: the value of patient-reported swollen joints

Early diagnosis and management of patients with inflammatory arthritis (IA) are critical to improve long-term patient outcomes. Assessment of joint swelling at joint examination is the reference of IA identification; early access clinics are constructed to promote this early recognition. Due to the COVID-19 pandemic, the face-to-face capacity of such services is severely reduced.[1] This raises the concern of a major step backward after the important progress that has been made in the past 15 years.[1] Telemedicine has recently become rapidly implemented. Although probably a valuable alternative in the management of established rheumatoid arthritis (RA), there is also the fear that this might cause delay in the speed of diagnosis.[2] A symptom that evidently raises suspicion for IA during remote evaluation is the presence of patient-reported swelling. This symptom is also included in triage tools.[3 4]

The accuracy of patient-reported swelling in comparison with joint examination has been extensively evaluated in established RA. Heterogeneous results are reported; correlation coefficients were higher when patient scored their swelling on mannequins (ρ: 0.31–0.67) than when determined with questions.[5] Hypothetically, the accuracy of patient-reported joint swelling for first recognition of IA is different than for flare detection in patients with established RA. To promote evidence-based care in the era of telemedicine, we determined the accuracy of patient-reported joint swelling for actual presence of IA in persons suspected of IA by general practitioners (GPs).

Data from two Dutch Early Arthritis Recognition Clinics were studied. These are screening clinics (1.5 lines setting) where GPs send patients in case of doubt on IA. At this clinic, patients were asked to mark the presence of swollen joints on a mannequin with 52 joints (42 joints were used for this analysis, see online supplemental text/figure S1). Subsequently, an experienced rheumatologist performed joint examination (see online supplemental text). Clinically apparent IA of ≥1 joint was the reference to calculate sensitivity, specificity, positive and negative likelihood ratios (LR+ and LR−) and positive and negative predictive value (PPV and NPV) on patient level. Pearson correlation coefficients (ρ) were determined. Predictive values depend on the prevalence of a disease in a population. Because the prevalence of IA in a 1.5 lines setting will differ from a primary care setting, post-test probabilities of IA were estimated for two lower prior-test probabilities as example, namely 20% (estimated probability in patients GPs believe IA is likely) and 2% (prior-test probability with less preselection by GPs), using likelihood ratios and nomograms (online supplemental figures S2 and S3).

A total of 1637 consecutive patients were studied. Patient characteristics are presented supplementary (online supplemental table S1). Median symptom duration was 13 weeks. Seventy-six per cent of patients marked ≥1 swollen joint at the mannequin. Forty-one per cent of patients had ≥1 swollen joint at examination by rheumatologists. ρ was 0.20 (patient level) to 0.26 (joint level).

The sensitivity of patient-reported joint swelling was high, 87%, indicating that the majority of patients with IA had marked swelling on the mannequin. However, the specificity was 31%, indicating that 69% of persons without IA had also done so (figure 1A). The LR+ was 1.25; the LR− 0.43. The PPV

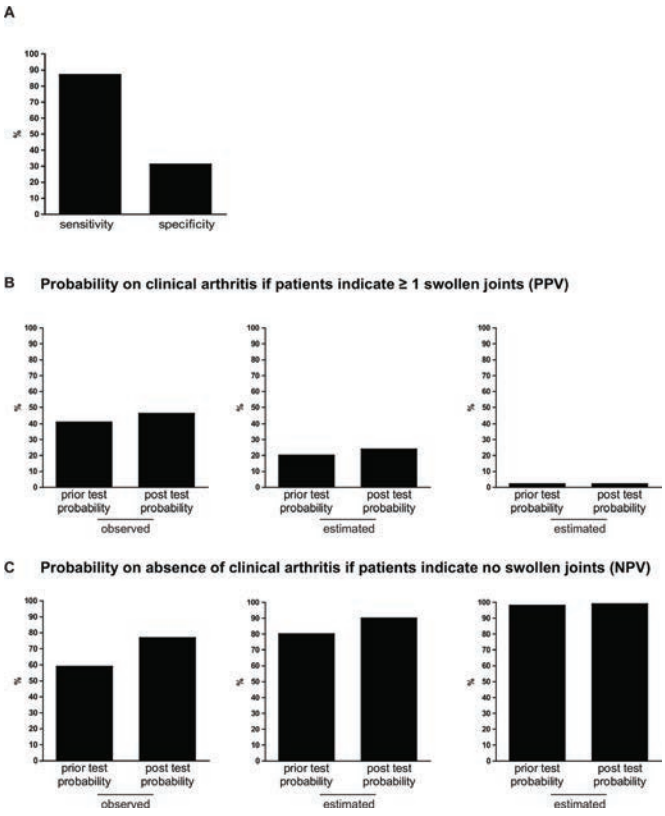

Figure 1 Test characteristics of patient-reported joint swelling (A) and predictive values (B and C), demonstrating the limited value of patient-reported joint swelling for detection of IA in three settings with different prior probabilities. (A) Sensitivity and specificity of patient-reported swollen joints with IA (joint swelling at physical examination as golden standard). (B) Prior probability on having IA of 41% (observed), 20% (estimated) and 2% (estimated) with corresponding post-test probabilities on having IA, if patients indicate to have ≥1 swollen joints (PPV). (C) Prior-test probability of not having IA 59% (observed), 80% (estimated) and 98% (estimated) with the corresponding post-test probability on not having IA, if patients indicate no swollen joints (NPV). IA, inflammatory arthritis; NPV, negative predictive value; PPV, positive predictive value.

was 46%, and the NPV was 77% (figure 1B,C). Thus, the PPV increased hardly (from 41% to 46%), and the NPV somewhat increased (from 59% to 77%). Also in settings with prior-test probabilities of 20% and 2%, estimated PPVs and NPVs hardly increased (figure 1B,C).

Thus, patient-reported joint swelling had little value in distinguishing patients with and without IA, for different prior-test probabilities. Correlations identified in this population were lower than known for established RA. When evaluating ≥1 self-reported swollen and tender joints, similar results were obtained (online supplemental table S2). Together this suggests that evaluation of patient-reported swelling is less valuable for early detection of IA than for flare detection in established RA.[5 6]

Thanks to the current pandemic, telemedicine has accelerated and will continue to grow in upcoming years.[1 2] The challenge is to continue to work in an evidence-based manner. Although inaccurate when assessed alone, patient-reported swelling may be helpful when combined with other characteristics (either clinical characteristics, such as published previously, and/or laboratory characteristics).[3 4 7 8] Other innovative tools, for example, imaging modalities that do not

require human-to-human contact, may also contribute to early identification of IA in a '1.5m society' with limited access to rheumatologists.

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What comes after the lockdown? Clustering of ANCA-associated vasculitis: single-centre observation of a spatiotemporal pattern

Antineutrophil cytoplasm antibodies (ANCA)-associated vasculitides (AAV) are characterised by a heterogeneous clinical phenotype.[1] We report a cluster of 15 patients diagnosed with AAV either de novo (n=11) or with relapsing disease (n=4) during COVID-19 pandemic between February and August 2020. During this period, we observed two major phenomena: (1) an incidence-shift with a 'COVID-gap' of no diagnosed AAV cases during the lockdown period (March and April), followed by a 'postlockdown cluster' of 14 active patients (8 myeloperoxidase-ANCA, 6 proteinase 3-ANCA vasculitis) in the subsequent 4 months and (2) an increased incidence rate (figure 1 and online supplemental figure S3). Mean creatinine at baseline was 3.66 mg/dL. Inflammatory markers were significantly elevated in most patients, with a mean C reactive protein value of 9.93 mg/dL and an erythrocyte sedimentation rate of 83 mm per first hour. Despite detrimental effects on humoral immunity,[2] most patients received two doses of rituximab and methylprednisolone. No severe treatment complications occurred. SARS-CoV-2 PCR and serology were negative in tested patients. Further clinical and radiological characteristics are provided in online supplemental tables S1 and S2).

Comparable observations were recently reported from Italy, where a cluster of nine patients with AAV was detected in the second trimester of 2020. Kidney replacement therapy was necessary in seven of nine patients and one patient died.[3] In contrast to these findings, disease courses in our cohort were comparably mild with a mean creatinine of 2.48 mg/dL at last follow-up and only one patient requiring intermittent kidney replacement therapy after spontaneous kidney bleeding from disseminated pseudoaneurysms.

The clustering of AAV in our centre may be attributable to a delayed presentation to our clinic. Containment measures may cause deferral of initial presentation after onset of symptoms by several means: (1) patients may be frightened of demanding healthcare services and (2) infrastructural cutbacks, such as restricted availability of public transports and an overall reduced access to healthcare institutions, further exacerbate this situation. Such delayed diagnoses may have significant impacts. For example, fast-track services facilitating immediate treatment have been

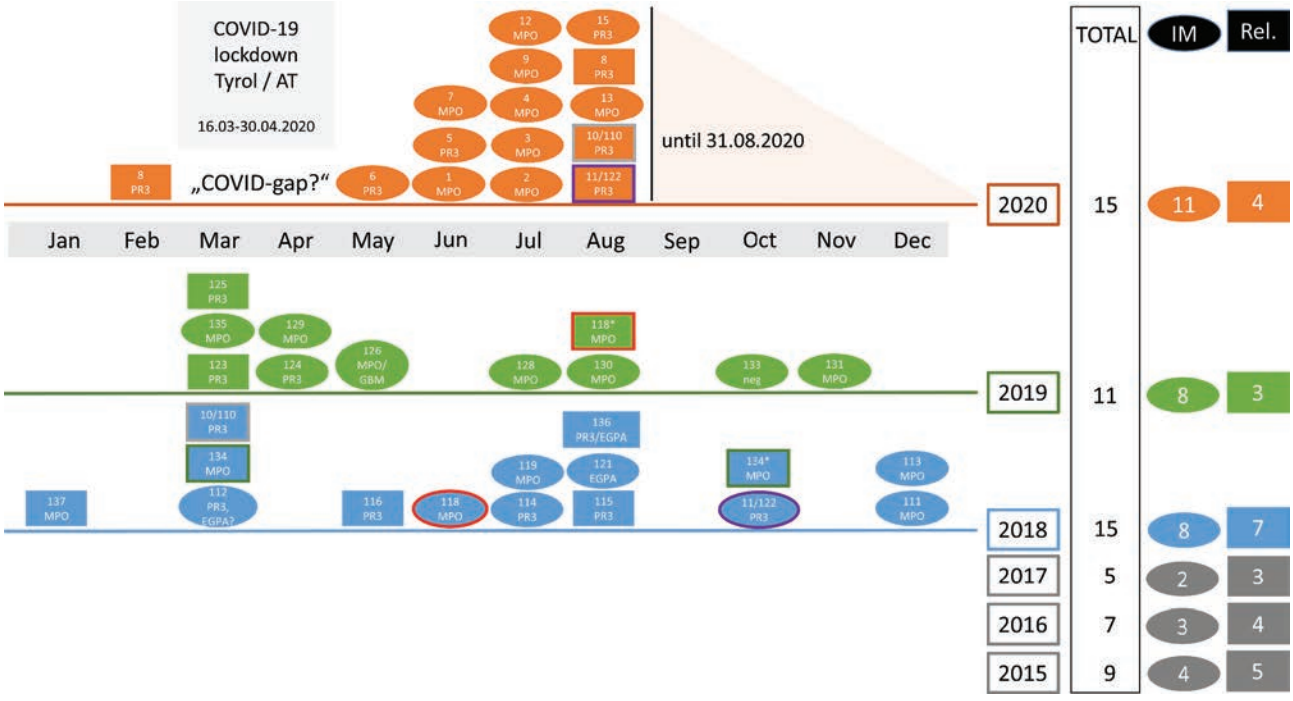

Figure 1 Timeline of incident ANCA-associates vasculitis cases in 2018, 2019 and 2020. Incident cases are posed on a timeline at the time of diagnosis of either initial manifestation (oval) or disease relapse (box). Duplicates are marked as such by a coloured frame. Numbers in boxes/ovals match with respective identity (ID) in online supplemental table S1. ANCA, antineutrophil cytoplasmic antibody; AT, Austria; IM, initial manifestation; MPO, myeloperoxidase; PR3, proteinase 3; Rel, relapse.

established for patients with giant cell arteritis who are at risk of blindness. A reduction of 75% in the request for such fast-track assessment, compared with the same time frame in 2019, was recently reported from a centre in Italy. Two cases of irreversible bilateral visual loss were attributed to a delayed diagnosis and deemed preventable.[4]

Our findings underline previous observations that the COVID-19 pandemic has significant impact on patients with diseases other than COVID-19.[5] Although definite conclusions on clinical outcomes cannot be yet drawn, our observations indicate no detrimental effects of COVID-19 on clinical outcomes of non-infected patients with AAV. Nonetheless, prompt diagnoses and referrals currently affected by the ongoing global pandemic are crucial in the disease management. Compared with the previous years, we observed an over twofold increased incidence rate of AAV diagnoses (1.9 cases per month in 2020 vs 0.8 cases from 2015 to 2019) and almost threefold increased incidence rate of de novo AAV manifestations (1.2 de novo cases per month in 2020 vs 0.4 de novo cases from 2015 to 2019). This may not only be attributed to deferral of symptoms and delayed diagnoses, as patients showed significant overall improvement following initiation of immunosuppression. Though geographical clustering of AAV may be attributed to certain environmental factors,[6] the impact of such factors on disease incidence remains elusive thus far. Finally, whether COVID-19 could be a trigger of regional clustering either directly (infection) or indirectly (effects of containment measures, eg, decreases in carbon dioxide emissions due to reduced air/ground travel or psychosocial consequences of a lockdown) is speculative and should be subjected to further investigation.

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Prevalence, admission rates and hypoxia due to COVID-19 in patients with rheumatic disorders treated with targeted synthetic or biologic disease modifying antirheumatic drugs or methotrexate: a nationwide study from Iceland

Susceptibility and tolerance to COVID-19 of patients with rheumatic disorders remains poorly understood. A recent meta-analysis did not demonstrate any considerably worse outcomes.[1] Sufferers from inflammatory rheumatic disorders are, however, known to be more prone to infections than the general population and this risk is increased by targeted biologic therapy.[2 3] Therefore, we were interested in examining the risk of admission and respiratory failure in patients with rheumatic disorders with COVID-19 being treated with targeted synthetic or biologic disease-modifying anti-rheumatic drugs (ts/bDMARDs), comparing them to matched comparators and methotrexate (MTX) users within Iceland. Unique conditions exist in Iceland for this study, as the island nation is naturally isolated and performed extensive screening, tracing and systematic registration of all PCR-confirmed cases.[4 5] All diagnosed individuals received regular follow-up by a COVID-19 outpatient clinic.[6]

ICEBIO is a nationwide registry of patients with inflammatory arthritis treated with ts/bDMARDs. We included all patients in ICEBIO undergoing treatment at the start of the domestic outbreak. From the Icelandic Medicine Database we extracted all MTX prescriptions filled in the 9 months before Iceland's first recorded case of COVID-19. Each individual from the ICEBIO and MTX groups was randomly matched with up to ten controls based on age, sex and geographic location. Individuals in ICEBIO or with MTX prescriptions from haematologists and oncologists were excluded from the MTX group, although their comparator group remained unaltered. The Icelandic Directorate of Health provided data on all PCR tests and hospital admissions in our study population. Data were extracted on 3 June 2020, when the first domestic outbreak ended: 1796 individuals had been diagnosed with COVID-19, with two active cases remaining. At that time, 61 639 tests had been administered in a nation of roughly 360 000 people, and the Directorate of Health reports a successful infection tracing rate of over 95%.[4]

We identified 1438 individuals from ICEBIO, 13 815 ICEBIO comparators, 1746 individuals with an MTX prescription and 22 962 MTX comparators, see table 1. The relative risk (RR) for the ICEBIO group to undergo testing was 1.35 (1.23–1.48; p<0.001), compared with their comparators and the RR for the MTX group at 1.05 (0.96–1.15; p=0.28) compared with theirs.

Nine from ICEBIO, eighty-four ICEBIO comparators, five MTX treated and one hundred and thirty-four MTX comparators were SARS-CoV-2 positive. All infected patients from ICEBIO had received tumour necrosis factor inhibitors (online supplemental tables S1 and S2). Two of three hospitalised patients from ICEBIO, three of three ICEBIO comparators, one of one from the MTX group and ten of thirteen hospitalised MTX comparators received oxygen supplementation. One of three admitted patients from the ICEBIO comparators and two of thirteen MTX comparators received mechanical ventilation (table 1). The RR for infected patients from ICEBIO to be admitted was 9.33 (2.20–39.6; p<0.001) and 6.22 (1.19–32.46; p=0.02) to be admitted with hypoxia. The RR for hypoxia following admission was 0.67 (0.30–1.48) for patients from ICEBIO and 0.77 (0.57–1.4) for patients taking MTX. The mean length of admission for the patient from ICEBIO was 4.7±3.6 days, while it was 20.2±12.7 days for their comparators (p=0.16). As no patients with rheumatic disorders in any group required mechanical ventilation, neither OR nor RR can be calculated for that outcome.

We found that patients with COVID-19 with rheumatologic disorders on bDMARDs are at a higher risk of hospitalisation than matched comparators. This might be explained by a lower threshold for admitting patients on biologics, as hospitalised rheumatology patients on bDMARDs fared numerically better, although the small numbers prevent meaningful statistical analysis. Further studies in larger populations are needed to better quantify the risk and severity of COVID-19 in patients with rheumatic disorders treated with bDMARDs.

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Table 1 Demographics, proportion and the number of individuals who underwent nasal swab PCR test for SARS-CoV-2; numbers of hospital admission and prevalence of COVID-19 in studied groups

	ICEBIO group	ICEBIO comparators	MTX group	MTX comparators
No.	1438	13 815	1746	22 962
Mean age ±SD	54.9±14.9	54.7±15.1	60.4±14.6	59.6±14.5
% females	59.0%	58.7%	61.9%	62.0%
No. of nasopharyngeal swaps	427 (297 per 1000 population)	3016 (218 per 1000 population)	426 (244 per 1000 population)	4565 (199 per 1000 population)
No. of tested individuals (%)	383 (26.6%)	2728 (19.7%)	385 (22.1%)	4838 (21.1%)
No. of SARS-CoV-2-positive individuals (%)	9 (0.6%) *	84 (0.6%)	5 (0.3%)	134 (0.6%)
Prevalence of COVID-19 in tested subjects	2.3%	3.1%	1.3%	2.8%
No. of hospital admissions (% of infected)	3 (33%)	3 (3.6%)	1 (20%)	13 (9.7%)
No. of admitted patients with hypoxia	2	3	1	10
No. of admitted patients intubated and on mechanical ventilators	0	1	0	2
Mean length of admission ±SD	4.7±3.6	20.2±12.7	14	10.8±7.8
Mean age of admitted patients ±SD	64.7±6.1	70±14	68	62.3±9

*All were treated with tumour necrosis factor (TNF) inhibitors (85.2% of patients in ICEBIO are on TNF inhibitors).

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Declining in-hospital mortality gap between systemic lupus erythematosus (SLE) and non-SLE hospitalisations: a national study

Mortality in systemic lupus erythematosus (SLE) is twofold to threefold higher compared with the general population.[1] Overall SLE mortality decreased over time,[1 2] but in-hospital mortality has increased.[3] We hypothesised an increase in in-hospital mortality rates in SLE and SLE–non-SLE mortality gap over time.

We included discharges from US National Inpatient Sample (NIS) from 1998 to 2014, the last year of the use of the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM). The NIS is a 20% stratified sample of hospital discharges. We identified primary SLE hospitalisations based on the presence of an ICD-9-CM code of 710.0x in the primary position; this diagnostic code has a sensitivity of 98% and specificity of 72%.[4]

We calculated the unadjusted, age-adjusted and age–sex-adjusted in-hospital mortality rates per 1000 primary SLE hospitalisations versus general non-SLE hospitalisations. We used the Cochran Armitage test to analyse mortality time trends.

The 241 130 primary SLE hospitalisations in 1998–2014 included predominantly black (34.3%), young (mean age, 36 years) and female (86.9%) patients. Deyo-Charlson Comorbidity Score was ≥2 for 47%; one-third were receiving Medicaid and 1.5% died in-hospital (table 1).

Unadjusted mortality in primary SLE hospitalisations wavered over time and decreased significantly from 17.9 per 1000 in 1998 to 9.5 in 2014, versus 28.1 per 1000 to 21.2 in non-SLE (45.2% vs 25.9% reduction; p<0.01 for both; figure 1); SLE hospitalisations occured in much younger people than non-SLE hospitalisations. Age, sex, race/ethnicity did not change over time, but Deyo-Charlson Comorbidity Score increased in 2009–2014 (online supplemental appendix 1).

Age- and sex-adjusted mortality gap by SLE also decreased over time and the mortality curves overlapped in 2014: primary SLE hospitalisations, 17.6 per 1000 claims in 1998 to 13.8 in 2014 *versus* 15.1 per 1000 claims in 1998 to 13.2 in 2014 in non-SLE hospitalisations (21.5% vs 12.5% reduction, respectively; p<0.01 for both; figure 1; online supplemental appendix 2). Annual rates of primary SLE hospitalisations (i.e., 0.04% of all NIS claims) and total NIS hospitalisations remained fairly constant from 1998 to 2014 (online supplemental appendix 3; SLE hospitalisation rates were 40.3 per 100 000 NIS claims in 1998–2000 vs 34.9 in 2013–2014). The age- and sex-adjusted mortality rates in hospitalised people in SLE increased for infections as secondary diagnoses and decreased for non-infectious causes over time (online supplemental appendix 4). In-hospital mortality reduction over time in SLE for Caucasians versus non-Caucasians was: (1) unadjusted, 32% vs 40%; and (2) age–sex-adjusted, 24% vs 3%, respectively (online supplemental appendix 5).

Age–sex-adjusted in-hospital mortality decreased more rapidly in SLE versus non-SLE hospitalisations from 1998 to 2014, and the mortality reduction was greater for Caucasians compared to non-Caucasians in SLE hospitalisations. In-hospital mortality is likely a small proportion of all-cause mortality. The SLE to non-SLE mortality rate ratio was 1.17 in 1998 and 1.04 in 2014, validating previous findings[2 5] and extending to current. The in-hospital SLE mortality decreased from 1998 to 2014, likely due to an earlier diagnosis of SLE and comorbid cardiac disease, and more frequent use of immunosuppressives (and biologics)[1 6] over time. Our in-hospital SLE versus non-SLE mortality gap was narrower than all-cause mortality ratio reduction from 13.5 in 1970s to 2.2 recently.[1] A further reduction in the twofold to threefold higher all-cause mortality in SLE versus the general population[5] is now needed.

Study strengths were the use of a national representative database, a long study period and a large sample size. We used data to 2014, since ICD-9 changed to ICD-10 in 2015 in the USA.

Study limitations included misclassification bias due to the use of diagnostic code for SLE (over-reporting and under-reporting), the lack of medication and laboratory data and the lack of out-of-hospital mortality data in the NIS.

Table 1 Characteristics of hospitalisations in people with SLE in the USA from 1998 to 2014*

	People with SLE; n (%), unless specified otherwise
Age in years, mean (SE)†; median	36.15 (0.18); 33.8
Age category, years	
<50	192 251 (79.76)
50–<65	34 660 (14.38)
65–<80	11 673 (4.84)
≥80	2464 (1.02)
Sex	
Male	31 477 (13.06)
Female	209 458 (86.94)
Race	
White	61 201 (25.38)
Black	82 667 (34.29)
Hispanic	37 979 (15.75)
Other/missing	59 266 (24.58)
Deyo-Charlson Index Score	
0	0 (0.0)
1	128 510 (53.29)
≥2	112 620 (46.71)
Income category	
0–25th percentile	66 760 (28.43)
25–50th percentile	58 832 (25.05)
50–75th percentile	53 690 (22.86)
75–100th percentile	55 556 (23.66)
Insurance	
Medicare	51 983 (21.61)
Medicaid	68 681 (28.55)
Private	91 736 (38.14)
Self	16 343 (6.79)
Other	11 803 (4.91)
Hospital bed size	
Small	22 226 (9.25)
Medium	54 693 (22.76)
Large	163 345 (67.99)
Hospital region	
Northeast	48 566 (20.14)
Midwest	42 510 (17.63)
South	100 208 (41.56)
West	49 846 (20.67)
Hospital location/teaching	
Rural	13 447 (5.60)
Urban	66 476 (27.67)
Urban teaching	160 342 (66.74)
Died during hospitalisation	3559 (1.48)

*We used US National Inpatient Sample (NIS) trend weights to allow analyses across multiple years accounting for sampling redesign in 2012.
†All numbers are national estimates.
SLE, systemic lupus erythematosus.

In conclusion, we found the SLE–non SLE in-hospital mortality gap decreased from 1998 to 2014. Future interventions should be targeted at the all-cause mortality gap between SLE and the general populations, which is still significant.

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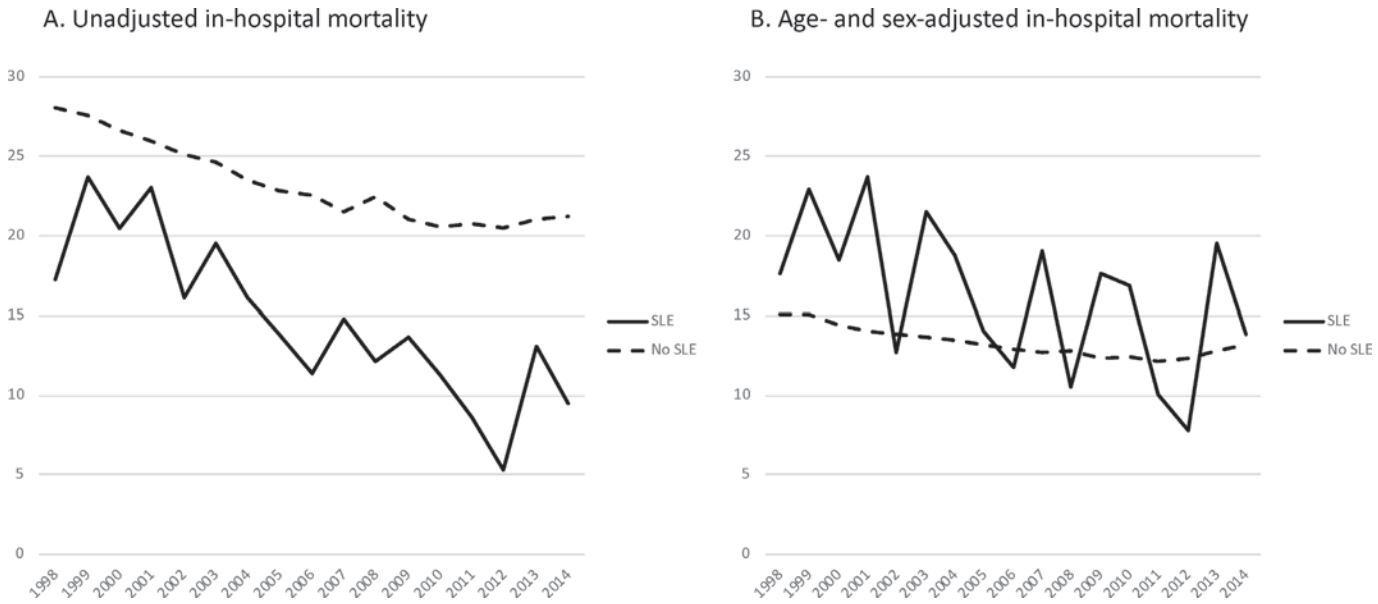

Figure 1 Time trends in unadjusted (A) and age- and sex-adjusted (B) in-hospital mortality rates per 1000 population of hospitalisations in people with systemic lupus erythematosus (SLE) compared with people without SLE. X-axis shows the calendar year. Y-axis shows the mortality rate per 1000 hospitalisations with the respective denominators of primary SLE diagnosis hospitalisations for SLE and all other hospitalisations for non-SLE in the general US population. The solid line shows mortality in people with SLE, and the dashed line shows the mortality in the general population.

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The virtual fishbowl: bringing back dynamic debates to medical conferences

'A good discussion increases the dimension of everyone who takes part'—Randolph Bourne (American writer, 1886–1918).

COVID-19 is changing clinical routines of healthcare providers worldwide. This also affects communication and knowledge exchange. Webinars and virtual conferences have become the 'new normal'.[1] Although digital conferences enable the transfer of research results and medical knowledge, opportunities for participation and discussion are very limited.[2] Dynamic debates and panel discussions have become scarce during COVID-19 lockdown.

Recently, the fishbowl technique has been successfully implemented in rheumatology conferences.[3] It is a validated method, fostering open group discussions and engagement of all audience members.[3 4] By including an expert panel (inner circle) and one empty chair for an alternating audience member, the technique promotes a dynamic and direct exchange with the audience.

To determine whether the fishbowl technique is also feasible virtually, it was piloted at the first virtual congress of the German Society for Rheumatology 2020.

A virtual fishbowl (figure 1) was conducted with the theme 'How does the internet affect the doctor–patient relationship?' using a Zoom-based software. The inner circle of the fishbowl discussion consisted of five experts and a free slot, representing the empty chair for participants from the audience (outer circle) to join the discussion. Participants could join at any time, by registering in the chat. Once it was their turn, their camera and microphone were unlocked by tech support. After each statement, the participants had to leave the inner circle of the discussion again for new participants. If they wished to provide a further comment, they had to reregister.

The virtual fishbowl discussion was evaluated based on attendance and verbal participation, as well as an online polling at the end of the event. The polling questions were adopted from Mucke *et al*[3] to create comparability between face-to-face and virtual fishbowl.

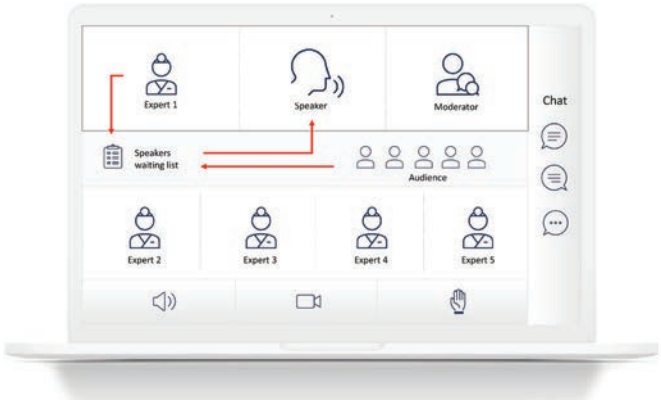

Figure 1 Set up of a virtual fishbowl discussion.

Table 1 Evaluation of the fishbowl—polling results

'Have you participated in a fishbowl discussion before?'	n=30*
Yes	8 (26.6%)
No	22 (73.3%)
'Would you recommend the virtual fishbowl technique for medical conferences?'	n=22*
Yes	21 (95.5%)
No	1 (4.5%)
'Does the virtual fishbowl technique allow the audience to continuously participate in the discussion?'	n=31*
Yes	29 (93.5%)
No	2 (6.5%)
'Compared with other discussion methods, was the virtual fishbowl discussion more multifaceted?'	n=49*
Yes	39 (79.6%)
No	10 (21.3%)

*The polling questions were displayed individually in different lengths at the end of the event. Hence n is rather low and differs by question.

A total of 476 delegates attended the 90-minute session. The actual discussion lasted 56 min. In total, there were 19 content contributions, of which 7 contributions came from audience members (37%). The polling results (table 1) reflect a generally positive impression of the participants towards the fishbowl discussion. This positive assessment is in line with the previous positive face-to-face fishbowl evaluation,[3] highlighting the future potential of this virtual approach. However, due to short response periods only a limited and varying number of delegates answered the polling questions.

Yet, there are also certain limitations and pitfalls to the technique. The virtual fishbowl requires teamwork on both sides (experts and auditorium), as the processes are prone to technological disruption. Accordingly, experienced tech support and a moderator are crucial. The fishbowl resembles a black box, as the direction of the discussion stays unpredictable. This makes it a double-edged sword. During COVID-19 times, we experienced this discussion format as a refreshing relief from strictly scheduled prerecorded presentations.

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COVID-19 in patients with rheumatological diseases treated with anti-TNF

We have read with great interest the recent article from Silva *et al* about the clinical course of COVID-19 in rheumatic disease.[1] In this matched cohort study of patients with COVID-19 infection, although the authors found a similar proportion of symptoms, risk of hospitalisation and mortality between patients with and without rheumatic disease, there was a threefold higher odds of intensive care admission/mechanical ventilation in the former. The authors considered that certain immunosuppressive medications could explain the higher risk of respiratory complications. However, the risk associated with severe infections differs among immunosuppressive medications; therefore, the analysis of clinical disclosures must be individualised according to therapeutic class.[2–4] In the study by Silva *et al*, there was no detailed comparison of the clinical behaviour of patients using different immunosuppressive medications. There is a record of corticosteroid use in 37 of 52 patients, probably combined with the use of other immunosuppressive medications.[1] The use of corticosteroids in patients with rheumatological disease has been associated with a higher risk of infections for different aetiologic agents, including respiratory infection.[2] Studies in patients infected with coronavirus and influenza virus treated with corticosteroids show a higher risk of complications and deaths.[5]

In the study, the second most common group of drugs used by the patients was biological disease-modifying antirheumatic drugs, with 60% of the patients using this therapy, and among them, a tumour necrosis factor (TNF) inhibitor was the most used. Patients with rheumatological diseases using immunosuppressive drugs, including biological therapy, have been considered to potentially be an at-risk group for COVID-19 infection and for complications.[6] Some medical specialty societies have recommended postponing the start or extending the use of biological therapy, including anti-TNF treatment, in areas of sustained community circulation of COVID-19, though the use of interleukin 6 (IL-6) inhibitors is considered safer.[7 8]

Recently, there have been case reports of patients infected with COVID-19 who were using TNF inhibitors and experienced no respiratory complications or death.[9–11] In the clinical practice of this group, we reported three patients with rheumatological diseases using anti-TNF who were infected with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). One of the patients had been diagnosed with Behçet's disease 8 years prior, with a history of several manifestations of vasculitis, including multiple painful and recurrent oral ulcers, recurrent abdominal pain and distension, peripheral venous thrombotic phenomena, neurological manifestations and human leukocyte antigen (HLA)-B51 positivity. Past use of azathioprine 100 mg/day, oral anticoagulant and mycophenolate sodium was recorded. Treatment with infliximab started 9 months prior due to a neurological condition. The second patient had ankylosing spondylitis (AS) and had used golimumab. The third patient had rheumatoid arthritis for 12 years and had used infliximab for 4 years.

The patients had a mild form of COVID-19, not presenting with dyspnoea and not requiring hospitalisation; outpatient follow-up was sufficient. They were treated only with symptomatic medication (paracetamol). None of the three patients used antivirals or hydroxychloroquine, and only the patient with AS was prescribed azithromycin. All had taken regular doses of anti-TNF before the COVID-19 infection, and the patient with Behçet's disease used it 1 day before the onset of symptoms.

Table 1 Demographic data, clinical characteristics and treatment of the patients with confirmed and clinical COVID-19

	Patient 1	Patient 2	Patient 3
Diagnosis	Behçet's disease	Rheumatoid arthritis	Ankylosin spondylitis
Age, years	40	60	65
Sex	F	M	F
Comorbidities	–	Hypertension	Hypertension Hashimoto's disease
Disease status at last visit	Remission	Remission	Remission
Diagnosis of the disease in years	8	12	4
Use of corticosteroids	No	No	No
Biological therapy: anti-TNF	Infliximab	Infliximab	Golimumab
Date of the last infusion of anti-TNF	03/16/2020	03/24/2020	03/31/2020
Symptom onset date—COVID-19	03/17/2020	04/31/2020	04/17/2020
Time interval between infusion and symptom onset in days	1	38	17
RT-PCR COVID-19 (data)	03/24/2020	05/11/2020	04/22/2020
Therapy instituted during treatment	Symptomatic medications	Azithromycin	Symptomatic medications
Symptoms (duration of symptoms in days)			
Fever	No	Yes (1)	No
Maximum temperature	37.2	38.5	36.5
Non-productive cough	No	Yes (4)	Yes (10)
Sputum production	No	No	No
Rhinorrhea	Yes (1)	No	No
Nasal congestion	Yes (1)	Yes (2)	Yes (8)
Sore throat	No	No	Yes (2)
Anorexia	Yes (2)	No	No
Fatigue	Yes (4)	No	Yes (12)
Myalgia	No	Yes (2)	Yes (5)
Arthralgia	No	No	No
Anosmia	Yes (9)	No	Yes (7)
Dysgeusia	Yes (4)	No	Yes (8)
Headache	Yes (2)	Yes (2)	Yes (2)
Diarrhoea	No	No	Yes (2)
Nausea	Yes (3)	No	No
Vomiting	No	No	No
Chest X-ray or CT scan	Not done	Not done	Not done

Twenty-one days after overcoming the resolution of symptoms, they were allowed to continue the anti-TNF treatment (table 1).

Since anti-TNF has been associated with an increased risk of infections, often severe, patients using anti-TNF have been considered a high-risk group for COVID-19 infection.[7 8] Despite the increased risk associated with anti-TNF, infections are selective, likely involving some types of viral intracellular pathogens (hepatitis B, varicella zoster, human polyomavirus JC virus) and bacteria (*Listeria monocytogenes* or *Salmonella* spp), especially granulomatous infections such as tuberculosis, which mechanism for combating infection is partially dependent on TNF, with no evidence at the moment of risk for infection by a coronavirus, including SARS-CoV-2.[4]

A cytokine storm has been associated with the immunopathogenesis of COVID-19 infection, including the participation of TNF, which has pro-inflammatory activities that can lead to extensive tissue damage, including pulmonary injury and shock by vascular leakage.[12 13] In vitro studies have shown that TNF

facilitates the SARS-CoV-2 interaction with ACE2, which is involved in viral entry.[14] Increased levels of cytokines can be a risk factor for severe forms of the disease. In a study conducted with 548 COVID-19 patients, Li *et al* demonstrated that increased levels of IL-2R, IL-6, IL-10 and TNF-α cytokines were significantly higher in critically ill patients than in non-critically ill patients (all $p<0.01$).[15]

Rheumatological diseases may be associated with an increased risk of severe infections associated with underlying diseases, chronic inflammatory processes and the use of immunosuppressive drugs. However, the case reports have shown a mild form of the disease, and the use of anti-TNF seems to have had a protective effect on the evolution to severe forms, thereby preventing the damaging effects of the high levels of cytokines associated with the immunopathogenesis of infection. In addition to having a mild form of infection, the reported cases did not experience recurrence of their rheumatological disease during the COVID-19 infection. Further clinical trials may help define the real benefit of anti-TNFs and their applicability to reduce the incidence of severe forms of COVID-19.

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Clinical characteristics and outcomes of patients with COVID-19 and rheumatic disease in China 'hot spot' versus in US 'hot spot': similarities and differences

We read with great interest the article by D'Silva *et al* concerning clinical characteristics and outcomes of patients with COVID-19 and rheumatic disease.[1] In this study, the authors mentioned that patients with and without rheumatic disease had similar symptoms and laboratory findings, but those with rheumatic disease were more likely to require mechanical ventilation.

We analysed our data of 3059 patients with confirmed COVID-19, including 29 cases in combination with rheumatic diseases from Huoshenshan Hospital in Wuhan, which was a 'hot spot' of COVID-19 in China, from 4 February 2020 to 9 April 2020. There were 15 rheumatoid arthritis, 5 systematic lupus erythematosus, 1 Rhupus, 2 myasthenia gravis, 1 Sjögren's syndrome, 1 ankylosing spondylitis, 1 dermatomyositis, 1 autoimmune liver disease and 2 undifferentiated connective tissue disease cases (figure 1). The study population encompassed 4 men and 25 women, with median age of 61 years. Twenty-one patients presented with cough, 21 patients had fatigue, 3 had diarrhoea, 14 had varying degrees of difficulty in breathing and fever was observed in all cases.

Along with the results reported by D'Silva *et al*, the main manifestations and laboratory findings (figure 2) in patients with rheumatic disease were similar to patients with COVID-19 in the general population. Nevertheless, the need for mechanical ventilation was much lower in our study (2/29 vs 7/52). We hypothesised that part of the differences cross studies could be explained by medication and therapeutic strategy of the rheumatic population.

First, we note that the proportion of rheumatic diseases (0.95%) in our hospital was relative lower than reported by D'Silva (2.2%). And the most common comorbidity in patients with rheumatic disease was diabetes (6/29) in our study, whereas hypertension (34/52) was the most common in D'Silva's report. It is indeed that different ethnicity, different regional prevalence

Figure 1 Basic situation and medication of patients with rheumatic disease with COVID-19 during the course of the disease. Heatmap in the panel records the patient's disease classification, severity grade of COVID-19, number of underlying diseases, duration of disease, special treatment, inpatient and long-term medication, oxygen therapy, intensive care and clinical outcomes. Rheumatic immune diseases including systemic lupus erythematosus (SLE); rheumatoid arthritis (RA); myasthenia gravis (MG); Sjogren's syndrome (SS); dermatomyositis (DM); ankylosing spondylitis (AS); autoimmune hepatitis (AIH); undifferentiated connective tissue disease (UCTD). Basic diseases include hypertension, diabetes, coronary heart disease, lung disease, kidney disease, anaemia, thyroid disease and so on. Diabetes is the most combined basic disease, a total of six people. CPT, convalescent plasma therapy; ICU, intensive care unit.

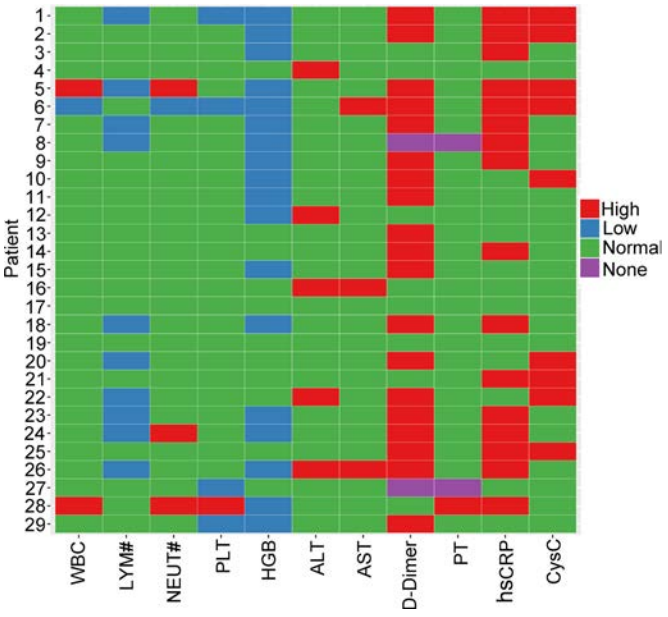

Figure 2 Laboratory index of COVID-19 in patients with rheumatism. Test items include white blood cells (WBC); lymphocytes (LYM); neutrophils (NEUT); platelets (PLT); haemoglobin (HGB); alanine aminotransferase (ALT); aspartate aminotransferase (AST); D-dimer; prothrombin time (PT); high sensitivity C-reactive protein (hsCRP); cystatin c (CysC). Colours indicate the rise and fall of the indicator; there are four missing values. # defined as absolute count.

of rheumatic diseases, different varieties and proportion of these diseases, and also different burden of comorbidities may contribute to the different outcome.

Second, medication is another important issue. In our study, five patients were treated with hydroxychloroquine (HCQ) for long term, and seven took corticosteroids as a regular prescription before the diagnosis of COVID-19, whereas eight received corticosteroids during hospitalisation. HCQ and chloroquine have been successfully used to treat variety of rheumatic diseases, and the sudden outbreak raises many questions concerning the potential benefit on protecting or antiviral potency of severe acute respiratory syndrome coronavirus 2 infection.[2–4] None of the five cases with long-term HCQ treatment progressed to critical severe cases. Notably, the traditional Chinese medicines such as Lianhuaqingwen capsule were also confirmed to have the potency of ameliorating clinical symptoms of COVID-19.[5]

Lastly, we also use convalescent plasma therapy in three patients, and tocilizumab in one patient due to their immunocompromised status. All patients recovered within 1 week after administration of immunotherapy.

In conclusion, we observed similar clinical manifestations of patients with COVID-19 and rheumatic disease in line with D'Silva's report. However, the need for mechanical ventilation was much lower in our study, and this remains inconclusive due to different ethnicity, different regional prevalence of rheumatic diseases and also concomitant treatments. Immunotherapy as an alternative therapy might play a role in maintaining the immune function, delaying or preventing the worsening of the disease and further minimising the need for mechanical ventilation.

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Response to: 'COVID-19 in patients with rheumatological diseases treated with Anti-TNF' by Brito *et al* and 'Clinical characteristics and outcomes of patients with COVID-19 and rheumatic disease in China 'hot spot' versus in US 'hot spot': similarities and differences' by Zhao *et al*

We appreciate the comments by Brito *et al*,[1] and Zhao *et al*,[2] in response to our manuscript evaluating outcomes among a cohort of patients with rheumatic diseases and COVID-19.[3] We were interested to read the reports of their patients during the COVID-19 pandemic and would like to reply to some of their queries.

Brito *et al* raised the important point that the risk of severe infections may vary with therapeutic class of immunosuppressive therapy. We agree that further studies are needed to assess this important question, and we plan to investigate this by therapeutic class for our cohort in future studies as our sample size grows. We agree that studies of therapeutic class will have to account for potential confounding factors, including glucocorticoid exposure, in their design. A recent report performed among patients with rheumatic diseases suggested lower odds of hospitalised infection for biological/targeted disease-modifying antirheumatic drugs (DMARDs) and higher odds for glucocorticoid use.[4] However, adjustment for immunosuppressive medication use is not possible when comparing patients with rheumatic disease to those without rheumatic disease who would not be expected to use these medications. While we await additional studies, we support the American College of Rheumatology's recommendations for management of rheumatic diseases during the pandemic, which do not recommend preemptively discontinuing immunosuppression.[5]

Brito *et al* also reported on three patients who did well clinically after developing COVID-19 while maintaining treatment with tumour necrosis factor (TNF) inhibitors for rheumatic disease. It is unclear how these cases of COVID-19 were identified. Given the small sample size, high variability in COVID-19 presentations (ie, many patients in a general population experience only mild illness), possibility of reporting bias (ie, clinicians may have been more likely to see patients with more mild symptoms in clinic) and lack of a comparison group, further studies will be needed to determine the relationship of TNF inhibitors to severe outcomes in COVID-19.

Zhao *et al* described a cohort of 29 patients with rheumatic diseases from Wuhan, China, the first epicentre of the COVID-19 pandemic. Compared with findings in our study, they observed a lower rate of mechanical ventilation (2/29 (7%) in theirs vs 7/52 (14%) in ours). As they suggest, these differences may be related to differences between the two cohorts, including the distribution of age, rheumatic diseases, comorbidities, race/ethnicity and regional variations in the management of rheumatic disease and COVID-19. However, another recent investigation from Wuhan, China showed that the rate of mechanical ventilation was 38% in patients with rheumatic disease versus 10% in patients with non-rheumatic disease, a statistically significant difference and similar to our findings.[6]

As investigators report their experience with COVID-19 in patients with rheumatic diseases from around the world, we believe it is important for all to report the proportions of patients on conventional, biological and targeted synthetic DMARD, prior to the development of COVID-19. This information will help readers understand the generalisability of each study's observations to their patients. In the context of a growing body of literature suggesting that hydroxychloroquine may not have efficacy in COVID-19,[7–9] we would urge caution with regards to interpreting a protective effect from hydroxychloroquine based on the favourable outcomes of five patients with unknown COVID-19 disease severity. We await final data from ongoing randomised controlled trials evaluating the safety and efficacy of hydroxychloroquine in COVID-19.

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Experience of telemedicine use in a big cohort of patients with rheumatoid arthritis during COVID-19 pandemic

We have read with interest the work of Bozzalla-Cassione *et al*[1] published recently in your journal regarding the implementation of a telemedicine programme for patients with lupus in northern Italy. It is logical to suppose that the risk of patients with rheumatic diseases of having a more severe clinical course if they become infected with the COVID-19 infection is very high; however, although some of the reports show that there seems to be a low incidence of COVID-19 infection in patients with rheumatic disease, collaborative work with large cohorts is needed, which could show us the real incidence of COVID-19 infection in these patients and what happens with the establishment of telemedicine programmes.[2–6]

We show an experience in a specialised centre in Bogota, Colombia; currently, we have a cohort of 5597 patients with rheumatoid arthritis (RA) in exclusively ambulatory care. On 12 March 2020, in Colombia, the health emergency by COVID-19 was established and a week later the Ministry of Health ordered the outpatient care procedure for the population in isolation. From that moment on, our institution, carrying out the proper logistical and legal processes, proceeded to convert its ambulatory care services into care through telemedicine.

By telecounselling, patients were offered consultation by telemedicine due to the high epidemiological risk of COVID-19; the patient gave informed consent to accept it or otherwise to request a face-to-face consultation despite the epidemiological risk warning; a third option was that the patient did not accept telemedicine or face-to-face consultation for personal reasons.

Here, we report the outcomes since 21 March–16 May (8 weeks later). For rheumatology care, the doctor must request informed consent for the consultation; then a standardised protocol was applied both for RA and also for suspected symptoms of COVID-19; as a measure of disease activity Patient Activity Score (PAS) was applied, and Health Assessment Questionnaire (HAQ) was also evaluated. When during the consultation the doctor finds that there is potentially high activity of the disease, a face-to-face consultation was ordered. In case of need, patients are sent to telemedicine consultation with the physiatrist or psychologist. For face-to-face consultation, standardised clinimetry instruments are used.

Until May the 16 (8 weeks later), 3503 patients have been followed up; 3228 (92%) have been seen by telemedicine and 275 (8%) by conventional face-to-face consultation; of these patients, 55 (20%) men and 220 (80%) women attended the face-to-face consultation; of patients attended by telemedicine, 567 (17.5%) were men and 2661 (82.5%) were women. Regarding COVID-19 infection, in 3 of the 275 patients who attended an in-person consultation, COVID-19 infection was suspected due to respiratory symptoms, but was finally ruled out. None of the patients seen so far by telemedicine had suspected COVID-19 by clinic or had contact with COVID-19 confirmed patients.

At first glance, these results seem surprising; there are zero incidences of COVID-19 infection in this large cohort of patients with RA, which we believe is due to the sanitary measures imposed by the country and to the adequate and standardised use of telemedicine. At first sight, we found that almost 75% of patients are well controlled regarding disease activity; however, the centre has started a mixed methodology study that includes a cohort study and a qualitative study to evaluate, whether telemedicine is effective in controlling disease activity of RA, such as the usual outpatient consultation. In this regard, there are some publications, but in the conditions of a pandemic like the present one, we do not have the evidence; on the other hand, it is necessary to evaluate the real incidence of COVID-19 infection in this group of patients and its clinical course.

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Response to: 'Experience of telemedicine use in a big cohort of patients with rheumatoid arthritis during COVID-19 pandemic' by Santos-Moreno *et al*

We thank Dr Santos-Moreno *et al*[1] for their interest in our paper[2] and for sharing their experience on telemedicine use in patients with rheumatoid arthritis (RA). We agree on the fact that a preliminary consultation is necessary to assess the willingness of the patients to be evaluated by telemedicine and, possibly, to explain them how the platform works. However, given the different setting in which we applied telemedicine—a connective tissue diseases (CTDs) outpatient clinic—our approach was slightly different. In fact, unlike inflammatory arthritis, in which disease flares are in most cases symptomatic and impacting on patients' quality of life and functionality, CTDs like systemic lupus erythematosus (SLE) or systemic sclerosis may give rise to more insidious manifestations. In our opinion, this makes these diseases less suitable for an assessment based on patients' reported outcomes (PROs). This led to two major differences in our telemedicine assessment protocol:

- The preliminary phone call was aimed also at identifying symptomatic patients requiring to be addressed to immediate face-to-face evaluation for suspected life-threatening or organ-threatening manifestations.
- Patients who did not refer any major symptom were scheduled for telemedicine assessment. The platform at our disposal allows both visual interaction and real-time sharing of test results.[3]

Following this approach, from 24 February to 17 April, we evaluated in person 47 patients against the 315 visits planned before the severe acute respiratory syndrome coronavirus 2 outbreak (15%). Most of these patients were treated with infusive drugs: prostanoids in 21 patients (44%) for uncontrolled Raynaud's phenomenon or for ulcer healing, 4 patients with cyclophosphamide (9%) for interstitial lung disease (ILD) (n=1), myocarditis (n=2) and SLE-related enteric vasculitis (n=1), and 2 patients with rituximab for antisynthetase ILD (n=1) and cryoglobulinaemia-related neuropathy (n=1). The remaining patients were evaluated for clinical assessment and treatment modification due to arthritis (n=8, 17%), ILD (n=5, 10%), nephritis (n=2, 4%), and neuropathy, angio-oedema, myositis, haemolytic anaemia and myocarditis (1 case each, 2%).

Unquestionably, telemedicine has proven itself as a valuable tool at these difficult times, and its diffusion will probably move faster than expected before the pandemic. In order to make telemedicine even more reliable, several digital applications for monitoring disease activity are already available for RA,[4] and efforts are being made in order to evaluate their impact on disease control and treatment adherence.[5 6] However, a treat-to-target strategy exclusively based on PROs is debated also for RA and might be problematic especially for patients in a near-remission status or with established disease.[7 8] In conclusion, we believe that the identification of the most suitable subsets of patients and the development of flexible approaches—allowing a prompt switch to inperson evaluation when necessary—are of utmost importance to provide good-quality healthcare assistance.

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Increased risk for severe COVID-19 in patients with inflammatory rheumatic diseases treated with rituximab

It is currently unknown whether immunosuppressive and/or immunomodulating agents such as biological disease-modifying antirheumatic drugs (bDMARDs) affect the rate and the outcome of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections of patients with inflammatory rheumatic diseases (IRDs). While several national authorities have defined patients under immunosuppressive therapy as at risk for severe COVID-19,[1] accumulating data from individual cases and also from case series, such as a series from Italy published in the *Annals of the Rheumatic Diseases* by Monti *et al*[2] and a report about patients with immune-mediated inflammatory diseases from New York,[3] suggest that baseline use of bDMARDs is not associated with worse COVID-19 outcome. Although the idea of a potentially protective effect of bDMRADs in COVID-19 is intriguing, we feel that extrapolation of these initial data is dangerous and potentially harmful. In particular, some caution may have to be applied when employing rituximab (RTX), a B-cell depleting bDMARD, in patients with immune-mediated disease. This notion may be illustrated by the following observations:

We recently lost two patients with rheumatoid arthritis (RA) treated with RTX to lethal COVID-19. The first patient, a 71-year-old man with rheumatoid factor positive, erosive RA and a history of mild chronic obstructive pulmonary disease was admitted to the hospital with symptoms of severe COVID-19. His RA was well controlled by RTX (2×1000 mg within 14 days every 6 months since 2015) in combination with methotrexate (MTX) 15 mg subcutaneously per week and he has been off daily glucocorticoids since 2017. RTX was well tolerated, no increased infection rate was noted and serum IgG was always within normal limits. As required by label, RTX was always administered with premedication including 50 mg prednisolone. Two weeks after the second RTX infusion in March 2020, the patient presented with a 2-day history of fever (up to 39.5°C), cough and chest pain. SARS-CoV-2 was proven and bilateral COVID-19 pneumonia was diagnosed by clinical examination and chest X-ray. Due to rapidly increasing dyspnoea and renal failure, the patient was transferred to the intensive care unit. Despite antibiotic treatment (piperacillin/tazobactam, followed by meropenem) and nasal high flow therapy, no improvement of the respiratory condition could be achieved. CT scan at that time showed bilateral pneumonia and reticular densifications. Invasive ventilation and increasing inotropic support were subsequently required due to further deterioration. Continuous veno-venous haemofiltration dialysis with cytosorb therapy was initiated. Despite all efforts, the patient died 12 days after admission in multiorgan failure.

The second patient, an 80-year-old woman with erosive RA and a history of mild hypertension and osteoporosis was started on treatment with RTX (2×1000 mg within 14 days) 6 months ago in combination with MTX 10 mg subcutaneously per week and 5 mg/day prednisolone. Her serum IgG was within normal limits. The patient presented to the hospital with sudden onset of fever (up to 39.5°C), dry cough, fatigue and dizziness. SARS-CoV-2 was proven and the patient rapidly deteriorated, requiring invasive ventilation. She developed acute respiratory distress syndrome and passed away despite intensive efforts 17 days after admission in multiorgan failure.

Sustained treatment of IRD with RTX is associated with a decrease in serum IgG and with an increased incidence of certain viral infections. However, COVID-19 has a mild clinically course in patients with agammaglobulinaemia,[4] suggesting that protection from severe COVID-19 may be rather independent of serum IgG. In this regard, our patients' serum IgG always was within normal limits. The lesson from our patients may rather argue that they might have been severely immunocompromised by the depletion of B cells and the application of prednisolone (as part of the premedication in patient 1 and as part of the daily treatment in patient 2). Supportive of this assumption is the aggressive course of COVID-19 in patients with common variable immunodeficiency[4] and the recent observation that glucocorticoids may impose a risk for requiring hospitalisation in patients with IRD infected with SARS-CoV-2.[3]

Our patients are not the unfortunate exceptions in that a substantial proportion of patients with IRD treated with RTX require hospitalisation when infected with SARS-CoV-2 (eg, 67% of the patients in the National Registry for patients with IRD infected with SARS-CoV-2 in Germany) (Hasseli *et al*, submitted for publication, 2020). Although successful treatment of granulomatosis with polyangiitis in a patient with COVID-19 with RTX has been reported,[5] RTX may need to be applied with particular caution in patients with IRD. Consequences for future management of patients with RTX therapy could be to perform a SARS-CoV-2 test before applying RTX, to consider reducing the dose of glucocorticoids during application of RTX (despite the requirement noted in the label) and to instruct the patient to strictly follow the measures in place to avoid contact for several days following RTX application.[1] The fatal outcome of COVID-19 in our patient illustrates the need to be extremely vigilant for the potential of complications associated with immunosuppressive therapy in patients with immune-mediated diseases.

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Response to: 'Increased risk for severe COVID-19 in patients with inflammatory rheumatic diseases treated with rituximab' by Schulze-Koops *et al*

We appreciated the comment from Schulze-Koops *et al*[1] in response to our paper on the clinical course and outcome of COVID-19 in a cohort of patients treated with biological and targeted synthetic disease-modifying antirheumatic drugs (b/tsDMARDs).[2] The authors stated that the message conveyed by our report or other similar observational data or clinical studies is potentially harmful for patients with rheumatic diseases who might think they are protected against complications of COVID-19 by their immunomodulatory drug. Nevertheless, in our publication, we clearly stated that our findings did not allow any conclusions on the overall outcome of immunocompromised patients affected by COVID-19 and that a high level of vigilance and strict follow-up should be maintained on these susceptible patients. Moreover, our findings supported the observation that patients with chronic arthritis treated with the reported b/tsDMARDs described in our cohort (therefore excluding rituximab) did not seem to be at increased risk of severe complications compared with the general population, which does not imply that they would be protected against the virus and that patients should reduce hygiene precautions and social distancing in a highly lethal condition even in the general population.

According to our report and further evidence accumulating since the pandemic outbreak confirming similar risks of incidence and complications between immunocompromised patients with rheumatic diseases and the general population, it seems reasonable to suggest that preventive withdrawal of bDMARDs in the absence of ongoing infections should be avoided as this would expose our patients to the risk of disease relapses.[3 4]

Nevertheless, we agree with Schulze-Koops that the available evidence should be interpreted critically and that evidence supporting the relative safety of one class of bDMARDs should not be inferred to all types of biologics or to different diseases treated with the same drug. Particularly, rituximab, by its long-term action on the humoral response might indeed impair the ability of the subject to effectively recover from COVID-19. The evidence regarding outcomes of patients with rheumatic diseases contracting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection while receiving treatment with rituximab is controversial.[5 6] We had previously commented on a correspondence supporting the favourable outcome of COVID-19 in a patient with granulomatosis with polyangiitis treated with rituximab by discussing the uncertainty existing around the effects that immunosuppressive agents with different mechanisms of action might play on SARS-CoV-2 infection.[6] While it is hypothesised that some bDMARDs might not contribute to a worsening of the clinical course of these patients, or might even attenuate its severity in the context of an aberrant inflammatory cytokine production triggered by SARS-Co-2, other treatments, especially those acting on B-cells and antibody production might turn out to be particularly detrimental. In conclusion, despite some reassuring observational evidence, until data from large cohorts and controlled studies become available, high vigilance and caution should always be applied when managing our patients with chronic rheumatic conditions.

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COVID-19 among Malaysian patients with systemic lupus erythematosus on hydroxychloroquine

We read with interest the letter by Mathian A *et al*[1] describing the clinical course of COVID-19 in a series of 17 patients with systemic lupus erythematosus under long-term treatment with hydroxychloroquine. The COVID-19 Global Rheumatology Alliance registry[2] shows that 19 (17%) of 110 patients with rheumatic diseases who have been diagnosed with COVID-19 as of 1 April 2020 were patients with lupus. Bozzalla Cassione *et al*,[3] Romão *et al*[4] and Ye *et al*,[5] respectively, described patients with SLE in their papers. The latest paper by D'Silva *et al* reported ten cases of lupus in their cohort.[6] We would like to share the clinical course of COVID-19 among patients with SLE in Malaysia.

As of 30 March 2020, there were five cases of SLE from a total of 2626 cases of COVID-19 in Malaysia. Clinical data were obtained through a review of medical records. COVID-19 was diagnosed in the patients based on a positive result on a reverse transcriptase PCR testing that detected severe acute respiratory syndrome coronavirus 2 from nasopharygeal swab specimen. All five patients were women with a mean age of 52.80±4.46 years and a mean disease duration of 13.20±3.92 years. All the patients were on long-term hydroxycholoroquine at baseline. Two patients were on conventional disease modifying antirheumatic drugs (DMARDs) (sulfasalazine and azathioprine), and one patient was on biological DMARDs (belimumab). Only one patient was on prednisolone during the diagnosis of COVID-19 infection. Majority of the patients were hypertensive and obese; 60% of them were on ACE inhibitor or angiotensin II receptor blocker treatment. The most common presentations were fever and cough. One patient was having active lupus shortly before COVID-19 diagnosis, while two patients were having flares of disease concurrently with COVID-19. Most patients have lymphopenia (lymphocyte count $<1500\,mm^3$). Radiologically,

Table 1 Demographic, clinical characteristics, outcome and laboratory findings

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Demographic					
Age (years)	46	50	57	53	58
Gender	Female	Female	Female	Female	Female
SLE					
SLE manifestations	Malar rash, arthritis, ANA, dsDNA	Malar rash, photosensitivity arthritis, Raynaud's phenomenon, ANA, dsDNA	Arthritis, autoimune haemolytic anaemia, low complement, ANA, dsDNA	Discoid rash, photosensitivity, oral ulcer, arthritis, leucopenia	Arthritis, pancytopenia, oral ulcer, ANA
Disease activity	Stable	Flare during admission	Flare during admission	Active	Stable
Hydroxychloroquine at baseline	Yes, 8 years	Yes, 12 years	Yes, 12 years	Yes, 20 years	Yes, 14 years
Prednisolone at baseline	No	No	No	Yes, 10 mg daily	No
Medications	Sulfasalazine	Azathioprine	No	Intravenous belimumab	No
ACE-I/ARB	Losartan	No	Losartan	Perindopril	No
Comorbidities	Hypertension, Obesity	No	Hypertension, dyslipidaemia, obesity	Hypertension, diabetes, obesity	Hypertension, Graves' disease postradioactitive iodine, obesity
Body Mass Index (kg/m^2)	35.2	20.5	32.3	36.8	30.4
Clinical					
Symptoms at disease onset	Fever, diarrhoea, cough, runny nose, dyspnoea	Fever, cough, multiple cervical lymph nodes	Lethargy, loss of appetite, arthralgia, haemolytic anaemia	Fever, cough dyspnoea	Fever, cough, myalgia
Imaging features	Bibasal lungs consolidation	Bilateral lower zone recticular opacities	Focal air space opacities in the right middle and lower zones	Bilateral air space opacities in the midzone and right base	Right perihilar infiltrates
Clinical stage of COVID-19	5 (ARDS)	3B	3B	5 (intubated)	3A
Treatment					
Hydroxychloroquine	Yes	Yes	Yes	Yes	Yes
Lopinavir/ritonavir	No	Yes	Yes	Yes	Yes
Subcutaneous interferon beta	No	No	No	Yes	No
Intravenous antibiotic	Yes Augmentin/azithromycin/ piperacillin/tazobactam	Yes Ceftriaxone	Yes Piperacillin/tazobactam	Yes Ceftriaxone	No
Intravenous hydrocortisone/intravenous methylprednisolone	No	Yes	Yes	Yes	No
Outcomes	Death	Home well	Home well	Home well	Home well

Clinical staging of COVID-19: 1, asymptomatic; 2, symptomatic, no pneumonia; 3, symptomatic, pneumonia; A, without fever; B, with fever; 4, symptomatic, pneumonia, requiring supplemental oxygen; 5, critically ill with multiorgan failure.
ACE-I, ACE inhibitor; ANA, antinuclear antibody; ARB, angiotensin II receptor blocker; ARDS, acute respiratory distress syndrome; dsDNA, double-stranded DNA; od, once a day; SLE, systemic lupus erythematosus.

Table 2 Laboratory findings

Laboratory findings	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
White cell count (10^3/µL)	7.90	2.95	4.20	7.9	5.50
Differential count (10^3/µL)					
Absolute lymphocyte count	0.72	0.50	1.87	0.80	1.50
Platelet count (10^3/µL)	296	353	332	151	232
Haemoglobin (g/L)	139	94	73	129	129
Albumin (g/L)	40	34	42	24	44
Alanine aminotransferase (U/L)	24	13	29	19	37
Aspartate aminotransferase (U/L)	42	30	36	24	27
Lactate hydrogenase (U/L)	344	420	496	–	–
Sodium (mmol/L)	136	133	138	135	136
Potassium (mmol/L)	4.2	3.9	4.4	3.6	3.7
Urea (mmol/L)	3.5	2.9	10.2	3.5	8.3
Creatinine (µmol/L)	70	55	117	56	81

all the patients had pneumonia in the chest X-rays. Demographic characteristics, clinical features, treatments and outcomes of five cases are illustrated in tables 1 and 2.

The ongoing, rapidly evolving COVID-19 pandemic poses a real threat to patients with SLE. As illustrated in the cases, COVID-19 is fast becoming a cause for morbidity and mortality in patients with SLE who are immunosuppressed. COVID-19 might mimic SLE flare as well as occur concurrently with SLE flare as demonstrated by cases 2 (arthritis) and 3 (autoimmune haemolytic anaemia).

Early diagnosis and treatment of COVID-19 is of paramount importance to ensure a good outcome. As illustrated by the cases, all patients presented with moderately severe to severe COVID-19 but responded well to treatment except the first case, which presented very late and was diagnosed posthumously. Only case 4 needed invasive ventilation and this patient received intravenous belimumab 1 week prior to contracting COVID-19. She needed more intensive treatment compared with the others. The role of belimumab in the clinical course of COVID-19 awaits further research. Three of them were also received intravenous corticosteroids during their hospitalisations; two received intravenous hydrocortisone as treatment of their concurrent SLE flares. Interestingly, case 3 received intravenous methylprednisolone for severe haemolytic anaemia at a very early stage; her COVID-19 was not worsening with this early use of corticosteroids but responded to standard treatment and she recovered well. The role of corticosteroids in the treatment of COVID-19 is controversial.

In summary, COVID-19 among our patients with SLE on hydroxychloroquine has a severe disease course needing aggressive therapy. Background hydroxychloroquine treatment for SLE did not prevent COVID-19 among our patients. Early diagnosis and treatment of COVID-19 resulted in good outcome in our patients with SLE.

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Response to: 'COVID-19 among Malaysian patients with systemic lupus erythematosus on hydroxychloroquine' by Teh *et al*

We thank Teh *et al* for their interest in our study reporting on the course of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) COVID-19 in a case series of patients with systemic lupus erythematosus (SLE) under long-term treatment with hydroxychloroquine (HCQ).[1 2] Teh *et al* report in detail the clinical course of COVID-19 in five patients with SLE in Malaysia. All patients were women, 50 years old on average, under long-term HCQ treatment, and the majority were suffering from comorbidities such as hypertension, obesity or diabetes. All patients presented with moderately severe to severe COVID-19; one patient died and another needed invasive ventilation. In this respect, the series of patients with SLE infected with SARS-CoV-2 described by Teh *et al* resemble other series previously reported by our group and others, encompassing a low number of patients, often hospitalised, with a majority of women in their 50s suffering from various comorbidities and an occasional severe or even lethal clinical evolution.[1 3] However, these case series do not allow drawing of conclusions on the incidence rate and severity of COVID-19 in SLE, because they most likely over-represent the most symptomatic and severe cases, resulting from a selection bias because of clinicians tending to report the most dramatic cases. The results from these series nevertheless point to a lack of a preventive effect of HCQ, at least in these patients, and suggest, similar to what has been observed in the general population, that comorbidities favour the severity of COVID-19 in patients with SLE.[1–3] Recent investigations with different study designs now complete these data by giving an estimation of the incidence of COVID-19 and its severity in patients with SLE. In a cohort of 165 patients with SLE in Italy, Bozzalla Cassione *et al* reported a prevalence of 7.2% of confirmed or suspected COVID-19, with a disease course that was generally mild with only one patient requiring intensive care, subsequent to the development of acute respiratory distress syndrome.[4] In another cohort of 225 patients with SLE in Belgium, Gendebien *et al* reported a prevalence of 8% of confirmed or suspected COVID-19, with only two patients hospitalised without the need for intensive hospital care.[5] A third case series from New York City has suggested that 18 (4%) of the 450 patients with SLE, followed-up in the Colombia Lupus Cohort, developed symptomatic confirmed or clinically suspected COVID-19, as compared with the suggested 2% community risk in New York City.[6] Clinical symptoms of COVID-19 were more pronounced than in the previous two studies with seven patients being hospitalised and three suffering from severe hypoxaemic respiratory failure, two of whom required non-invasive ventilation and one required invasive mechanical ventilation.[6] In this cohort, 83% and 39% of the patients with COVID-19 with SLE were taking immunosuppressants or steroids, respectively, prior to infection with SARS-CoV-2, which was substantially more than in the cohorts reported by Bozzalla Cassione *et al* and Gendebien *et al*.[6] The authors also noted a high frequency of lupus nephritis. In these three studies, no risk factors for contracting COVID-19 or developing a severe form of the disease were clearly identified. However, given that the vast majority of the patients with SLE included in these three cohorts were taking either HCQ or chloroquine, the effectiveness of these treatments to prevent symptomatic COVID-19 in SLE has been questioned.[4–6]

We believe the available information from the studies published as yet warrants the conclusion for now that the incidence of COVID-19, both severe and non-severe, is not dramatically increased in patients with SLE, as compared with the general population or with patients with rheumatic diseases. Furthermore, the first results obtained from patients with COVID-19 with immune-mediated inflammatory disease seem to indicate that exposure to prednisone without dose precision,[7] or at doses exceeding 5 mg/day[8] or 10 mg/day,[9] as well as the use of methotrexate[7 8] and rituximab[8] are associated with hospital admission. In one study, a prednisone dose exceeding 5 mg/day was reportedly associated with mortality.[8] However, at present, these observations cannot be extended to SLE. Future studies dedicated specifically to this disease will eventually determine the non-specific and specific risk factors that contribute to the development of a severe form of COVID-19 in SLE.

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Impact of COVID-19 pandemic on patients with SLE: results of a large multicentric survey from India

We have read the recent report by Mathian *et al* with great interest where they described the clinical course of COVID-19 in 17 patients with systemic lupus erythematosus (SLE).[1] The COVID-19 pandemic has caught the attention of the rheumatology fraternity due to a variety of reasons, such as the in vitro inhibition of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by hydroxychloroquine (HCQ),[2] use of tocilizumab in the treatment of cytokine storm[3] and concerns regarding cardiac toxicity due to HCQ.[4] Patients with SLE are routinely prescribed HCQ and other immunosuppressants. The clinical picture of COVID-19 (such as pneumonia, cardiac injury, renal injury, venous thrombosis and septic shock) in patients with SLE on long-term HCQ described by Mathian *et al* intrigued the global rheumatology community.[1] We assessed the impact of the pandemic on Indian patients with SLE in a larger multicentric survey. We expected that differences in disease expression, ethnicity and treatment may possibly alter the impact of the pandemic in contrast to the aforementioned study. We included patients who had visited their rheumatologist at least once in the preceding 1 year and surveyed them telephonically.

Twenty rheumatology centres across 18 cities in India collaborated, and 845 patients (women 92%, mean (±SD) age 34.8±12 years) with SLE were surveyed. Among these, 9.7% had hypertension; 3.8% had diabetes; and 0.9% had both. At the time of the survey, 813 (96.2%) patients were on HCQ (mean (±SD) dose 257.9±99 mg per day, mean (±SD) duration 30.8±30.7 months). Two-third of patients (559) were on glucocorticoids at a mean (±SD) dose of 6.9±6.8 mg prednisolone equivalent per day.

India reported its first COVID-19 case on 30 January 2020,[5] and at the time of the completion of this 3-day survey (on 5 May 2020), there were 46 711 positive cases and 1583 deaths in the country.[6] Of the 845 patients surveyed, two had tested positive for SARS-CoV-2. A total of 17 patients reported fever (more than 100°F), cough and/or shortness of breath in the preceding 3 months. The symptoms in these patients were not attributable to SLE. Two of these 17 patients were tested for SARS-CoV-2, and one was found to be positive. The patient who tested positive had been hospitalised for 2 days at the time of the completion of the survey. Symptoms of the remaining 16 patients resolved without any complications (see online supplementary figure S1). Five patients in our cohort, of whom three were healthcare workers, had been traced as close contacts of diagnosed COVID-19 cases. Three of these patients were advised only isolation, whereas two were also tested for SARS-CoV-2. One of these two patients tested positive but remains asymptomatic. Table 1 shows the characteristics of patients with confirmed and suspected COVID-19. For clinical features of patients with confirmed COVID-19, state-wise data of patients surveyed, summary of survey findings and comparisons between groups, see online supplementary tables S1–4.

The patients were also asked if they had palpitations or other cardiac problems ever since initiation on HCQ. None of the 845 patients reported any such symptoms or instances where a symptom was attributed to HCQ by any other doctor.

While in our survey, use of various immunosuppressants by patients with SLE did not result in a high incidence of COVID-19 and a worse outcome, more extensive studies are required to answer this question satisfactorily. Another possible explanation is the fact that a majority of the patients participating were ensuring all possible measures to protect themselves from infection while being on immunosuppressants. While we must monitor all our patients closely during this pandemic, there appears to be neither rationale nor evidence for withdrawing immunosuppressant medications as a preventive strategy for COVID-19. Lack of appropriate comparator group with patients not on HCQ and a low number of either confirmed or suspected COVID-19 patients did not allow us to draw meaningful conclusions regarding the role of HCQ in COVID-19. We plan to follow-up these patients and resurvey the same cohort once the pandemic settles.

Table 1 Demographics and clinical characteristics of patients with SLE with confirmed or suspected COVID-19

	COVID-19-like clinical picture (group A)*	Contact with patient with COVID-19 (group B)*	Confirmed COVID-19 (group C)*
Number of patients	17	5	2
Age (years) (mean±SD)	29.3±7.0	33.4±10.8	34.5±13.4
Female, n (%)	17 (100)	5 (100)	2 (100)
Organ systems involved, n (%)			
Musculoskeletal	14 (82.4)	4 (80)	1 (50)
Mucocutaneous	10 (58.8)	2 (40)	1 (50)
Haematological	9 (52.9)	2 (40)	1 (50)
Renal	7 (41.2)	0	1 (50)
Neuropsychiatric	3 (17.6)	1 (20)	0
Serositis	3 (17.6)	0	0
Constitutional	2 (11.8)	0	0
Others	4 (23.5)	3 (60)	1 (50)
Comorbidities, n (%)			
Hypertension	1 (5.9)	1 (20)	1 (50)
Diabetes	1 (5.9)	1 (20)	0
Hypothyroidism	5 (29.4)	0	0
Other	1 (5.9)	0	0
Medications, n (%)			
Hydroxychloroquine	17 (100)	5 (100)	2 (100)
Dose (mg per day) (mean±SD)	258.8±79.5	300±0	300±0
Duration (months) (mean±SD)	32.6±21.6	20.4±17.7	26±31.1
Glucocorticoid	14 (82.4)	4 (80)	2 (100)
Dose (mg per day)† (mean±SD)	12.6±11.0	17.5±21.9	35±21.2
Mycophenolate	7 (41.2)	2 (40)	2 (100)
Methotrexate	3 (17.6)	1 (20)	0
Azathioprine	4 (23.5)	1 (20)	0
Rituximab	3 (17.6)	0	0
Symptoms			
Fever (>100°F)	14 (82.4)	0	0
Dyspnoea	11 (64.7)	0	1 (50)
Dry cough	10 (58.8)	0	0
Contact with patient with COVID-19	0	5 (100)	1 (50)

*Group C comprised one patient each from groups A and B.
†Glucocorticoid dose is expressed in prednisolone equivalent.
SLE, systemic lupus erythematosus.

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Response to: 'Impact of COVID-19 pandemic on patients with SLE: results of a large multicentric survey from India' by Goyal *et al*

We thank Goyal *et al* for their interest in our study reporting on the course of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease 2019 (COVID-19) in a case series of patients with systemic lupus erythematosus (SLE) under long-term treatment with hydroxychloroquine.[1 2] Goyal *et al*, on a series of 845 patients with SLE, reported symptoms compatible with COVID-19 in 17 (2.0%) and confirmed COVID-19 in only 1 (0.1%) of the patients. The very low frequency of COVID-19 in these patients reflects the low incidence of SARS-Cov-2 infection in the Indian population at the completion of their study. However, since then, the epidemic has unfortunately progressed and the number of deaths from COVID-19 in India has multiplied nearly 10-fold. Thus, the number of patients with SLE affected by COVID-19 is probably much higher now than that reported by Goyal *et al* at the beginning of the epidemic. The network of rheumatology centres that was created during this multicentric survey will make it possible to collect new information concerning the occurrence of SARS-CoV-2 infection in patients with SLE in India. However, we would like to insist on the necessity to use reliable markers to establish the diagnosis of COVID-19 such as viral detection by real-time reverse transcription-PCR analysis and/or the detection of anti-SARS-CoV-2 serum antibodies. In addition, a chest CT scan suggestive of SARS-CoV-2 pneumonia will also allow, in the context of a COVID-19 outbreak, to confirm the diagnosis of SARS-CoV-2 infection. Unfortunately, Goyal *et al* reported that only 2 out of 17 patients who reported fever, cough or shortness of breath were tested for infection with SARS-CoV-2. In the context of the low attack rate, observed at the beginning of the pandemic in India, the occurrence of this symptomatology is not synonymous with COVID-19 because infection with virus strains other than SARS-CoV-2 may cause similar, indistinguishable symptoms. We therefore insist on the use of the aforementioned diagnostic methods to improve the reliability of this type of study.

To conclude, if the attack rate in the general population remains low in the foreseeable future, it will be useful to regroup the different observational cohorts from various countries and continents permitting to better identify, and with a gain of statistical power, the potential risk factors for severe COVID-19 in patients with lupus, provided that the diagnosis of COVID-19 is certain.[3–6]

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Presence of antiphospholipid antibodies in COVID-19: a case series study

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and its associated coagulopathy are particularly worrisome in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS), as these diseases carry an increased risk of thrombotic complications. Mathian *et al* recently reported the clinical course of COVID-19 in a series of 17 patients with SLE under chronic hydroxychloroquine therapy.[1] Of note, only one patient (6%) presented thrombosis despite the fact that four patients (24%) had a history of secondary APS, and five patients (29%) were receiving oral anticoagulants. Antiphospholipid (aPL) antibodies were not measured in these patients during active SARS-CoV-2 infection.[1]

The American Society of Hematology recently stated that 'at the current time, there are only very limited data on aPL antibodies in COVID-19 and it is unclear if they represent an epiphenomenon or are actually involved in any haemostatic abnormalities seen in COVID-19 disease'.[2] Furthermore, almost all the available information refers to the lupus anticoagulant, with frequencies ranging from 45% to 87%.[3 4] This paucity of data led us to test a panel of aPL antibodies in blood specimens from 21 patients hospitalised in the intensive care unit between 12 and 19 April, due to severe or critical COVID-19, and received at our laboratory on 20 April to measure interleukin-6 levels. Anticardiolipin, anti-β_2 glycoprotein I, antiprothrombin, antiphosphatidylserine, antiphosphatidylinositol and antiannexin V antibodies were measured, each in IgM and IgG isotypes. Subsequently, demographic and clinical data were obtained from electronic medical records. Sera collected before the SARS-CoV-2 pandemic from 12 healthy individuals, matched for age and sex, were tested as controls.

Pertinent results are summarised in table 1. The median age of patients was 62 years; 43% were men; and a high number of comorbidities were observed (median Charlson Comorbidity Index of 3). A total of 19 patients (90%) had shortness of breath on admission, and 12 (57%) eventually required invasive mechanical ventilation during hospitalisation. Elevated levels of D-dimer, ferritin and C reactive protein were found at presentation.

Of the 21 patients with COVID-19 studied, 12 had at least one circulating aPL antibody, whereas only 1 of the 12 controls yielded a positive result (57% vs 8%; Fisher's exact test, p=0.009). The most frequently detected aPL antibodies were antiannexin V IgM (19%), anticardiolipin IgM (14%), antiphosphatidylserine IgM (14%), anticardiolipin IgG (10%) and antiphosphatidylserine IgG (10%) antibodies. One patient had triple positivity (8%); three patients had double positivity (25%); and the remaining eight had a single positivity (67%). Age and number of comorbidities tended to be lower in patients with aPL antibodies. In contrast, levels of D-dimer, ferritin and C reactive protein were higher both on admission and throughout the hospital stay in these patients. Elevated levels of interleukin-6 (>40 pg/mL) were found only in patients with aPL antibodies. The type of therapies administered in both groups was similar, except for a greater number of patients with aPL antibodies who received glucocorticoids (50% vs 0; Fisher's exact test, p=0.018).

The occurrence of hospital outcomes was followed up to 30 days after aPL antibody measurement. Two patients presented pulmonary thromboembolism despite being on heparin: a 28-year-old man with a previous diagnosis of

Table 1 Main clinical and laboratory data of 21 patients with severe or critical COVID-19

	Total (N=21)	Positive aPL antibodies (n=12)	Negative aPL antibodies (n=9)
Age (years)	62 (54–67)	55 (49–63)	67 (62–68)
Male sex, n (%)	9 (43)	6 (50)	3 (33)
Days of symptom onset	7 (5–9)	7 (4–8)	7 (5–9)
Charlson Comorbidity Index	3.0 (1.0–4.0)	1.5 (1.0–3.0)	4.0 (2.0–5.0)
Coexisting conditions, n (%)			
Hypertension	12 (57)	5 (42)	7 (78)
Diabetes mellitus	8 (38)	3 (25)	5 (56)
Dyslipidaemia	7 (33)	3 (25)	4 (44)
Obesity	7 (33)	5 (42)	2 (22)
Coronary artery disease	3 (14)	2 (17)	1 (11)
Stroke	1 (5)	0	1 (11)
Current smoker	2 (10)	2 (17)	0
Pulmonary disease	2 (10)	2 (17)	0
Chronic kidney disease	3 (14)	1 (8)	2 (22)
Chronic heart failure	2 (10)	0	2 (22)
Cancer	1 (5)	0	1 (11)
Main findings at hospital admission			
Fever, n (%)	13 (62)	7 (58)	6 (67)
Shortness of breath/respiratory distress, n (%)	19 (90)	12 (100)	7 (78)
White cell count (×10^3 per mm^3)	6.5 (4.9–10.4)	7.0 (5.4–12.1)	6.2 (4.9–9.6)
Platelet count (×10^3 per mm^3)	179 (146–198)	179 (156–193)	171 (143–240)
D-dimer (ng/mL)	339 (177–484)	387 (207–484)	303 (132–446)
Ferritin (µg/L)	557 (156–882)	677 (490–1249)	199 (112–326)
C reactive protein (mg/L)	139 (57–210)	200 (95–256)	86 (57–144)
Intubation, n (%)	12 (57)	7 (58)	5 (56)
Laboratory values at the time of aPL measurements			
White cell count (×10^3 per mm^3)	7.8 (6.9–10.6)	8.6 (6.7–13.2)	6.4 (5.7–9.8)
Platelet count (×10^3 per mm^3)	260 (212–349)	262 (201–332)	259 (229–349)
D-dimer (ng/mL)	417 (216–613)	437 (206–601)	403 (278–621)
Ferritin (µg/L)	604 (365–1353)	1038 (580–1392)	443 (237–547)
C reactive protein (mg/L)	90 (17–219)	140 (60–270)	39 (17–129)
Serum interleukin-6 levels>40 pg/mL, n (%)	2 (10)	2 (17)	0
Treatment, n (%)			
Heparin	18 (86)	9 (75)	9 (100)
Glucocorticoids	6 (29)	6 (50)	0
Hydroxychloroquine	15 (71)	9 (75)	6 (67)
Azithromycin	18 (86)	10 (83)	8 (89)
Lopinavir plus ritonavir	11 (52)	6 (50)	5 (56)
Positive aPL antibodies, n (%)			
Anticardiolipin IgM	3 (14)	3 (25)	0
Anticardiolipin IgG	2 (10)	2 (17)	0
Anti-β_2 glycoprotein I IgM	0	0	0
Anti-β_2 glycoprotein I IgG	1 (5)	1 (8)	0
Antiprothrombin IgM	1 (5)	1 (8)	0
Antiprothrombin IgG	0	0	0
Antiphosphatidylserine IgM	3 (14)	3 (25)	0
Antiphosphatidylserine IgG	2 (10)	2 (17)	0
Antiphosphatidylinositol IgM	0	0	0
Antiphosphatidylinositol IgG	0	0	0
Antiannexin V IgM	4 (19)	4 (33)	0
Antiannexin V IgG	1 (5)	1 (8)	0
Pulmonary thromboembolism	2 (10)	2 (17)	0
Major bleeding, n (%)	1 (5)	1 (8)	0
Ventilator-associated pneumonia, n (%)	3 (14)	1 (8)	2 (22)
In-hospital deaths, n (%)	4 (19)	2 (17)	2 (22)
Discharged, n (%)	13 (62)	9 (75)	4 (44)

Data are presented as median (IQR) unless otherwise specified.
aPL, antiphospholipid.

idiopathic pulmonary hypertension who had anticardiolipin IgG antibodies and a 63-year-old woman with a history of Fahr syndrome and hypoparathyroidism who had antiannexin V IgM antibodies. Both patients had extremely high levels of D-dimer and C reactive protein throughout the follow-up and eventually died of haemodynamic complications. Necropsy studies were not performed. Despite the fact that most patients received heparin, the only clinically significant bleeding was spontaneous retroperitoneal haematoma in a 44-year-old man with antiphosphatidylserine IgM and antiannexin V IgM antibodies who recovered with conservative management. Two patients in whom no aPL antibodies were observed eventually died of multisystem organ failure. As of 18 May, 13 patients (62%) had been discharged from the hospital; 4 (19%) remained hospitalised; and 4 (19%) died.

In this case series study, a high frequency (57%) of both 'criteria and non-criteria' aPL antibodies was found in patients with severe and critical COVID-19. These aPL antibodies appear to be associated with a hyperinflammatory state characterised by extremely high levels of ferritin, C reactive protein and interleukin-6; meanwhile, an association with pulmonary thromboembolism may be suggested. During acute infection, thrombosis or inflammation, different aPL antibodies may transiently arise, and it should not be assumed that a patient with COVID-19-associated coagulopathy and aPL antibodies has catastrophic APS.[5] Indeed, although COVID-19-associated coagulopathy and catastrophic APS may share clinical and laboratory features, both diseases are likely to have a different underlying pathophysiology. However, the high frequency and wide variety of aPL antibodies observed in patients with COVID-19 cannot be ignored.

Currently, there are limited data on the occurrence of aPL antibodies during SARS-CoV-2 infection, and further studies are required to determine whether these represent a simple epiphenomenon or are actually involved in COVID-19-associated coagulopathy.

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Response to: 'Presence of anti-phospholipid antibodies in COVID-19: a case series study' by Amezcua-Guerra *et al*

We thank Amezcua-Guerra *et al* for their interest in our study reporting on the course of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease 2019 (COVID-19) in a case series of patients with systemic lupus erythematosus under long-term treatment with hydroxychloroquine.[1 2] Complementary to our work, Amezcua-Guerra *et al* address the issue of anti-phospholipid antibodies (anti-PL abs) during the course of COVID-19. Indeed, despite adequate thromboprophylaxis, COVID-19 is associated with a high rates of venous, as well as arterial, thromboembolic events, in particular in patients hospitalised in an intensive care unit.[3–5] This state of hypercoagulation has been linked to an important systemic inflammatory response syndrome, with elevated serum levels of fibrinogen, factor VIII and D-dimers.[6 7] Several reports, including the study by Amezcua-Guerra *et al*[2] have emphasised the high frequency of serum anti-PL abs and lupus anti-coagulant (LA) in a case series of patients with severe COVID-19, however with surprisingly heterogeneous results.

Amezcua-Guerra *et al* report a high frequency (57%) of both conventional (ie, those included in the antiphospholipid syndrome (APS) classification criteria) and non-conventional anti-PL abs in patients with severe and critical COVID-19, which appear to be associated with a hyperinflammatory state. An association with pulmonary thromboembolism has also been suggested although this concerned only 2 (17%) of the 12 patients who had at least one type of circulating anti-PL abs.[2] More recently, Zuo *et al*, measuring serum levels of eight different types of anti-PL abs in 172 patients hospitalised with COVID-19, detected anti-cardiolipin (anti-CL) IgM in 23%, anti-PS/PT IgG in 24% and anti-PS/PT IgM in 18% of the patients, with at least one type of anti-PL abs present in 52%.[8] In contrast, Galeano-Valle *et al* reported a very low prevalence of conventional serum anti-PL abs among patients experiencing venous thromboembolism during the course of severe COVID-19.[9] The results from their study were confirmed and extended by Borghi *et al* who also reported a low prevalence of anti-β_2GPI IgG, IgA and IgM in patients with COVID-19 at a frequency of 15.6, 6.6 and 9.0%, respectively, as well as anti-CL IgG (5.7%) or IgM (6.6%), that was not associated with major thrombotic events.[10] In the latter study, anti-PL abs were mainly directed against β_2GPI, but they displayed an epitope specificity different from that of anti-PL abs present in APS.[10] The explanation for the observed discrepancy between the rates of anti-PL abs reported in these studies might rely on the possibility that their generation is linked to the severity of COVID-19. In this respect, Bertin *et al* reported in a cohort of 56 patients with COVID-19 that such differences were found for anti-CL IgG whose presence were significantly associated with a severe form of the disease.[11] This observation was confirmed by Xiao *et al*, who showed that anti-PL abs, mostly anti-β_2GPI and aCL IgA, were detected in 47.0% of critically ill patients, but not in patients with non-critical conditions.[12] Surprisingly, in the latter study, LA was detectable in only 2 of 66 critically ill patients. The presence of multiple anti-PL abs with a moderate serum titres of at least one type of anti-PL ab, was found to be statistically associated with a higher incidence of cerebral infarction.[12] Of note, anti-PL abs were mainly of the IgA isotype which suggest a cross-reactivity and/or breakdown of mucosal immune tolerance induced by SARS-CoV-2 because of the pulmonary and intestinal mucosal tropism of this virus.

Meanwhile, many studies have shown a significant presence of LA in patients with severe COVID-19, mainly in critically ill conditions. In the study of Bowles *et al*, 31 patients (14%) were shown to be positive for an LA assay in a series of 216 patients with severe COVID-19 with only two patients having a confirmed or suspected venous thrombosis.[7] Harzallah *et al* reported 25 patients (45%) positive for LA in a series of 56 critically ill patients with COVID-19,[13] whereas in the study of Helm *et al*, 50 patients (33%) tested positive for an LA assay in a series of 150 patients with COVID-19-related acute respiratory disease syndrome (ARDS).[3] These important rates of LA in critically ill patients with COVID-19 should however be interpreted with caution. Indeed, the extrapolation of LA results from patients receiving anticoagulants, which is now current clinical practice in the vast majority of patients hospitalised for COVID-19, is subject to discussion.[14] Furthermore, one should be aware of false-positive LA testing results in patients with COVID-19 because many assays are sensitive to the presence of C-reactive protein resulting in false positives.[15]

We recently reported, on a series of 25 patients with refractory COVID-19-related ARDS, 23 cases (92%) of LA. Anti-CL or anti-β_2GPI abs were observed in 13 (52%) and 3 (12%) cases, respectively.[16] Three patients (12%) were triple positive for LA, anti-CL and anti-β2GP1 abs, whereas massive pulmonary embolism was diagnosed in six patients, all positive for the presence of anti-PL abs.

During acute infection, thrombosis or inflammation, serum levels of different anti-PL abs may transiently arise. Strikingly, however, this elevation of anti-PL abs and/or LA titres reported in a major proportion of patients with severe COVID-19 has rarely been observed in other pathologies. Nevertheless, the involvement of anti-PL abs in the induction of a hypercoagulable state and the possibility that SARS-Cov-2 may trigger the development of 'COVID-19-induced APS-like syndrome' have to be confirmed in large clinical series. Notwithstanding, the high frequency and wide variety of anti-PL abs observed in patients with COVID-19 cannot be ignored.

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Role of antimalarials in COVID-19: observational data from a cohort of rheumatic patients

The potential role of chloroquine (CQ) and hydroxychloroquine (HCQ) in the management of COVID-19 is certainly of relevance in this health emergency scenario. For this reason, we read with great interest the letter published by Romão and colleagues highlighting the need for more definite evidence on the role of antimalarial drugs in both preventing severe acute respiratory syndrome coronavirus-2 infection and making COVID-19 clinical course milder.[1] While several ongoing clinical trials are progressively providing controversial data about the real efficacy and safety of antimalarials in the treatment of overt COVID-19,[2–4] rheumatological patients already taking CQ or HCQ for the treatment of chronic inflammatory diseases are an excellent bench for testing the potential effect in preventing the contagion.[5] Being operative in the Research Center for Adult and Paediatric Rheumatic Diseases of the ASST Gaetano Pini-CTO in Milan (Lombardy), we had the opportunity to deal with a large cohort of rheumatic patients living in one of the regions most affected by the outbreak.[6] In the period between 25 February and 16 April 2020 we circulated among our patients a survey designed to investigate the incidence of COVID-19 (defined as nasopharyngeal swab positivity) and symptoms consistent with viral infection, and to clarify how our patients had changed their treatment and behaviour due to the outbreak. The survey was administered face-to-face to all patients evaluated during an outpatient visit or by telephone to those who missed a scheduled appointment during the period under review. The rate of non-responders to the survey was very low (1.85%) and unlikely to significantly affect the overall results. The final study population included 914 patients stratified in HCQ-users (n=112) and non-HCQ-users (n=802), whose demographic and clinical characteristics are detailed in table 1. Briefly, mean age, mean disease duration and prevalence of comorbidity were overlapping in the two groups. Conversely, significant differences were observed in the distribution according to gender (female prevalence was greater in HCQ-users) and diagnosis (rheumatoid arthritis and connective tissue diseases were more frequent in HCQ-users, whereas spondyloarthritis in non-HCQ-users). Moreover, the prevalence of concomitant biological/targeted synthetic drugs was higher in non HCQ-users, while corticosteroids were more frequently reported in HCQ-users. The vast majority of patients in both groups had strictly adhered to the norms for the prevention of contagion (use of masks and gloves, social distancing, home-working) since the beginning of the epidemic. The frequency of definite contact with COVID-19 positive subjects was similar in both groups. In the overall population, six patients with COVID-19 positive swab were observed, five of whom had a complete recovery (four required hospitalisation with low-flow oxygen therapy), while a 32-year-old woman suffering from systemic sclerosis with lung involvement (taking HCQ) died. The incidence of COVID-19 positive subjects was comparable in the two groups (0.89% in HCQ-users vs 0.62% in non HCQ-users; p=0.64). This result did not change either by broadening the definition of COVID-19 to include patients who had not had access to the swab but who presented symptoms consistent with COVID-19 (at least one between fever >37.5°C, cough, or dyspnoea of recent onset), or were living in a highly endemic area (COVID-19 incidence ≥1%), according to WHO criteria (16% HCQ users vs 14.6% non-users; p=0.67). In conclusion, our preliminary data does not appear to support the use of antimalarials as prophylactic therapy for COVID-19, although the lack of a complete matching between the two groups under analysis does not allow definitive conclusions to be drawn.

Table 1 Demographic and clinical characteristics of study population

	HCQ users (n=112)	Non-HCQ users (n=802)	P value
Age (mean±SD)	57.3±14.2	54.5±16	0.06
Female	96 (85.7%)	528 (65.8%)	<0.0001
Disease duration (mean±SD)	11.4±10	13.4±9	0.07
Diagnosis			
Rheumatoid arthritis	79 (70.6%)	401 (50%)	<0.0001
Spondyloarthritis	8 (7.1%)	307 (38.2%)	<0.0001
Connective tissue diseases	21 (18.7%)	52 (6.5%)	<0.0001
Other	4 (3.6%)	42 (5.3%)	0.64
Concomitant bDMARD	50 (44.6%)	605 (75.4%)	<0.0001
Concomitant corticosteroids	70 (62.5%)	253 (31.5%)	<0.0001
Comorbidities (≥1)	39 (34.8%)	302 (37.6%)	0.60
COVID-19			
Contagion prevention	93 (83%)	700 (87.2%)	0.23
Treatment discontinuation	4 (3.6%)	42 (5.2%)	0.54
Definite contact with COVID-19 positive subjects	2 (1.8%)	14 (1.7%)	0.90
COVID-19 positive swab	1 (0.89%)	5 (0.62%)	0.64
Respiratory symptoms (no swab)	18 (16%)	117 (14.6%)	0.67

P value was calculated by using Fisher's test for categorical variables and t-test for continuous variables. Respiratory symptoms, at least one between fever >37.5°C, cough, or dyspnoea of recent onset.
bDMARD, biologic disease-modifying antirheumatic drugs; HCQ, hydroxychloroquine.

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Response to: 'The role of antimalarials in COVID-19: observational data from a cohort of rheumatic patients' by Favalli *et al*

We thank Favalli *et al* for their comment on our letter[1] regarding the effect of antimalarials for the prevention of coronavirus disease 2019 (COVID-19).[2] The authors provide updated information on a cohort of 914 patients with rheumatic and musculoskeletal diseases (RMDs), 112 of whom were treated with hydroxychloroquine (HCQ). The prevalence of confirmed or suspected COVID-19 cases was similar in both groups, and a patient with systemic sclerosis-associated lung disease treated with HCQ had a fatal outcome. Notably, 87% of patients reported rigorous compliance with contagion prevention measures.

These data are in accordance with accumulating evidence published over the last 2 months, indicating the occurrence of mild and severe cases of infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in patients with RMDs treated with antimalarials (table 1).[1–16] Out of 869 reported patients with RMDs with confirmed COVID-19, 190 (22%) were taking long-term antimalarials prior to the infection.[1–16] Of those, 99 out of 181 (55%) with available data developed severe disease that required hospitalisation. While these figures are limited by confounding by indication and selection bias, they attest to the fact that RMD patients treated with antimalarials can indeed be infected by SARS-CoV-2 and develop severe disease. Further, this seems to occur at a similar rate to patients not previously exposed to these drugs, considering the lack of association of previous antimalarial treatment with disease outcome in the studies that specifically assessed this aspect.[11–14] Of note, this conclusion persisted even after adjustment for other clinically relevant variables, including sex, age, comorbidities and concomitant immunosuppressive and glucocorticoid therapy.[11–13]

Table 1 Reported frequency of COVID-19 in patients with RMDs treated with antimalarials

	Total confirmed cases	Cases on previous HCQ/CQ (% total)	Severe course* if on previous HCQ/CQ (%)
Avouac *et al*[8]	3	0	–
Bozzalla Cassione *et al*[10]	4	3 (75)	1 (33)
Benoy *et al*[3]	27	5 (19)	NR
D'Silva *et al*[11]	52	9 (17)	5 (56)
Favalli *et al*[2]†	6	1 (17)	1 (100)
Gianfrancesco *et al*[13]‡	600	130 (22)	66 (51)
Haberman *et al*[14]	50	8 (16)	3 (38)
Mathian *et al*[15]	17	17 (100)	14 (82)
Michelena *et al*[4]	11	0	–
Monti *et al*[9]	4	1 (25)	0
Romão *et al*[1]	2	2 (100)	0
Sanchez-Piedra *et al*[7]	41	4 (10)	NR
Wallace *et al*[16]	31	4 (13)	3 (75)
Ye *et al*[6]	21	6 (29)	6 (100)
Total	869	190 (22)	99 (55)§

*Defined as those requiring hospitalisation.
†Data also include patients reported by Favalli *et al*[5] in a separate publication.
‡Data also include patients reported by Konig *et al*[12] in a separate publication.
§Percentage calculated based on patients with available data (n=181).
CQ, chloroquine; HCQ, hydroxychloroquine; NR, not reported.

At this point, although several questions regarding the risk and impact of COVID-19 for patients with RMDs remain unanswered, a few aspects begin to emerge. There is so far no convincing evidence to support the fact that patients with RMDs have an increased risk or worse prognosis of COVID-19.[17] The reported incidence of SARS-CoV-2 infection and hospitalisation seems to be in line with that observed for the general population in various severely hit western countries.[3–5 9 14] Moreover, hospitalisation rates are not increased, and most RMD patients on immunosuppressive therapies seem to have a favourable disease course,[11 13 14] although three studies did report a high frequency of intensive care unit admission.[6 11 15] Concomitant demographic (older age), clinical (comorbidities), and treatment (moderate-to-high-dose glucocorticoids) factors are likely more important detrimental prognostic factors.[11–14]

As noted by Konig *et al*, dosing and blood concentration issues may hamper an eventual beneficial antiviral effect of HCQ on patients with RMDs.[12] Nevertheless, high-dose antimalarials have been shown to be ineffective and even potentially harmful in randomised controlled trials (RCTs).[18 19] Large observational studies also failed to demonstrate a positive effect of HCQ for the treatment of COVID-19,[20–22] but overall evidence is conflicting and insufficient.[23] The results of ongoing large RCTs (eg, SOLIDARITY, ISRCTN83971151; DisCoVeRy, NCT04315948) are thus warranted for definite conclusions.

Regarding prophylaxis, a recently published RCT (n=821) failed to demonstrate a benefit of starting HCQ within 4 days after moderate-to-high-risk exposure to SARS-CoV-2, for preventing the development of COVID-19.[24] As alluded to by Moiseev *et al*, additional RCTs are currently enrolling impressive numbers of patients to investigate the efficacy of pre-exposure and postexposure antimalarials in the prophylaxis of COVID-19.[25] Though the findings of these trials may not be entirely generalisable to patients with RMDs on long-term treatment with antimalarials, they will definitely contribute to better define what role, if any, do these drugs play in the protection against SARS-CoV-2.

In conclusion, following the initial enthusiasm on the prospect of a putative preventive effect of antimalarial drugs in patients with RMDs, emerging data suggest otherwise. We are certainly still far from a final verdict, but RMD patients treated with antimalarials do develop mild-to-moderate and severe COVID-19. Thus, for now, focus should remain on advising patients to reinforce effective infection control measures, such as social distancing, hand/respiratory hygiene and face mask use.[17 26]

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Exacerbation of immune thrombocytopenia triggered by COVID-19 in patients with systemic lupus erythematosus

We read the article regarding COVID-19 in patients with systemic lupus erythematosus (SLE) by Mathian *et al*[1] with great interest. We would like to report a case with SLE with COVID-19 who presented severe relapse of thrombocytopaenia. Mild thrombocytopaenia during COVID-19 is frequently observed, and immune thrombocytopaenia (ITP) has also been reported.[2 3] Management of ITP during active COVID-19 can be difficult as immunosuppressive therapies can exacerbate infections, and the recovery of platelet count may lead to thrombosis due to coagulopathy caused in COVID-19.[4] Here, we report a case with severe ITP relapse in patients with SLE during a course of COVID-19.

A 58-year-old woman with a nearly 20-year history of SLE was admitted to our hospital with COVID-19. Her main manifestation of SLE was ITP, and her platelet count was low but stable at approximately 60×10^9/L with 5 mg of prednisolone (PSL) since the administration of 12 years before. On 2 days before admission, she presented with chest discomfort, and her chest CT scan showed patchy ground-glass opacities in the both lungs. Her oxygen saturation was 95% on room air. A reverse transcription PCR test of a nasopharyngeal swab for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was positive. Laboratory examinations on admission revealed normal white cell count (6020/μL) with lymphocytopaenia (650/μL) and slightly elevated C reactive protein (0.5 mg/dL). Her platelet count was 10×10^9/L, which was acutely decreased from 6.1×10^9/L of 3 months before, with a highly elevated immature platelet fraction (36.0%). Her lupus anticoagulant test was positive with a prolonged activated partial thromboplastin time of 41.3 s, and her rapid plasma regain test showed a biologic false-positive with a negative *Treponema pallidum* hemagglutination test, which had never been observed at previous examinations. On day 3, her platelet count decreased to 8×10^9/L, and the PSL dose was increased from 5 mg/day to 10 mg/day. On day 6, her platelet count was further decreased to 5×10^9/L with continued gingival bleeding. Daily 20 g doses of intravenous immunoglobulin (IVIg) were administered for 5 days, and her platelet count increased to 121×10^9/L on day 13 with cessation of bleeding. The patient's COVID-19 remained mild throughout her clinical course with ciclesonide inhalation, and no thrombosis was developed.

To our knowledge, this is the first description of a case with severe ITP exacerbation induced by COVID-19. She was successfully treated with IVIg without worsening of respiratory symptoms and thrombosis. Mild thrombocytopaenia is a common feature of COVID-19, while a count below 100×10^9/L has been found only in only 5% of hospitalised patients.[5] Our case's chronic ITP, although stable, might have been volatile to such a viral infection; however, rheumatologists should be awere that autoimmune disease flares can be triggered by COVID-19. Standard first-line therapy for new or relapsed acute ITP is usually the use of PSL; however, concerns remain that the COVID-19 disease may worsen. Thrombopoietin receptor agonists are alternative therapeutic options for ITP; however, they have a potential to increase the risk of thrombosis in patients with COVID-19, which causes vascular endothelial damage. Therefore, as our case suggests, IVIg would be a good option for patients with severe ITP who acquire COVID-19, although accumulation of more cases is needed.[6] Interestingly, our patient showed newly detected lupus anticoagulant and biological false positivity, indicating the presence of antiphospholipid antibodies. Antiphospholipid antibodies are frequently observed in patients with SLE, but our patient previously presented as negative. Since the presence of antiphospholipid antibodies with coagulopathy has been reported in patients with COVID-19,[7] special attention should be provided to patients with SLE and COVID-19.

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Response to: 'Exacerbation of immune thrombocytopenia triggered by COVID-19 in patients with systemic lupus erythematosus' by Kondo *et al*

We thank Kondo *et al*[1] for their interest in our study reporting on the course of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease (COVID-19) in a case series of patients with systemic lupus erythematosus (SLE) under long-term treatment with hydroxychloroquine.[2] Currently, there are no data that identify SLE as a risk factor for COVID-19-related immunological complications. Kondo *et al*[1] report a patient with SLE experiencing an exacerbation of immune thrombocytopaenic purpura (ITP) likely triggered by COVID-19. This case raises several subjects for discussion. In the cohort of patients with SLE that we reported, there were no manifestations of lupus activity during the course of COVID-19, except for one patient who had a tenosynovitis at the onset of SARS-CoV-2 infection.[2] Similar to our observations, other studies reported that the occurrence of COVID-19 in patients with SLE was not accompanied by a flare of the autoimmune disease, at least in the short term.[3–5] This finding is rather surprising considering the fact that SARS-CoV-2 is clearly a type I interferon-inducing virus,[6] and that this family of cytokines has a key role in the pathogenesis of SLE flares.[7] The latter consideration notwithstanding, it is expected that the secretion of interferons over a relatively short period of time, as in the course of COVID-19, is unlikely to be sufficient to induce a sustained activation of the immune system leading to an SLE flare. In this regard, we noticed that thrombocytopaenia was the only clinical manifestation of lupus activity in the case reported by Kondo *et al*.[1] This observation is reminiscent of cases of ITP in patients without SLE, reported recently in the context of SARS-CoV-2 infection[8 9] and previously for coronavirus HKU1 infections,[10] which suggests an immunological mechanism common to patients with or without lupus that might be directly linked to the viral impact on the immune response mediated by a type I interferon-independent mechanism. An example for such a mechanism is the increased platelet clearance caused by coating of these cells with non-specific immune complexes or platelet antibodies produced during the immune response against SARS-CoV-2.

While thrombocytopaenia is prominent in severe cases of COVID-19 and clearly associated with poor outcomes and mortality,[11] the occurrence of an ITP during the course of this disease does not seem to be linked with the severity of the viral infection, given that most of the reported cases of COVID-19 in this condition were mild or moderate.[1 8 9] Finally, it is important to note that in the course of this infectious disease, which presents a major thrombotic risk,[12] glucocorticoids and/or eltrombopag were used, without complications, neither infectious nor thrombotic, in patients who were poorly responsive or non-responsive to intravenous immunoglobulin.[8 9] In conclusion, we recommend that new-onset thrombocytopaenia in SLE should be screened for the presence of SARS-CoV-2 during the current COVID-19 epidemic.

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Correspondence on 'Recovery from COVID-19 in a patient with spondyloarthritis treated with TNF-alpha inhibitor etanercept. A report on a patient with COVID-19 with psoriatic arthritis receiving ustekinumab'

We have read with great interest the case of a recovery from COVID-19 in a man with spondyloarthritis treated with etanercept and methotrexate, described by Duret *et al*.[1]

We describe the case of a 53-year-old man suffering from psoriatic arthritis and psoriasis since he was 22, with no other medical conditions. He was previously treated with phototherapy, cyclosporine, methotrexate, acitretin, efalizumab and etanercept.

Since 2010, he was treated with ustekinumab (90 mg) with complete remission of both psoriatic arthritis and psoriasis. On 10 February 2020, he administered an injection of 90 mg ustekinumab. On 28 February, the patient developed low-grade fever, cough and general malaise. On 2 March, he was tested positive for Severe Acute Respiratory Syndrome (SARS) - Coronavirus (CoV)-2, after his office manager was discovered to be affected by COVID-19.

On 5 March, the body temperature rose to 39.5°C, cough and fatigue worsened and he was hospitalised in the infectious disease unit.

On the day of hospitalisation his blood tests revealed leucopenia, anaemia, thrombocytopenia, increased C reactive protein (28 mg/L), ferritin (508 ug/L) and lactate dehydrogenase (228 U/L) while fibrinogen and D-dimer were normal. Chest X-rays were suggestive for SARS-CoV-2 infection. He was treated with chloroquine, lopinavir and ritonavir, methylprednisolone, paracetamol and azithromycin. He initially received high-flow oxygen; however, since dyspnoea was worsening and inflammatory markers were rising, he was transferred to the subintensive pulmonary unit, where he received non-invasive ventilation.

From 13 March, the patient showed a progressive improvement of chest X-rays and respiratory symptoms and, on 19 March, he was discharged. After being tested negative twice, on 2 April, he was considered healed.

To date, only few reports have described the outcome of COVID-19 in rheumatological patients treated with biological agents.[1 2] Notably, an isolated case of COVID-19 in a patient with psoriatic arthritis treated with guselkumab has been reported to occur with only mild symptoms.[2]

To our knowledge, this is the first description of SARS-CoV-2 infection in a patient with psoriatic arthritis and psoriasis treated with ustekinumab.

Ustekinumab targets both interleukin (IL)-23 and IL-12 by binding to their common subunit p40.[3]

Available data currently suggest that targeting the IL-23/IL-17 axis may be beneficial in COVID-19, dampening the systemic inflammation known as 'cytokine storm' which can lead to multiorgan failure and death.[2]

Confirming this hypothesis, a real-life database collecting patients with inflammatory bowel disease affected by COVID-19 reported a lower incidence of adverse outcomes in patients treated with ustekinumab.[4] Likewise, tumour necrosis factor inhibitors seem to be associated with lower admission to intensive care units and lower fatality rates, in agreement with the case described by Duret.[1 4]

Since IL-12 leads Th1 polarisation, which is needed for efficient viral clearance, its inhibition may be deleterious in COVID-19. In fact, polymorphisms of IL-12 receptor less responsive to IL-12 signalling have been associated with increased susceptibility to SARS infection.[5] On the other hand, IL-12 seems to contribute to the inflammatory manifestations of the disease, thus exerting also a detrimental role.[5]

Overall, despite the potential increased risk of infection, ustekinumab may exert a protective action in COVID-19, due to the anti-inflammatory effect of the double neutralisation of IL-12 and IL-23. Further data are warranted to clarify this issue.

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Response to: 'Correspondence on Recovery from COVID-19 in a patient with spondyloarthritis treated with TNF-alpha inhibitor etanercept. A report on a COVID-19 patient with psoriatic arthritis receiving ustekinumab' by Messina *et al*

We thank Messina *et al* for their interest in our article reporting a benign evolution of COVID-19 in a patient with spondyloarthritis (SPA) treated with a combination of disease-modifying antirheumatic drugs (DMARDs), methotrexate and a tumour necrosis factor (TNF)-alpha inhibitor, etanercept.[1 2] We read with deep interest their report on a patient with psoriatic arthritis treated with ustekinumab, an antagonist of the interleukin (IL)-12/23 axis, who developed a moderate form of COVID-19, with full recovery.[1]

Of interest, a Th17 immune response with overexpression of IL-17, among other cytokines, has been shown elsewhere associated with COVID-19-related cytokine release syndrome (CRS).[3–6]

The authors are balancing an apparent paradox of an increased risk of infection, because of a potential compromised viral clearance, in patients exposed to ustekinumab, contrasting with a potential 'protective' effect of IL-12/23 inhibition, which might limit a deregulated Th1 and Th17 immune response leading to CRS associated with severe evolution of COVID-19.[1 6 7]

Presumably, this phenomenon might not be restricted to IL-12/23 inhibitors, but is likely to be extended for a broader panel of conventional, biological or targeted synthetic DMARDs, particularly anticytokine drugs, echoing our observation.[2] In addition, recent registry data have shown lower hospitalisation rates in patients treated with TNF-alpha inhibitors.[8] Conversely, concerns have recently been raised suggesting a detrimental effect, with an increased risk of severe evolution and mortality, in patients treated with B-cell depleting monoclonal antibodies, such as the anti-CD20 rituximab (RTX).[9–11]

Collectively, these emerging data, along with the experience of rheumatologists regarding the risk of infection in patients undergoing immunosuppressive therapies, support the current European League Against Rheumatism (EULAR) recommendations relative to the management of patients with rheumatic and musculoskeletal diseases (RMDs) in the context of COVID-19.[12]

We report here characteristics and outcomes of patients with RMDs followed and treated in our centre (Hôpitaux civils de Colmar; France) who developed COVID-19 under DMARDs (table 1).

A total of 17 patients, including 8 patients with rheumatoid arthritis (RA), 6 patients with SPA, 2 patients with primary Sjögren's syndrome and 1 patient with Still's disease, were confirmed for COVID-19, either by real-time retrotranscription (RT)-PCR on nasopharyngeal swabs performed in 10 symptomatic patients (58.8%), or retrospectively, by serology for detection of SARS-CoV-2 IgM and/or IgG in 5 patients (29.4%) with a history of symptoms highly suggestive of SARS-CoV-2 infection, or based solely on a typical bilateral pneumonia pattern on CT-scan for 2 patients (11.8%).

Immunomodulatory treatment regimens consisted on csDMARDs for 13 patients (76.5%), including methotrexate in 9 patients (52.9%), leflunomide in 3 patients (17.6%), azathioprine in 1 patient (5.9%) and hydroxychloroquine in 1 patient (5.9%). A total of 10 patients (58.8%) were treated with bDMARDs including TNF inhibitors in 6 patients (35.3 %), IL-6 receptor inhibitor, tocilizumab, in 3 patients (17.6 %) and anti-CD20 monoclonal antibody, rituximab, in 1 patient (5.9%). One patient (5.9%) was treated with a tsDMARD, the Janus Kinase (JAK) inhibitor baricitinib in combination with methotrexate 20 mg, subcutaneous, weekly. Seven patients (41.2%) were treated with an association of csDMARDs and bDMARDs/tsDMARDs. In our study, no patients treated with IL-17A or IL-12/23 inhibitors have been confirmed for SARS-CoV-2 infection.

Overall, 16 patients out of the 17 analysed (94.1%) fully recovered, regardless of the type of RMD and the type of immunomodulation considered. A benign evolution was observed in 13 patients (76.5%), while 3 patients (17.6%) developed a moderate form requiring hospitalisation but without the need for invasive ventilation.

Unfortunately, 1 patient (5.9%), an 83-year-old man with a spondyloarthritis treated with golimumab, a TNF-alpha inhibitor monoclonal antibody, experienced a severe form of COVID-19, complicated by a fatal acute respiratory distress syndrome. In accordance with data obtained in the general population, age and cardiovascular comorbidities appear to be the dominating risk factors of severe evolution and death related to COVID-19. We presume that in this patient, age and hypertension might have accounted, more likely than TNF-alpha inhibition, for the unfavourable outcome.

Furthermore, in our study, only one patient treated with the anti-CD20 monoclonal antibody RTX developed COVID-19, with mild symptoms and a favourable outcome. To date, no other patient treated with RTX from our centre was confirmed for COVID-19 either by RT-PCR, CT-scan or serology.

Data concerning COVID-19 outcomes in patients treated with RTX are still scarce. It is conceivable that while some immunomodulating agents (eg, anti-TNF; anti-IL6R; anti-IL1; anti-IL17A; anti-IL12/23; JAKi) might confer an advantage over the exaggerated immune response and cytokine storm triggered by SARS-CoV-2, other agents, including those acting on cells responsible for humoral immunity, would appear to be detrimental in the context of COVID-19. In addition, as suggested by Mathian *et al*[13] and Avouac *et al*[10] in their reports of COVID-19 in systemic lupus erythematosus and systemic sclerosis, respectively, severe autoimmune diseases, especially when associated with organ damages are probably at higher risk of developing severe forms of COVID-19, in comparison to patients with SPA or RA. Nonetheless, subgroups of patients with RA, especially those receiving RTX seem to be at higher risk than patients with RA treated with other cs/b/tsDMARDs.[11] Organ damages associated with age, a long-standing evolution of the disease, cardiovascular risk factors and interstitial lung disease (ILD) that are likely to be found in higher proportions in this population, along with the impaired B-cell response and reduced antibody production raised against the SARS-CoV-2, might explain the potential detrimental effect reported in the subgroup of patients with RA treated with anti-CD20. Clarifying whether this plausible increased risk related to RTX use is due to B-cell depletion or to comorbidities represents an important issue that is not fully resolved yet.

Our results, although collected on a limited number of patients, are consistent with the data in the literature so far available on COVID-19 and RMDs.[14–16] If it is not protective, at least no warnings suggestive of a pejorative evolution of COVID-19 have been detected. However, these studies do not fully clarify whether or not patients with RMDs are at increased risk of developing severe forms of COVID-19 compared with the general population.[17 18]

Table 1 Clinical characteristics of patients with rheumatic and musculoskeletal diseases (RMDs) treated with disease-modifying antirheumatic drugs (DMARDs) and COVID-19 outcomes

Variable	Spondyloarthritis	Rheumatoid arthritis	Sjögren's syndrome	Still's disease	All RMDs
Number of patients	6	8	2	1	17
Sex, female, n (%)	2 (33.3)	6 (75)	2 (100)	1 (100)	11 (64.7)
Age at COVID-19 onset, median (range)	54.5 (53–83)	57 (49–71)	63.5 (59–68)	35	55 (35–83)
Comorbidities and risk factors					
Hypertension, n (%)	2 (33.3)	4 (50)	1 (50)	0	7 (41.2)
Diabetes, n (%)	0	1 (12.5)	0	0	1 (5.9)
BMI, median (range)	28.6 (26.9–34)	26.2 (20.7–45.5)	39.4 (31.6–47.2)	18.97	28 (18.97–47.2)
COVID-19 diagnosis					
Positive SARS-CoV-2 RT-PCR, n (%)	3 (50)	4 (50)	2 (100)	1 (100)	10 (58.8)
Positive SARS-CoV-2 serology, n (%)	2 (33.3)	3 (37.5)	0	0	5 (29.4)
Positive CT-scan, n (%)	2 (33.3)	3 (37.5)	0	1 (100)	6 (35.3)
Positive CT-scan and serology, n (%)	0	1 (12.5)	0	0	1 (5.9)
Positive CT-scan and RT-PCR, n (%)	1 (16.7)	1 (12.5)	0	1 (100)	3 (17.6)
Positive CT-scan without RT-PCR or serology available, n (%)	1 (16.7)	1 (12.5)	0	0	2 (11.8)
Rheumatic disease treatment previous to COVID-19					
csDMARDs, n (%)	4 (66.7)	7 (87.5)	2 (100)	0	13 (76.5)
Methotrexate	4 (66.7)	5 (62.5)	0	0	9 (52.9)
Leflunomide	0	2 (25)	1 (50)	0	3 (17.6)
Hydroxychloroquine	0	1 (12.5)	0	0	1 (5.9)
Azathioprine	0	0	1 (50)	0	1 (5.9)
bDMARDs, n (%)	5 (83.3)	3 (37.5)	1 (50)	1 (100)	10 (58.8)
TNF inhibitors, n (%)	5 (83.3)	1 (12.5)	0	0	6 (35.3)
Adalimumab	1 (16.7)	0	0	0	1 (5.9)
Etanercept	1 (16.7)	1 (12.5)	0	0	2 (11.8)
Golimumab	2 (33.3)	0	0	0	2 (11.8)
Infliximab	1 (16.7)	0	0	0	1 (5.9)
IL-6 receptor inhibitors, n (%)	0	1 (12.5)	1 (50)	1 (100)	3 (17.6)
Tocilizumab	0	1 (12.5)	1 (50)	1 (100)	3 (17.6)
Anti-CD20 monoclonal antibodies, n (%)	0	1 (12.5)	0	0	1 (5.9)
Rituximab	0	1 (12.5)	0	0	1 (5.9)
tsDMARDs, n (%)	0	1 (12.5)	0	0	1 (5.9)
JAK inhibitors, baricitinib	0	1 (12.5)	0	0	1 (5.9)
Combination of csDMARD and bDMARD/tsDMARD, n (%)	3 (50)	3 (37.5)	1 (50)	0	7 (41.2)
COVID-19 evolution and outcomes, n (%)					
Benign	4 (66.7)	6 (75)	2 (100)	1 (100)	13 (76.5)
Moderate	1 (16.7)	2 (25)	0	0	3 (17.6)
Severe	1 (16.7)	0	0	0	1 (5.9)
Recovery	5 (83.3)	8 (100)	2 (100)	1 (100)	16 (94.1)
Death	1 (16.7)	0	0	0	1 (5.9)

bDMARDs, biological disease-modifying antirheumatic drugs; BMI, body mass index; csDMARDs, conventional synthetic DMARDs; IL-6, Interleukin 6; JAKi, Janus Kinase inhibitors; RMDs, rheumatic and musculoskeletal diseases; TNF-α, tumour necrosis factor-alpha; ; tsDMARDS, targeted synthetic DMARDs.

Given the limited data, case reports and case series contribute to increase knowledge on the impact of immunomodulatory treatments in patients with autoimmune and rheumatic diseases. Importantly, in this context of uncertainty, and while waiting for wider datasets, each patient may reveal unique features when exposed to COVID-19 and should be scrupulously collected and considered.

However, data derived from monocentric case series should be interpreted with care, given the limited number of patients, which is prompt to impair statistical power. In addition, selection bias and 'centre/hotspot-effect' might compromise extrapolation of the results. As a consequence, robust data collected on a large amount of patients, with a rigorous analysis adjusted on identified risk factors, especially age, body mass index, cardiovascular risk factors (eg, diabetes mellitus; hypertension), ILDs and treatment regimens are needed to draw further and reliable conclusions.

Collecting data globally is a major challenge. There is no doubt that the impressive responsiveness and collective effort of the rheumatology community, as reflected by the 'COVID-19-Global-Rheumatology-Alliance' and national/international registries (in France, the 'French RMD COVID-19 cohort' (FAI2R/SFR/SNFMI consortium)) and its future contribution to the 'EULAR-COVID-19-Database', will provide new insights regarding the course of SARS-CoV-2 infection occurring in patients with autoimmune and rheumatic diseases but also the impact of DMARDs on COVID-19 outcomes.

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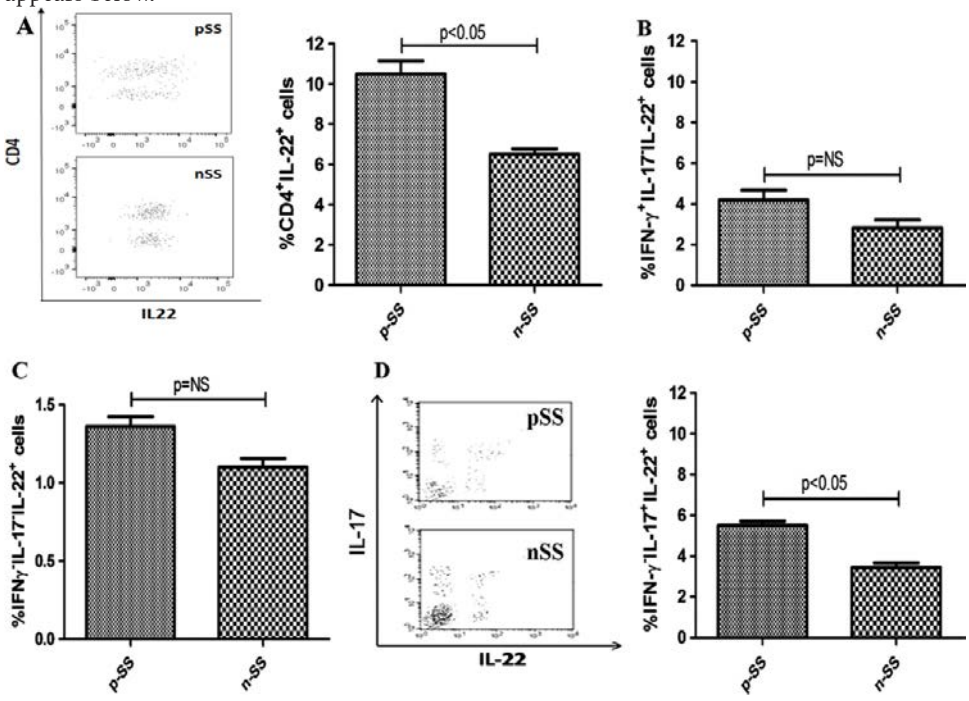
Correction: *Potential involvement of IL-22 and IL-22-producing cells in the inflamed salivary glands of patients with Sjogren's syndrome*

Ciccia F, Guggino G, Rizzo A, *et al*. Potential involvement of IL-22 and IL-22-producing cells in the inflamed salivary glands of patients with Sjogren's syndrome. *Ann of Rheum Dis* 2012;71:295–301.doi:10.1136/ard.2011.154013.

This article contains errors in figure 3A where, for an inadvertence in the preparation of the figure, authors have introduced similar plots derived from the preliminary analysis of the same raw data obtained from the analysis of a patient with pSS. The correct version of the figure appears below.



Ann Rheum Dis 2021;**80**:e81. doi:10.1136/ard.2011.154013.corr1

Correction: *Transcutaneous auricular vagus nerve stimulation reduces pain and fatigue in patients with systemic lupus erythematosus: a randomised, double-blind, shame-controlled pilot trial*

Aranow C, Atish-Fregoso Y, Lesser M, *et al*. Transcutaneous auricular vagus nerve stimulation reduces pain and fatigue in patients with systemic lupus erythematosus: a randomised, double-blind, shame-controlled pilot trial. *Ann Rheum Dis* 2021;80:203–8.

The second author should be Yemil Atisha-Fregoso and not Yemil Atish-Fregoso.
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Ann Rheum Dis 2021;**80**:e82. doi:10.1136/annrheumdis-2020-217872corr1

Correction: *Safety profile of upadacitinib in rheumatoid arthritis: integrated analysis from the SELECT phase III clinical programme*

Cohen SB, van Vollenhoven RF, Winthrop KL, *et al*. Safety profile of upadacitinib in rheumatoid arthritis: integrated analysis from the SELECT phase III clinical programme. *Ann of Rheum Dis* 2021;80:304–1. doi:10.1136/annrheumdis-2020-218510.

The link for reference 2 should be: https://www.accessdata.fda.gov/drugsatfda_docs/label/2020/211675s001lbl.pdf

The correct author name for reference 21 should be European Medicines Agency.

The correct citation details for reference 25 should be Arthritis Care Res 2013; 65:1600-7.

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Correction: *Methotrexate and BAFF interaction prevents immunization against TNF inhibitors*

Bitoun S, Nocturne G, Ly B, *et al*. Methotrexate and BAFF interaction prevents immunization against TNF inhibitors. *Ann of Rheum Dis* 2018;77:1463–70. doi:10.1136/annrheumdis-2018-213403.

The correct number of injections of etanercept in mice should be 3 times a week and not 2 times a week as stated in the article.

Ann Rheum Dis 2021;**80**:e84. doi:10.1136/annrheumdis-2018-213403corr1