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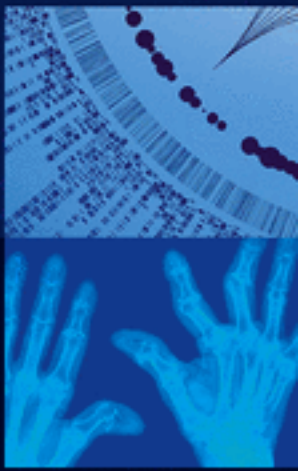

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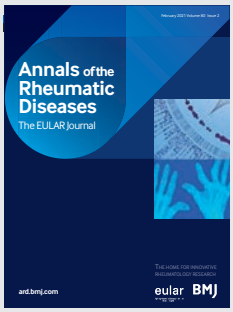

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Hydroxychloroquine shortages among patients with systemic lupus erythematosus during the COVID-19 pandemic: experience of the Systemic Lupus International Collaborating Clinics

Early scientific and public enthusiasm for hydroxychloroquine (HCQ) as a potential therapy for COVID-19 has prompted over 100 registered trials to date, although its efficacy remains to be demonstrated.[1] Unfortunately, accelerated demand for HCQ has the potential to diminish supplies for patients with systemic lupus erythematosus (SLE), which is worrisome due to the known risks of SLE flare after HCQ withdrawal.[2] We previously reported that rheumatologists in most Canadian provinces observed HCQ shortages early in the COVID-19 pandemic.[3] However, data are lacking on the global experience with HCQ access during the pandemic, specifically in SLE.

On 4 May 2020, we distributed an electronic survey to the 42 Systemic Lupus Erythematosus International Collaborating Clinics (SLICC) members affiliated with SLE referral centres (https://sliccgroup.org), with reminders after 1 and 3 weeks. Physicians were asked about experiences with HCQ shortages during the COVID-19 pandemic, and whether they

Table 1 Experience of HCQ shortages among patients with SLE during the pandemic and regional mitigation strategies

Country* (n responses)	Canada (n=7)	USA (n=8)	France (n=1)	UK (n=4)	Spain (n=1)	Italy (n=1)	Sweden (n=1)	Denmark (n=1)	Argentina (n=1)	Australia (n=1)	Turkey (n=2)	Singapore (n=1)	South Korea (n=1)
HCQ access issues													
Concerned about HCQ shortages, n													
Current	1	2	1	0	0	0	0	0	0	1	0	0	0
Resolved	1	5	0	3	1	1	0	0	1	0	0	0	0
Physicians contacted by patients re: HCQ access issue, n	3	8	1	3	1	1	0	0	1	1	1	0	0
Estimated % of patients with SLE affected (range)	3%–5%	5%–40%	70%	0%–5%	NR	20%	–	–	30%	50%	0%–1%	–	–
Regional mitigation strategies													
Limiting authorised prescribers	+		+			+	+	+		+		+	
Limiting HCQ to specific diagnoses	+	+			+	+		+		+	+		
Limiting dispensed supply	+												
Physician/patient association advocacy	+	+		+									
Hospital or pharmacies reserved supply for patients with SLE		+											

*One respondent did not indicate country of origin and is not included in this table.
HCQ, hydroxychloroquine; NR, not reported; SLE, systemic lupus erythematosus.

had been contacted by patients and/or pharmacists regarding difficulties accessing HCQ. Physicians who answered 'yes' to the latter question were asked to estimate how many and what proportion of their patients with SLE were affected. We inquired about regional measures taken that exacerbated or helped mitigate HCQ shortages for patients with SLE (free text responses).

We received 31 responses (rate 74%) from 13 of 15 countries represented in SLICC, mostly from Europe (29%), the USA (26%) and Canada (23%). Over half (55%) reported either previous (39%) or current (16%) HCQ shortages among patients with SLE during the pandemic (see table 1). Two-thirds (65%) were contacted by patients and pharmacies regarding difficulties accessing HCQ. Seventeen provided estimates of the number and proportion of their patients affected, which corresponded to a median of 40 (IQR 15–90) patients per physician representing 15% (IQR 5%–35%) of respective SLE populations. Seven physicians noted that shortages resolved within 2–8 weeks. Members from four countries (Sweden, Denmark, Singapore, South Korea) reported no HCQ access issues among their patients.

Physicians identified regional factors contributing to HCQ shortages, including diversion of HCQ to hospitals (n=3), for clinical trials (n=2) or off-label empiric prescribing for COVID-19 (n=1).

Twenty-three (74%) reported system-level measures taken during the pandemic to preserve HCQ access for patients with SLE, which included limiting prescribing capabilities to specific specialties (n=9) or diagnoses (n=10) and limiting dispensed supply (n=3). Some restrictions may have inadvertently delayed HCQ access for patients with SLE, who had to wait for physicians to update diagnostic codes in medical records, confirm diagnoses with pharmacies or apply for waivers. In some cases, patients had to register for pharmacy dispensing programmes or were subjected to general dispensing restrictions. In Canada, the USA and the UK, patient and physician organisations advocated to health authorities for the rapid resolution of HCQ shortages.

Currently, there is no substitution for antimalarials in SLE. HCQ reduces disease flares,[2] damage[4] and mortality,[5] with fewer adverse effects compared with glucocorticoids and immunosuppressants.[6] Regardless of the ultimate efficacy of HCQ for COVID-19, preserving patients' access to critical medications remains paramount. We observed that HCQ prescription restrictions were a common short-term strategy, although our cross-sectional survey was not intended to evaluate which mitigation strategies were most effective. Furthermore, physician estimates from single tertiary centres do not represent a comprehensive account of HCQ shortages or mitigation strategies and may not reflect the experience of an entire region or country.

According to this survey, HCQ access issues for patients with SLE occurred in multiple countries and continents during the COVID-19 pandemic. Because SLE can flare as little as 2 weeks after HCQ cessation,[2] further study of outcomes among patients who lost access to HCQ during the pandemic is warranted.

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Interleukin-6 receptor blockade with subcutaneous tocilizumab in severe COVID-19 pneumonia and hyperinflammation: a case–control study

Many patients with severe COVID-19 rapidly progress to critical disease with refractory hypoxemia requiring invasive mechanical ventilation (IMV).[1] Elevated levels of C reactive protein (CRP) and interleukin-6 (IL-6), reflecting an hyperinflammatory response, identify patients at risk of progression to refractory hypoxemia and death.[2] Recent evidences suggested that high-dose intravenous tocilizumab (TCZ), a humanised anti-IL-6 receptor antibody, may rapidly reduce fever and inflammatory markers, and improve oxygenation in severe to critical COVID-19.[3–5] Data on the safety and efficacy of subcutaneous TCZ, already approved for the treatment of rheumatoid arthritis, are limited. The aim of this study was to compare the clinical course and outcomes of patients treated with subcutaneous TCZ on top of standard of care (SOC) with those of patients receiving SOC only.

In this retrospective case–control study, we treated with TCZ 324 mg, given as two concomitant subcutaneous injections, all consecutive patients at Pescara General Hospital, Italy between 28 March and 21 April 2020, with laboratory-confirmed COVID-19 pneumonia (involving ≥20% of lung parenchyma on chest CT), hyperinflammation (CRP ≥20 mg/dL), hypoxemia (oxygen saturation <90% on room air) requiring supplemental oxygen through nasal cannulas or mask, who had no contraindications to treatment such as bacterial or fungal infection, neutropenia or liver injury. Patients signed an informed consent for the off-label use of TCZ. We reviewed all patients hospitalised for COVID-19 pneumonia when TCZ was not available in our centre and identified those matching the same treatment criteria: 40 subjects matched for sex and age were selected as SOC group (online supplementary table 1).

Clinical data were available for all patients until discharge or death, and for those discharged prior to day 35, additional clinical information was obtained by phone contact. Data are presented as median and IQR. Within-group changes were compared using the Wilcoxon test for paired analysis, and between-groups differences were analysed using the Mann-Whitney U test for unpaired test. Log-rank (Mantel-Cox) analysis was used to compare event-free survival between the two groups.

Treatment with TCZ was well tolerated, with no serious or clinically relevant adverse events. None of the patients experienced neutropenia (absolute neutrophil count <1000/mm^3), one (2.5%) developed bacterial pneumonia while on IMV, as compared with three (7.5%) in the SOC group, and one (2.5%) had transient moderate liver injury (elevation in alanine aminotransferase five times above the upper limit of normal value), as compared with none in the SOC group (online supplementary table 2).

Treatment with TCZ resulted in an improvement of oxygenation, as assessed by the ratio of partial pressure of oxygen to fraction of inspired oxygen (P/F), which increased at day 1 (+8%, IQR −9 to +25; p=0.005 for within-group and p<0.006 for between-group comparisons) and day 3 (+25%, IQR +10 to +52; p<0.001 for within-group and p<0.001 for between-group comparisons), whereas it continued to worsen in the SOC group (p<0.001, online supplementary figure S1).

When compared with SOC-treated patients, fewer TCZ-treated patients had disease progression, defined as requirement of IMV or death (2 (5%) vs 12 (30%), p=0.003), or died (2 (5%) vs 11 (27.5%), p=0.006) (figure 1). Online supplementary

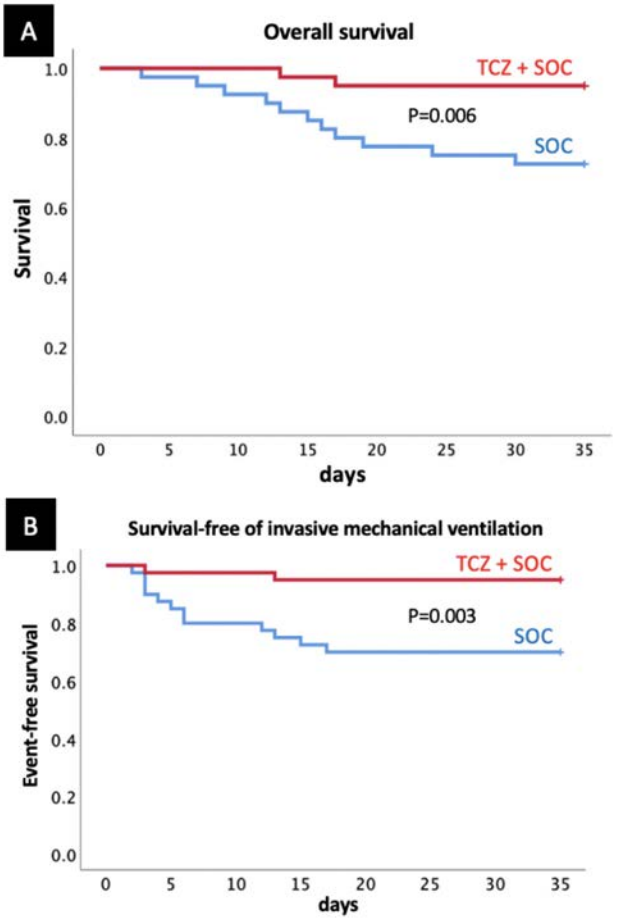

Figure 1 Survival and survival-free of invasive mechanical ventilation. Patients receiving tocilizumab (TCZ) on top of standard of care (SOC) were significantly less likely to die (A), or need invasive mechanical ventilation or die (B) than patients treated with SOC only matched for sex, age and severity of illness (log-rank Mantel-Cox χ^2 7.418, p=0.006 and χ^2 8.605, p=0.003 for panels A and B, respectively).

table 3 shows the WHO Ordinal Scale for Improvement for the two groups.

TCZ was associated with a reduction in CRP at day 1 (−32%, IQR −18 to −60) and day 3 (−83%, IQR −63 to −83; p<0.001 for within-group changes), whereas it increased in the SOC group (p<0.001 for between-group comparisons at both time points; online supplementary figure 2).

Our findings suggest that IL-6 receptor blockade with subcutaneous TCZ may reduce the risk of progression from severe to critical COVID-19 and mortality when administered on top of SOC. Infection from SARS-CoV viruses results in an inflammasome-mediated response characterised by elevated levels of interleukin-1β,[6] which trigger IL-6 release, promoting lung injury. TCZ is often provided intravenously on a compassionate-use basis to patients with COVID-19 with refractory hypoxemia on IMV. Randomised controlled trials are under way with IL-6 blockers. Nevertheless, many patients with COVID-19 are hospitalised with hypoxemia not requiring IMV. Hyperinflammation may promote disease progression as indicated by higher levels of inflammatory biomarkers being associated with increased risk for dire outcomes.[2] We herein report on the innovative use of early subcutaneous TCZ in a subgroup of patients with severe COVID-19 pneumonia who are at risk for progression to IMV and death. While limited by the small number of patients included and the non-random nature of the comparisons, data appear reassuring in terms of safety, and encouraging when compared with those of patients treated with SOC in our centre or other published cohorts.[1 3–5]

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The use of tocilizumab and tofacitinib in patients with resolved hepatitis B infection: a case series

The use of immunosuppressive medications in people with hepatitis B virus (HBV) infection is associated with an increased risk of HBV reactivation, which can lead to liver failure and death. Tumour necrosis factor inhibitors, rituximab and other biologic treatments have been associated with HBV reactivation in up to 24% of people with resolved HBV (positive core antibody (HBcAb), negative surface antigen (HBsAg) and positive or negative surface antibody (HBsAb)) and 34% of people with chronic HBV (positive HBsAg); reactivation risk varies based on HBsAb status.[1–3] Both tocilizumab (interleukin-6 (IL-6) receptor inhibitor) and tofacitinib (Janus kinase (JAK) inhibitor) interfere with IL-6 signalling, which moderates immune control of chronic HBV. Although HBV reactivation has been reported with tocilizumab and tofacitinib in Asia, limited data describe this risk in the USA.[4]

We performed a retrospective study of people who were prescribed tocilizumab or tofacitinib and had resolved or chronic HBV infection between 1995 and 2018 in the Partners Health Care System (PHS).[5] We extracted relevant variables from the electronic health record and defined HBV reactivation as: a greater than 10-fold increase or an absolute increase greater than 10^5 copies/mL in HBV DNA level from baseline or a positive HBsAg when previously negative. This study was considered exempt by the PHS Institutional Review Board.

Of the 20 people identified, all were HBcAb positive and HBsAg negative. Four received tofacitinib and tocilizumab sequentially such that there were 24 medication exposures. Sixteen patients (67%) received tocilizumab and eight patients (33%) received tofacitinib (table 1). Everyone treated with tocilizumab (16, 100%) and seven (88%) of those prescribed tofacitinib were HBsAb positive. The median age at treatment initiation was 59.4 years (tofacitinib) and 66.1 years (tocilizumab), and the majority were female in both groups. In each group, the most common diagnosis was rheumatoid arthritis, 75% received concurrent rheumatic disease medications and 25% received entecavir or tenofovir within 2 years of tocilizumab or tofacitinib (table 1).

Median follow-up time after treatment initiation was 4.0 years (IQR: 1.6–5.9) (tocilizumab) and 3.1 years (IQR: 0.9–5.7) (tofacitinib). During follow-up, all had aminotransferases measured at least once; in 63% (tocilizumab) and 38% (tofacitinib), aminotransferases were checked at least four times annually for 2 years. Six experienced mild, transient aminotransferase elevations and one had severe elevation (>10× normal) attributed to ischaemic injury; none were attributed to HBV reactivation. Among those with HBV DNA or HBsAg assessed after treatment initiation (88% in the tocilizumab group, median 3 tests; 75% in the tofacitinib group, median 2.5 tests), none were positive.

In conclusion, we observed no episodes of HBV reactivation in people with resolved HBV infection treated with tocilizumab or tofacitinib with over 3 years of follow-up time in a US healthcare system. The majority were HBsAb positive, which reduces but does not eliminate reactivation risk; HBV reactivation occurs in up to 6% of people with HBsAb/HBcAb positivity receiving

Table 1 Demographics, clinical characteristics and follow-up of study population

Characteristic	Tocilizumab-treated patients (N=16)	Tofacitinib-treated patients (N=8)
Age (years); median (IQR)	66.1 (45.4–71.3)	59.4 (42.4–70.9)
Female, n (%)	9 (56)	7 (88)
Race, n (%)		
White	7 (44)	3 (38)
Black or African–American	4 (25)	4 (50)
Asian	4 (25)	0 (0)
Unknown/other	1 (6)	1 (12)
Ethnicity, n (%)		
Non-Hispanic	15 (94)	8 (100)
Unknown	1 (6)	0 (0)
Diagnosis for medication indication, n (%)*		
Rheumatoid arthritis	10 (63)	7 (88)
Psoriatic arthritis	0 (0)	1 (13)
Giant cell arteritis	3 (19)	0 (0)
Lymphoma	2 (13)	0 (0)
Adult-onset Still's disease	1 (6)	0 (0)
Disease duration (years), median (IQR)	3.6 (1.1–10.5)	7.6 (2.9–17.5)
Baseline positive HBV serologies, n (%)		
HBcAb	16 (100)	8 (100)
HBsAg	0 (0)	0 (0)
HBsAb	16 (100)	7 (88)
Baseline HBV DNA assessed	10 (63)	6 (75)
Comorbidities†	1 (6)	1 (13)
Cirrhosis, n (%)	5 (31)	1 (13)
Diabetes	7 (44)	4 (50)
Hypertension	2 (13)	1 (13)
Coronary artery disease		
Time receiving medication (years), median (IQR)‡	1.4 (0.2–4.2)	0.8 (0.4–1.2)
Follow-up time (years), median (IQR)§	4.0 (1.6–5.9)	3.1 (0.9–5.7)
Concurrent immunomodulatory therapy, n (%)¶	12 (75)	6 (75)
Oral glucocorticoids	7/12 (58)	4/6 (67)
csDMARD	7/12(58)	4/6 (67)
Rituximab	1/12 (8)	0/6 (0)
Antiviral treatment,n (%)**	4 (25)	2 (25)
Reactivation of HBV during follow-up, n (%)*		
Yes	0 (0)	0 (0)
No	14 (88)	6 (75)
Unknown (no follow-up HBV DNA or HBsAg)	2 (13)	2 (25)
Number of repeat HBsAg and/or HBV DNA tests, median (IQR)	3 (1–6)	2.5 (0.5–7)

*Percentages do not add up to 100% due to rounding.
†Comorbidities were defined by presence of the diagnosis in the electronic health record.
‡Time receiving medication refers to the time from medication initiation to the discontinuation time as determined by electronic health record notes or the time of manuscript submission for patients still receiving the medication.
§Follow-up time refers to the time from the initial medication prescription to the most recent patient encounter in our healthcare system.
¶Percentages do not add up to 100% as some patients received multiple types of immunomodulatory medications within the 2 years following medication. csDMARDs included methotrexate, leflunomide and sulfasalazine in the tocilizumab group and methotrexate and sulfasalazine in the tofacitinib group.
**Refers to patients who received antiviral treatment at any point within the 2 years following medication. In the tocilizumab group, three patients received entecavir and one received tenofovir, one of which was after the study medication. In the tofacitinib group, one patient received tenofovir and one patient received entecavir, though both after study medication.
csDMARD, conventional syntheticdisease-modifying antirheumatic drug; HBcAb, hepatitis B core antibody; HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.;

rituximab.[1] Pretreatment HBV screening remains important because of the theoretical risk of reactivation.[1–3 6] A quarter of people in our study were prescribed antivirals, reflecting the uncertainty regarding best practices for patients with resolved HBV. Limitations of our study include no cases of chronic HBV, lack of HBV genotype data, small sample size, lack of a control group and use of antiviral therapy by 25%. Our findings suggest that tocilizumab or tofacitinib may be safely used in patients with resolved HBV infection, particularly in those who are HBsAb positive.

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Glucocorticoids: surprising new findings on their mechanisms of actions

Frank Buttgereit

Glucocorticoids are highly effective drugs that are used very widely to inhibit inflammation and to modulate the immune system.[1–3] Their great importance in the treatment of various rheumatic diseases is undisputed.[4–7] However, although their introduction into clinical medicine was more than 70 years ago, we have understood only a fraction of their mechanisms of action. This is mainly due to their highly pleiotropic effects.[8 9] A therapeutically used monoclonal antibody against tumour necrosis factor alpha (TNFalpha) neutralises this molecule, and that is it. In contrast, glucocorticoids as hormones regulate an estimated 20% of the entire genome.[10 11] The synthesis of cytokines, chemokines, adhesion molecules, receptors and many enzymes, mediators and other proteins is either upregulated or downregulated. In addition, various mechanisms of their action exist whereby we distinguish between genomic and non-genomic effects. These underlying mechanisms of action are known in principle and discussed in detail elsewhere,[3 12–14] so the current knowledge is only summarised here in the form of table 1. However, our understanding of glucocorticoid-mediated immunoregulation still has substantial gaps, not only but especially regarding effects of glucocorticoids in specific cell types and their key cellular targets in particular disease states, and the actions these hormones broadly induce in cells and tissues versus those that are unique to the immune system.[9 15]

GLUCOCORTICOID EFFECTS ARE HIGHLY DEPENDENT ON CELL TYPE

Given this background, research in the field of glucocorticoids—sometimes justifiably referred to as 'old friends'[1]—continues to be very active. If one enters the search term "glucocorticoids" in PubMed, you will get >230 000 hits. By comparison, the search term "TNF alpha inhibitors" gets only about 56 000 hits. It may be objected that research on glucocorticoids has been going on much longer than that on TNFalpha inhibitors. However, even if you only look at the year 2018, for example, you will find 7900 hits versus 2300 hits. Therefore, it is not surprising that we keep learning unexpected news. For example, the *Journal of Experimental Medicine* published in 2019 an outstanding paper describing surprising and for rheumatologists and clinical immunologists relevant news in research into the effects of glucocorticoids. Franco *et al* have investigated the transcriptional effects of glucocorticoids at the level of signalling pathways for nine primary human cell types obtained from healthy donors.[11] The cells studied included for example, B cells, CD4+ T cells, monocytes and neutrophils. The authors report that glucocorticoid effects are highly dependent on cell type with regard to the regulation of genes and signalling pathways. They found methylprednisolone to induce cell specific differences in the expression of more than 9000 unique genes or ~17% of the human transcriptome. I agree with the authors' interpretation that these results lead to a fundamentally new mechanistic understanding of the effects of glucocorticoids. It is clearly not a one-fits-all concept, but rather that these drugs trigger multifactorial, cell-specific effects. This finding has the potential to develop more selective, cell-specific immunoregulatory therapies.

11B-HYDROXYSTEROID DEHYDROGENASES REGULATE GLUCOCORTICOID EFFECTS

Another example of unexpected significant new findings is published in *Annals of Rheumatic Diseases*. Fenton *et al*[16] have dealt with a scientifically rather neglected area in glucocorticoid research, the pre-receptor metabolism. As a background, the type 1 and type 2 (11β-HSD1 and 2), act as key regulators by changing the balance between active and inactive glucocorticoids.[17] It is well known that in this way they have a major influence on the expression of glucocorticoid effects in the target cells. Endogenous (ie, physiologically produced) and exogenous (ie, therapeutically given) glucocorticoids circulate in both their active and inactive forms. The (mostly) hepatic 11βHSD1 facilitates the systemic regeneration of biologically active glucocorticoids (cortisol/hydrocortisone, corticosterone, prednisolone) from their inactive forms (cortisone, 11-dehydrocorticosterone, prednisone) by its oxidoreductase (11β reductase) action. Through 11-β dehydrogenation, this enzyme can also facilitate the reverse reaction to some small extent, but it is mainly the (renal) 11βHSD2, which unidirectionally inactivates glucocorticoids (figure 1A).[13 18]

Fenton *et al* focused their research not on this systemic, but on the peripheral, intracellular glucocorticoid metabolism in target cells. Both active and inactive glucocorticoid molecules are lipophilic substances that can easily penetrate the cell membrane and thus enter the cytosol of targets such as primary and secondary immune cells, fibroblasts and osteoblasts. However, before they can bind to the cytosolic glucocorticoid receptor alpha to trigger genomic effects (table 1), also in the target cell the activation status is determined by intracellular metabolism via the 11βHSD enzymes. As in the liver, the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme 11βHSD1 causes local glucocorticoid activation by reduction. In contrast, the 11βHSD2 enzyme catalyses a rapid inactivation by oxidation through the reverse reaction. In order to investigate the contribution of the local 11β-HSD1 enzyme to the local anti-inflammatory properties of glucocorticoids, the authors conducted carefully designed and performed experiments. The primary aim was to measure in murine polyarthritis models the anti-inflammatory properties of orally administered corticosterone in mice with global, myeloid and mesenchymal targeted transgenic deletion of 11β-HSD1. They show that the global deletion of 11β-HSD1 resulted in glucocorticoid treatment being ineffective, proven by findings of persistent synovitis, joint destruction and inflammatory leucocyte infiltration. This was partially reproduced with myeloid 11β-HSD1 deletion (targeted towards neutrophils, macrophages and granulocytes), but not with mesenchymal 11β-HSD1 deletion (targeting primary fibroblasts and osteoblasts). It was also found that paracrine GC signalling between cell populations can overcome targeted deletion of 11β-HSD1. Taken all observations together, the authors conclude that

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Table 1 Molecular mechanisms of GC actions[3 12–14]

Category	Molecular mechanism		Brief description
Genomic effects			***Mediated via direct or indirect interaction between the GC-GRα complex and DNA***
	Trans-activation		*Induction of gene expression*
		Dimer transactivation	GC-bound GRα homodimers bind to DNA GREs to positively regulate downstream gene expression (induction)
		Monomer transactivation	GC-bound GRα monomers bind to GREs and recruit co-activators to influence secondary transcription factor regulation, thereby positively regulating downstream gene expression
		Monomer tethering transactivation	GC-bound GRα monomers bind to a secondary transcription factor to positively regulate downstream gene expression
	Trans-repression		*Suppression of gene transcription*
		Dimer transrepression	GC-bound GRα homodimers bind to DNA GREs to negatively regulate downstream gene expression (suppression)
		Monomer transrepression	GC-bound GRα monomers bind to GREs and recruit co-repressors to influence secondary transcription factor regulation, thereby negatively regulating downstream gene expression
		Monomer tethering transrepression	GC-bound GRα monomers bind to a secondary transcription factor to negatively regulate downstream gene expression
Non-genomic effects			***Do not require direct interaction of the GC-GRα complex with DNA***
	Non-specific physicochemical interactions with membranes		GCs at high concentration intercalate into membranes thereby changing their physicochemical properties as well as activities of membrane-associated proteins
	Chaperone protein signalling		Chaperone proteins released from the multi-protein complex after the binding of GCs to the GRα modify signalling pathways
	Via cell membrane receptors		GCs bind to cell membrane-bound receptors and mediate transmembrane activity resulting in non-genomic signalling
	Competition for PI3K		GC-bound GRα sequestrates PI3K, thereby interfering with downstream protein kinase B signalling

GCα, glucocorticoid receptor alpha; GC(s), glucocorticoid(s); GREs, glucocorticoid response elements; PI3K, phosphoinositide 3-kinase.

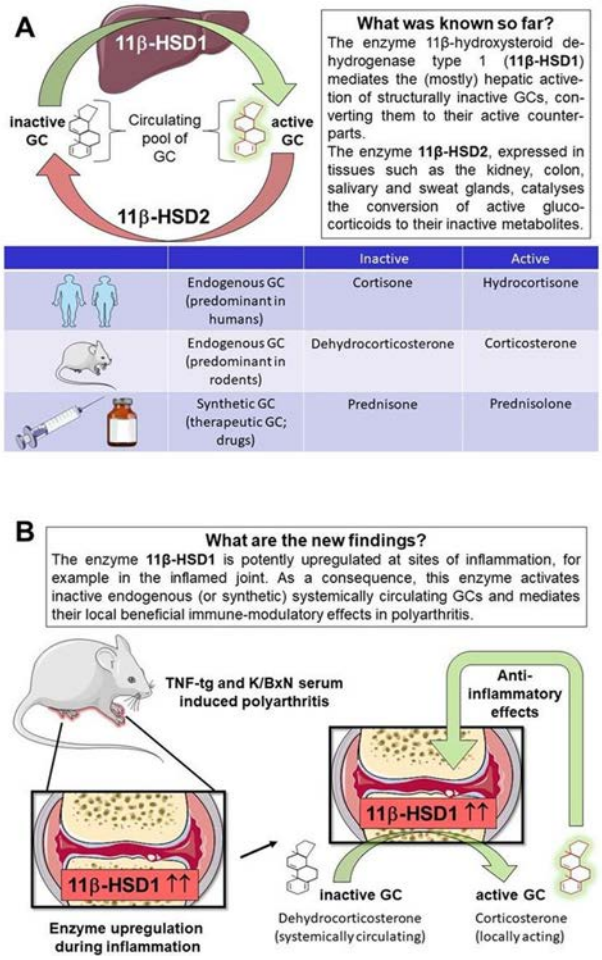

Figure 1 The functions of 11β-HSD enzymes in the glucocorticoid (GC) metabolism. (A) GC circulate in both their active and inactive forms. The shuttling between these forms is mediated by the actions of the 11β-HSD enzymes. While mainly the liver 11βHSD1 mediates activation, systemic inactivation is catalysed mainly by renal 11βHSD2. (B) Fenton *et al* show additionally in murine arthritis models that the peripheral upregulation of 11β-HSD1 in the inflamed joint leads to local reactivation of inactive GC molecules, which induces anti-inflammatory effects.

glucocorticoid molecules, which have undergone systemic inactivation, are peripherally, that is, locally in the inflammation area, reactivated by the enzyme 11beta-HSD1 to induce profound anti-inflammatory effects (figure 1B).

This study is certainly of great importance. It reveals a completely new, previously unknown component of the anti-inflammatory effect of glucocorticoids, namely and concisely, that systemically inactivated glucocorticoid molecules are peripherally reactivated by the enzyme 11beta-HSD1 to induce profound anti-inflammatory effects. It should be critically noted, however, that this study has some limitations. First, it must be stressed that results obtained in animal models cannot always be replicated in humans.[19 20] Confirmation of the significance of the observations made for clinical medicine is therefore still pending. Second, it is not yet clear whether the identified mechanisms are also applicable to the local activation of therapeutically applied glucocorticoids such as prednisone, prednisolone or methylprednisolone. Third, a more comprehensive scientific picture should be created by including so far previously unconsidered leucocyte subpopulations such as T cells into considerations. Finally, the question remains whether the observations made can be translated into new therapeutic approaches.

It is to be hoped that a deepening of the knowledge gained with regard to glucocorticoid treatment and the proof of its relevance to clinical medicine will in future lead to a further improvement

in the therapeutic options for treating patients with rheumatoid arthritis, and other rheumatic and inflammatory diseases, ultimately leading to a better benefit-risk ratio.[21 22]

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Johann Lucas Schoenlein (1793–1864): impact without publications

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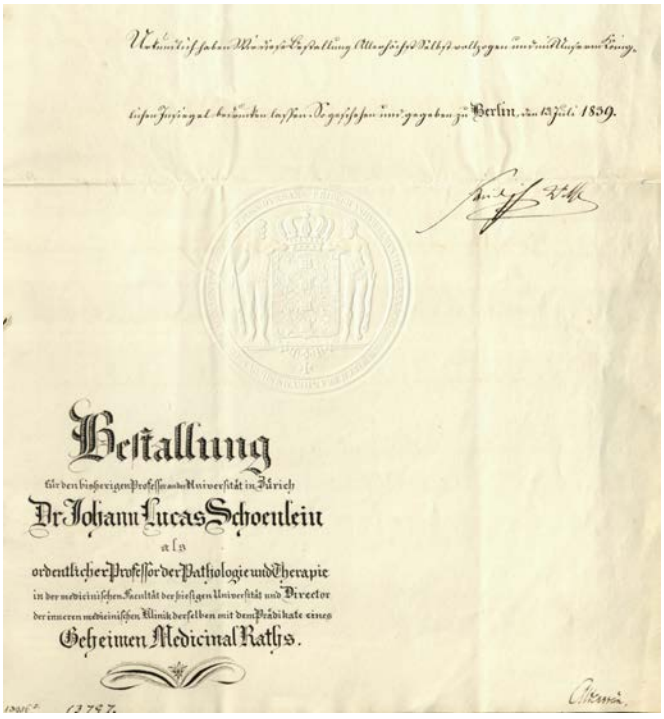
Johann Lucas Schoenlein was one of the most influential clinicians in Central Europe during the first half of the 19th century. However, today it is still not easy to retrace, how exactly he became such a celebrity, because with the exception of his doctoral thesis, all he published were two letters in a scientific journal, not even four pages in total.[1 2] Until recently, almost all we knew came from manuscripts, books and letters of his students. Nonetheless, these documents speak out for him: One of them, the famous surgeon Theodor Billroth, wrote, "Those, who felt spiritually close to Schoenlein raved and became enthusiastic about him and, through him, about medicine".[3] Wilhelm Griesinger, one of the founders of clinical psychiatry, described his impressions as follows: "It seemed to me that he knew everything; and that he could do everything at the bedside!".[4] Within the last years, two parts of his bequest of scientific and private correspondence comprising more than 1500 letters and notes have been rediscovered by chance and meanwhile partially edited.[5 6] This now allows a detailed insight into the life and scientific network of this clinician, about whom his most famous disciple, Rudolf Virchow said: "Thus he remained a colleague to his colleagues, a friend to his friends; thus he became a model of true humanity and liberality, in the correct classical sense of the word. Nothing human was foreign to him".[7] Virchows obituary, which is still the most detailed biography about Schoenlein, has also been translated into English.[8]

Schoenlein was born in 1793 as the only son of a rope maker in the romantic old Franconian city of Bamberg. After completing his medical education in Landshut and Würzburg, he served as Professor and Head of Medicine at the Julius-Maximilians-University of Würzburg from 1819 until 1832 (figure 1). Because of his liberal political attitude, he had to leave Bavaria and became the first Dean of Medicine of the newly founded Hochschule Zürich in Switzerland. In 1839, he was appointed by the Prussian king as 'Professor for Special Pathology and Therapy' and Director of Internal Medicine at the Charité in Berlin, where he worked until his retirement in 1859 (figure 2).[9] Besides those already mentioned, many more of his students pioneered in various areas of scientific medicine, for example, the anatomists Theodor Schwann and Carl Bruch, the physiologists Hermann von Helmholtz and Emil du Bois-Reymond, the internist Ernst von Leyden and the ophthalmologist Albrecht von Graefe.

Instead of giving elaborate philosophical lectures in Latin, he fascinated his students by teaching them the technique of acquiring practical information from questioning, examination and following-up their patients at the bedside. His excellence as a teacher created enormous attraction, as one of his disciples put it, "Has he not after all made Würzburg the place of pilgrimage for German doctors such as Rome is for artists? Has he not spellbound, by the mode of lecturing, foreigners from all nations?".[8] He vigorously adopted auscultation from René Laënnec in Paris and percussion from Leopold von Auenbrugger in Vienna[10] and integrated those in his lectures as well as the microscopic and chemical analysis of body fluids. He carefully documented the clinical diagnosis and course for more than 12 000 patients over a period of about 10 years in one of the largest hospitals in Central Europe[11] and without exception demanded a confirmation by autopsy in cases with lethal outcome. These innovative methods of gathering medical information, later designated 'Naturhistorische Schule', mark the transition from a purely speculative, philosophical approach to modern scientific medicine.[12]

Rheumatologists primarily associate his name with the eponym Henoch-Schoenlein Purpura. It does not come as a surprise that Schoenlein himself never published a word about this disease. However, a detailed description of knee and ankle arthritides with simultaneous appearance of confluent petechial skin eruptions at the lower

Figure 1 Johann Lucas Schoenlein as a young professor in Würzburg. Staatsbibliothek Bamberg, Germany.


Bestallung

Dr. Johann Lucas Schoenlein

als

ordentlicher Professor der Pathologie und Therapie

Geheimen Medicinal Raths.

Figure 2 Certificate of Schoenlein's appointment in Berlin signed by the Prussian king. Institute for History and Ethics of Medicine, Friedrich-Alexander-Universität Erlangen-Nuremberg, Germany.

extremities, frequently associated with haematuria, is given in a transcription of his lectures, published anonymously and never fully endorsed by him.[13] The first regular publication about this subject appeared more than 30 years later authored by his disciple, the paediatrician Eduard Henoch, who supplemented the gastrointestinal manifestations associated with this form of vasculitis.[14] In retrospect, it is remarkable that almost 200 years ago, a simple phenomenological observation was able to identify a constellation of symptoms, which under the name IgA-vasculitis is still considered a defined disease entity, according to the most recent immunopathology-based nomenclature.[15]

Schoenlein, however, contributed more to rheumatology than discovering just one form of vasculitis. He generally viewed disease not as a fixed and determined state, but a developing process, which requires a continuous follow-up of the patient's status. Virchow described his medical training in the winter semester of 1842/1843 at the Charité: "We went through the treatments of eighty-nine cases in detail, amongst which acute forms, especially typhuses, pneumonias, erysipelas, scarlet fever and rheumatisms were predominant".[8] A collection of 42 cases—including six with 'rheumatismus articulorum acutus'—, edited by yet another of his students also contains the first exact description of erosive atlanto-axial arthritis with subluxation in rheumatic disease: "The localisation of acute rheumatism in joints of the cervical spine is always ominous and demands the physician's full attention. I saw in one case, as a consequence of this localisation, an ulceration of the processus odontoides and thus a compression of the medulla oblongata, naturally with lethal outcome".[16]

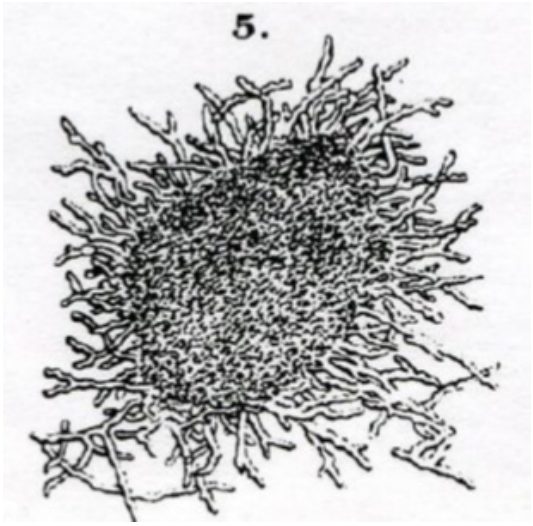


Figure 3 Original figure from Schoenlein's drawing of the microscopic aspect of a fungal colony of achorion (today: trichophyton) schoenleinii.[2]

In addition, Schoenlein was the first to recognise fever not as a separate disease entity, but a symptom of localised pathologies.[17] He also coined the term 'tuberculosis' for all microscopic tissue alterations associated with characteristic nodules, and saw their connection to caseating pneumonia and 'consumption'.[18] However, his greatest contribution to medicine, the second of his only two publications, was just half a page long with one drawing (figure 3).[2] It contains the first description of the fungal origin of favus, a contagious skin disease, making Schoenlein the founding father of medical mycology and giving birth to the rapidly evolving field of microbiology.[19 20]

During his time in Zürich, Schoenlein was increasingly consulted by members of the European aristocracy such as Napoleon Bonaparte III or the Russian Empress Alexandra, born as Charlotte of Prussia. After he had successfully assisted the Belgian Queen Louise in giving birth to the heir to the throne, King Leopold I offered him a generously doted position as personal physician at the royal court. However, Schoenlein refused, because he valued his position as an academic teacher more than any money. The English poet and physician Thomas Lovell Beddoes, who had been his student in Würzburg and Zürich, commented this decision: "Schoenlein will not stay in Brussels, because instead of becoming personal physician to a king, he could rather keep his own personal king".[21] This prediction, however, turned out to be wrong.

Not long after becoming Director of Medicine at the Charité, he was appointed personal physician to the Royal Prussian court. An anecdote tells that Schoenlein, who only few years earlier had had to flee from Germany because of his political convictions, told King Friedrich Wilhelm IV, beforehand: "Your majesty, I feel obliged to tell you that basically, I am a republican". The king answered: "This is pleasant, dear Schoenlein, now Humboldt will not be the only one at my court anymore".[22] Within his 20 years at the Charité, Schoenlein, together with the physiologist Johannes Müller and the surgeon Johann Friedrich Dieffenbach, formed the first generation of the prolific 'Berlin School of Medicine' in the 19th century, and Virchow stated: "In the long sequence of celebrated names which adorn the annals of this university over the first 50 years of its existence, his is one of the most celebrated".[8]

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Mechanisms of progressive fibrosis in connective tissue disease (CTD)-associated interstitial lung diseases (ILDs)

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ABSTRACT

Interstitial lung diseases (ILDs), which can arise from a broad spectrum of distinct aetiologies, can manifest as a pulmonary complication of an underlying autoimmune and connective tissue disease (CTD-ILD), such as rheumatoid arthritis-ILD and systemic sclerosis (SSc-ILD). Patients with clinically distinct ILDs, whether CTD-related or not, can exhibit a pattern of common clinical disease behaviour (declining lung function, worsening respiratory symptoms and higher mortality), attributable to progressive fibrosis in the lungs. In recent years, the tyrosine kinase inhibitor nintedanib has demonstrated efficacy and safety in idiopathic pulmonary fibrosis (IPF), SSc-ILD and a broad range of other fibrosing ILDs with a progressive phenotype, including those associated with CTDs. Data from phase II studies also suggest that pirfenidone, which has a different—yet largely unknown—mechanism of action, may also have activity in other fibrosing ILDs with a progressive phenotype, in addition to its known efficacy in IPF. Collectively, these studies add weight to the hypothesis that, irrespective of the original clinical diagnosis of ILD, a progressive fibrosing phenotype may arise from common, underlying pathophysiological mechanisms of fibrosis involving pathways associated with the targets of nintedanib and, potentially, pirfenidone. However, despite the early proof of concept provided by these clinical studies, very little is known about the mechanistic commonalities and differences between ILDs with a progressive phenotype. In this review, we explore the biological and genetic mechanisms that drive fibrosis, and identify the missing evidence needed to provide the rationale for further studies that use the progressive phenotype as a target population.

INTERSTITIAL LUNG DISEASES AND THE CURRENT TREATMENT LANDSCAPE

Interstitial (or diffuse parenchymal) lung diseases (ILDs) represent a large, heterogeneous group of several hundred generally rare pulmonary pathologies, some of which are associated with significant morbidity and mortality.[1–4] They are characterised by damage to the lung parenchyma and mediated by varying degrees of inflammation and fibrosis.[5] ILDs may arise from a broad spectrum of distinct aetiologies, both known and unknown. They can manifest as a pulmonary complication of an underlying connective tissue disease (CTD-ILD, such as rheumatoid arthritis (RA-ILD)[6–8] and systemic sclerosis (SSc-ILD)[9–11]), as a result of environmental exposure to antigens (eg, chronic hypersensitivity pneumonitis)[12 13] or due to unknown cause/s, as typified by idiopathic pulmonary fibrosis (IPF).[1 14 15] Patients with clinically distinct ILDs have different comorbidities and treatment profiles, and are heterogeneous in both their clinical course and pathophysiology. Nevertheless, a variable proportion of patients within each ILD subgroup can have a similar clinical lung phenotype characterised by declining lung function, worsening respiratory symptoms and health-related quality of life, and higher mortality. In recent literature, these have been termed 'progressive fibrosing ILDs', or 'fibrosing ILDs with a progressive phenotype' (in this review, we use the latter term).[16]

Phase II and III clinical trials have established the efficacy and safety of the antifibrotic drugs pirfenidone[17 18] and nintedanib[19 20] for the management of IPF (the archetypal ILD with a progressive phenotype), and both drugs are now approved for the treatment of IPF.[21 22] In the phase III SENSCIS trial, nintedanib proved efficacious in reducing the annual rate of decline in forced vital capacity (FVC) versus placebo in patients with SSc-ILD.[23] Post hoc analyses showed no heterogeneity in the treatment effect of nintedanib compared with placebo on the rate of FVC decline in subgroups defined by the presence or absence of ground-glass opacities.[24] Nintedanib was subsequently approved by the US Food and Drug Administration and the European Medicines Agency for the treatment of SSc-ILD in September 2019 and April 2020, respectively.[25 26]

Most recently, results from the phase III INBUILD study have shown that nintedanib is also efficacious in treating a pooled group of patients who have fibrosing ILDs with a progressive phenotype (consisting of several clinically distinct disease categories, including CTD-ILDs), by reducing the annual rate of decline in lung function after 52 weeks of treatment.[16] Of particular interest for rheumatologists are the proportions of patients in the nintedanib arm of INBUILD who have ILDs of autoimmune origin (24.7% in total): RA (12.7%), SSc (6.9%), mixed CTD (2.1%) and other autoimmune-related ILDs (3.0%). Subgroup analyses have indicated consistent efficacy across these autoimmune subgroups;[27] however, since INBUILD was not powered to assess efficacy by subgroup, the conclusions that can be drawn regarding the efficacy of nintedanib in individual autoimmune diseases are limited. For patients with unclassifiable ILD with a progressive phenotype, pirfenidone may

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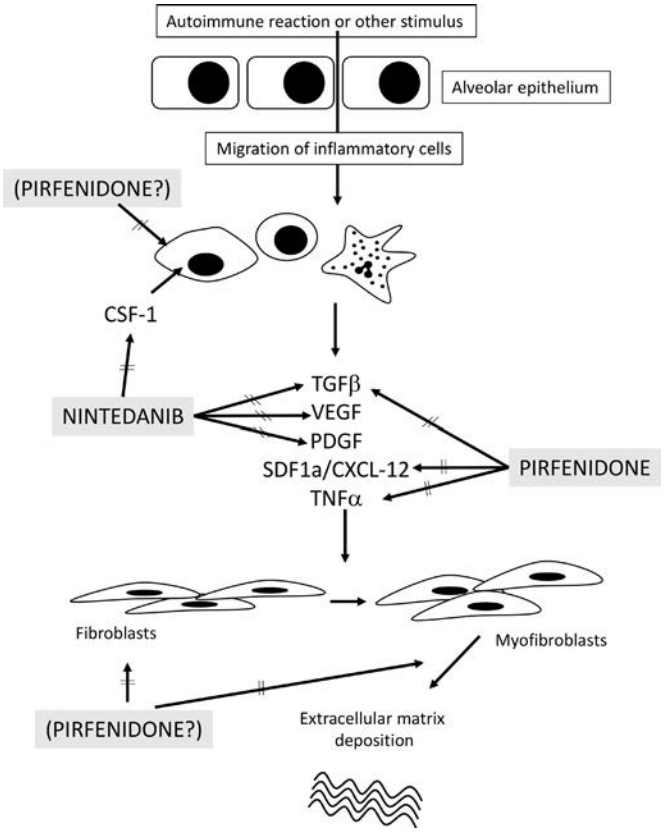

Figure 1 Known and proposed targets for the antifibrotic actions of nintedanib and pirfenidone. CSF, colony-stimulating factor-1; CXCL, C-X-C ligand; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumour necrosis factor; SDF, stromal cell-derived factor; VEGF, vascular endothelial growth factor.

have some clinical benefit. In one phase II study, mean change in FVC% predicted in patients with a range of unclassifiable idiopathic interstitial pneumonias, or interstitial pneumonia with autoimmune features (IPAF) showing a progressive fibrosing phenotype, was lower over 24 weeks in those who received pirfenidone compared with placebo (in this study, progression was defined as >10% fibrosis on high-resolution CT (HRCT) within the previous 12 months, and an annual decline in FVC predicted ≥5%); however, the planned statistical model could not be applied to these primary endpoint data.[28] In a separate phase II study, which was terminated early due to futility based on an interim analysis, patients with progressive forms of fibrotic ILD (annual decline in FVC predicted ≥5%) had a lower decline in FVC% predicted over 48 weeks when taking pirfenidone compared with placebo (after imputation of missing data). However, a major limitation of this study was its small sample size (collagen-vascular disease-ILD (n=37), fibrotic non-specific interstitial pneumonia (NSIP) (n=27), chronic hypersensitivity pneumonitis (n=57) and asbestos-related lung fibrosis (n=6)), and the full results have not yet been published.[29]

The immunosuppressive agents cyclophosphamide (CYC) and mycophenolate mofetil (MMF) have also been evaluated in SSc-ILD. In one study, CYC showed beneficial effects on lung function compared with placebo after 1 year of treatment, although these mostly dissipated after 2 years.[30] In a subsequent trial, 2 years of treatment with MMF did not significantly change the primary FVC endpoint compared with 1 year of CYC, though FVC improved in both groups, and MMF was better tolerated.[31] The anti-interleukin (IL)-6 receptor antibody tocilizumab has been evaluated in patients with SSc and demonstrated preservation of lung function in a phase II study,[32] although a phase III trial did not meet its primary modified Rodnan Skin Score endpoint.[33] The tyrosine kinase inhibitor imatinib is approved for the treatment of chronic myeloid leukaemia and targets the Bcr-Abl/c-Abl, a kinase downstream of transforming growth factor-β (TGF-β) signalling.[34] Imatinib also inhibits the platelet-derived growth factor (PDGF) receptor tyrosine kinase and has been evaluated in small open-label studies in SSc-ILD,[35 36] although no large randomised trials have been conducted and its efficacy is unclear.

Collectively, these trial results suggest that common fibrotic pathways in patients progressing to end-stage lung disease (involving the targets of nintedanib and, potentially, pirfenidone) may exist. The mechanisms of action of nintedanib and pirfenidone may therefore shed some light on the pathways involved in disease pathogenesis. Nintedanib is a small molecule tyrosine kinase inhibitor that targets receptor tyrosine kinases involved in fibrosis, including those for PDGF, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and TGF-β, as well as non-receptor kinases involved in inflammation and proliferation (Src family kinases), and activation and polarisation of macrophages (colony-stimulating factor-1).[37 38] Nintedanib also inhibits the proliferation of vascular cells[39] and modulates fibroblast activity.[40] The molecular mechanism of pirfenidone is not fully understood, but in preclinical models it reduces bleomycin-induced lung fibrosis in mice.[41] Pirfenidone inhibits stress-activated kinases[42] and modulates expression of several growth factors, as well as cytokines that are thought to be relevant to fibrosis, including TGF-β, PDGF, stromal cell-derived factor/C-X-C ligand 12 (SDF-1a/CXCL12) and tumour necrosis factor-α. It may also reduce fibroblast proliferation and alveolar macrophage activation, and modulate extracellular matrix (ECM) deposition.[43 44] Known and possible targets for the antifibrotic action of nintedanib and pirfenidone are shown in figure 1, although the relative weight or importance of specific pathways in different ILDs cannot reliably be made based on the current level of evidence. This review appraises current pathobiological concepts of fibrosis in ILDs exhibiting a progressive fibrosing phenotype, with a particular focus on some of the ILDs most commonly encountered by the rheumatologist, including ILDs associated with SSc, RA, inflammatory myopathy and Sjögren's syndrome.

Fibrosing CTD-ILDs with a progressive phenotype

Although IPF is the archetypal ILD with a progressive phenotype, a proportion of patients with non-IPF ILDs experience a disease course similar to that seen in IPF.[45] ILDs in which patients are at risk of developing a progressive fibrosing phenotype include chronic hypersensitivity pneumonitis, idiopathic NSIP (iNSIP), CTD-associated ILDs (including RA, SSc, mixed CTD, Sjögren's syndrome (though rarely) and inflammatory myopathies), pneumoconiosis (eg, asbestosis), drug-induced ILDs, unclassifiable ILDs, pulmonary sarcoidosis, and rare ILDs, such as pleuroparenchymal fibroelastosis (PPFE).[13 16 28 46 47] However, the proportion of patients who develop a progressive fibrosing phenotype varies by disease, and for many ILDs, the incidence is not known.

The term 'progressive' has been used for a long time in clinical and research settings; however, definitions of progression in the context of the fibrotic phenotype have varied and there are no definitive criteria. Most recently, the INBUILD study used a definition of progression based on fulfilment of ≥1 of the following

Table 1 Studies including patients that would meet the INBUILD criteria for progression

ILD subtype	Study size	Proportion of patients with a progressive phenotype
SSc-ILD	n=695	~33% of patients with DLco pred <50% within 3 years of the onset of Raynaud's phenomenon[121]
Limited cutaneous SSc	n=326	Worsening of ILD (>10% decline in FVC from baseline to second visit) observed in 19.9% of patients at 24 months follow-up[122]
RA-ILD	n=167*	14% of patients with FVC <50% pred at diagnosis, increasing to 22% after 5 years; 29% of patients with DLco <40% pred at diagnosis, increasing to 40% after 5 years[8]
Inflammatory myopathy-associated ILD	n=107	Worsening of pulmonary symptoms, deterioration on HRCT, and decline in lung function (≥10% in FVC or ≥15% in DLco) observed in 15.9% of patients (despite therapy), after a median 34 months of follow-up (range 4–372 months)[89]
Sjögren's syndrome-associated ILD	n=18†	5 patients (28%) had a decline in FVC pred of ≥10% or a decline in DLco pred of ≥15%, despite immunosuppression (median follow-up: 38 months)[123]

*167 patients encountered in clinical practice and referred for multi-specialty evaluation in a tertiary care centre (potential centre bias: severe cases are more often encountered at a specialised centre).
†18 patients selected over a 13-year period.
DLco, diffusing capacity of the lung for carbon monoxide; FVC, forced vital capacity; HRCT, high-resolution CT; ILD, interstitial lung disease; pred, predicted; RA, rheumatoid arthritis; SSc, systemic sclerosis.

criteria for progression of ILD within a 24-month period (despite management with standard treatments, excluding nintedanib or pirfenidone): relative decline in FVC predicted ≥10%; relative decline in FVC predicted ≥5–<10% with either worsened respiratory symptoms or increased extent of fibrosis on chest HRCT; or a combination of worsened respiratory symptoms and an increased extent of fibrosis on HRCT. This definition did appear to enrich for patients with progressive disease in the overall population, as demonstrated by the decline in patients in the placebo arm.[16] However, small patient numbers and the lack of a comparator group without enrichment criteria mean it is not possible to draw definite conclusions regarding enrichment in certain subgroups, including the CTD-ILDs.

In our review of the literature, we found only a small number of studies that included patients that would meet the INBUILD inclusion criteria of a progressive phenotype. These studies, which include SSc-ILD, RA-ILD, ILD associated with inflammatory myopathy (polymyositis and dermatomyositis), and Sjögren's syndrome-ILD, are summarised in table 1 and reviewed in further detail elsewhere.[48] Although these studies give an approximate indication of the proportion of patients who may develop a progressive fibrosing phenotype in certain ILDs, further longitudinal studies are needed to expand the evidence base.

In patients with certain ILDs, a specific radiographic pattern of fibrosis (usual interstitial pneumonia, UIP) identified by HRCT is often associated with more rapid disease progression compared with other fibrotic patterns. This association has been observed in patients with a range of ILDs, including IPF, chronic hypersensitivity pneumonitis, RA-ILD[45 49–53] and, though rarely, sarcoidosis.[54] In patients with SSc-ILD, the most common pattern of fibrosis on HRCT is NSIP.[55] However, radiographic patterns appear not to be related to a progressive fibrosing phenotype in SSc-ILD,[56] indicating that while fibrosing ILDs with a progressive phenotype share some similarities, differences also exist. In CTD-ILDs, NSIP is generally the most frequently observed pattern (with the exception of RA).

Biological mechanisms driving progressive pulmonary fibrosis

Broadly, fibrosis is characterised by the overgrowth, stiffening and/or scarring of tissues due to excess deposition of ECM components, notably collagen.[57] In fibrotic lung diseases, repetitive cycles of alveolar epithelial injury and attempted repair are thought to lead to the gradual destruction of functional lung parenchyma and its replacement by increasing deposits of non-functional connective tissue (fibrosis). This loss of functional alveoli due to sustained fibrosis leads to respiratory insufficiency and early mortality.[58 59]

In addition to epithelial lung injury, other forms of initial lung injuries (depending on the disease) might contribute to progression of the fibrotic phenotype. These include cellular and/or humoral autoimmunity (as in all CTD-ILDs, but to a varying degree),[55] endothelial cell dysfunction (as in SSc or asbestosis),[60–62] granuloma formation (as in sarcoidosis)[63] or alveolar macrophage activation (as in asbestosis).[64] For some ILDs, the initiating event may be hard to identify, such as in RA, where infections, cigarette-smoking, mucosal dysbiosis, immune response (including autoantibodies against citrullinated proteins), host genetics and premature senescence have all been proposed to play a role.[55 65–67] Chronic microaspiration secondary to gastro-oesophageal reflux, a common complication of SSc due to oesophageal motor dysfunction, can lead to persistent alveolar epithelial injury, potentially accelerating the progression of lung fibrosis.[68] Moreover, the increased negative intrathoracic pressure during inspiration caused by lung fibrosis may aggravate gastro-oesophageal reflux in a vicious circle.[68]

Following the injury, wound-healing responses are induced. If sustained and dysregulated, pathological fibrogenesis then occurs, whereby the rate of new collagen synthesis exceeds the rate of collagen degradation, culminating in the accumulation of collagen over time.[57] The principal cellular mediators of fibrosis, regardless of the initial injury, are collagen-secreting myofibroblasts.[57]

Both the innate and adaptive immune system contribute towards the development of fibrosis. This is mediated by cellular and humoral components, underpinning the rationale for immunomodulatory therapies.[69] Preclinical studies have identified profibrotic (Th2, Th17), antifibrotic (Th1, Th22 and γδ-T) and pleiotropic (T_{regs} and Th9) T cells as mediators of fibrosis,[69] and the profibrotic action of PD-1+ CD4+ T cells (targetable by currently available immunomodulatory therapies) has been specifically demonstrated in models of pulmonary fibrosis associated with IPF and sarcoidosis.[70] B cells also play a role, having been detected at higher levels in the lungs of patients with IPF, RA-ILD and Sjögren's syndrome, among others.[71 72] Other innate immune cells implicated in the process of fibrosis include neutrophils and macrophages, the profibrotic effects of which are mediated via secretion of TGF-β, PDGF and IL-6.[69 73] Blood monocytes are recruited to the lung during the fibrotic process, where they have been shown in both IPF and SSc to differentiate into fibrocytes[74 75] and into myofibroblasts in SSc.[76] Macrophages can undergo polarisation to become either 'proinflammatory'

classical M1 macrophages, which secrete proinflammatory and/or profibrotic cytokines (IL-1β, IL-8, IL-10 and CXCL13), or 'profibrotic' alternative M2a macrophages, which secrete profibrotic cytokines (CCL22, PDGF-BB and IL-6).[69 73] Neutrophils have pleiotropic effects within the fibrotic milieu, including the secretion of elastase and matrix metalloproteinases, which degrade ECM and activate accumulation of ECM driven by TGF-β.[69] Neutrophil extracellular traps play a key role in the development of fibrosis, having been detected in close proximity to alpha-smooth muscle actin-expressing fibroblasts in biopsies from patients with fibrotic ILD.[77] Finally, mast cells are increased in fibrotic areas of alveolar parenchyma in patients with a range of fibrotic lung diseases, with strong evidence for important bidirectional interactions between mast cells and myofibroblasts in fibrotic tissues.[78]

Our current understanding is that immune cells are profibrotic, though there is mounting preclinical and clinical evidence that the composition of the inflammatory infiltrate determines its fibrotic activity, and that some immune/inflammatory cells may even exert direct antifibrotic effects depending on the local environment.[79 80] T cells, for example, have been shown to inhibit fibroblast-to-myofibroblast differentiation in vitro through the secretion of inhibitory prostaglandins.[81] Adoptive transfer of splenic T_{reg} cells has been shown to attenuate bleomycin-induced lung fibrosis in vivo,[82] and global impairment of CD4+CD25+-FOXP3+ T_{reg} cells has been found to correlate strongly with disease severity in IPF, suggesting a role for T_{regs} in the fibrotic process.[83] B cells may also contribute to the formation of an antifibrotic 'shield', acting as regulators of polymorphonuclear cells and restraining the ability of these cells to cause ILD.[84] Gene knockout studies have identified a gene in B cells that appears to regulate lung fibrosis.[85] Interestingly, in an experimental model of cardiac fibrosis, engineered T cells targeting the Fibroblast activation protein protected against cardiac fibrosis,[86] providing proof of principle for the development of immunotherapeutic drugs for the treatment of fibrotic disorders.

Several humoral mediators also play a role in fibrogenesis. IL-13 is known to stimulate differentiation of lung fibroblasts to myofibroblasts via c-Jun N-terminal kinase-signalling, whereas IL-17 acts in concert with TGF-β-mediated pathways to promote pulmonary fibrosis. TGF-β itself promotes epithelial-to-mesenchymal transition, induces fibrosis through canonical and non-canonical pathways such as mitogen-activated protein kinase, extracellular signal-regulated kinases and PI3K/Akt signalling, and modulates fibroblast differentiation into myofibroblasts that drive ECM accumulation. PDGF is known to activate and promote ECM gene expression in fibroblasts, and CCL2 may increase fibrocyte recruitment and differentiation into fibroblasts (in addition to its role in monocyte chemotaxis). In some ILDs, antibodies may play a key role. In SSc, for example, anti-topoisomerase I antibodies are associated with the presence and severity of ILD at baseline.[11 87] In RA-ILD, IgA anti-citrullinated protein antibodies (ACPAs) (commonly found in synovial and articular sites) have been identified in sputum from individuals at risk of RA, suggesting that the lung may be the primary site of ACPA generation.[55] The presence of anti-Sjögren's-syndrome-related antigen A antibodies is a predisposing factor for ILD in patients with Sjögren's syndrome.[88] In myositis-associated ILD, however, one study found no correlation between the deterioration of ILD and the presence of antinuclear antibodies, anti-Jo-1 antibodies or anti-PM-Scl antibodies.[89] While an association between antibodies and certain forms of ILDs has been identified, a causal pathogenetic relationship has not.

Little is known about how the mechanisms of fibrosis differ across distinct ILDs, and even less is known about whether progressive fibrosis is driven by a different set of mediators than non-progressive fibrosis. The most studied ILDs from a mechanistic perspective are IPF and SSc-ILD. Common to both diseases are activation of macrophages with a similar chemokine expression profile (M2 profibrotic phenotype), and similar T-cell profiles (Th2-increased T_{regs}, Th22, Th17, increased ratio of CD4 to CD8 T cells).[90] However, the B-cell profiles of patients with IPF and SSc-ILD differ, as do their T-cell chemokine profiles (IL-4, IL-5, IL-10 and IL-17 for IPF, and IL-4, IL-5, IL-6, IL-10, IL-13 and IL-22 for SSc-ILD).[90] In particular, IL-6 is known to play a key role in SSc by increasing collagen production through fibroblast stimulation, myofibroblast differentiation and inhibiting the secretion of metalloproteinase.[91] In one study, serum IL-6 levels appeared to be predictive of early disease progression in patients with mild (FVC >70%) SSc-ILD,[92] yet were not in another study of SSc-ILD,[93] and CXCL4 has also been correlated with the presence and progression of lung fibrosis in SSc.[94] In RA-ILD, as in IPF and SSc-ILD, Th-17-cell-mediated immunity is involved in pathogenesis (the IL-17 receptor is upregulated in both RA-ILD and IPF).[55 66] In addition, lung tissue from individuals with RA-ILD has substantially greater numbers of B cells and CD4+ T cells than lung tissue from individuals with idiopathic UIP, implying that immune dysregulation might be more prevalent in RA-ILD than in idiopathic UIP.[95] Biomarkers of fibrosis could provide an important clue, but to date no serum biomarker has been identified as a sufficiently robust prognostic marker to justify its use in clinical practice. In studies in lung transplantation, it has also been shown that the concentrations of PDGF, FGF-2, VEGF and colony-stimulating factor-1 were significantly increased in lungs with progressive ILDs, including IPF, SSc-ILD and other ILDs, compared with donor lungs.[96]

Genetic mechanisms driving progressive pulmonary fibrosis

Certain genetic mutations are implicated in the aetiology of ILDs. Mutations in telomere-related genes (*TERT*, *TERC*, *RTEL1*, *PARN*, *TINF2*, *NAF1* and *DKC1*) have been associated with a broad range of ILDs, including IPF, iNSIP, RA-ILD, acute interstitial pneumonia, cryptogenic organising pneumonia, chronic hypersensitivity pneumonitis and PPFE.[97–99] Telomeres are distal regions of chromosomes associated with specific protein complexes, which protect the chromosome against degradation and aberration. It is believed that loss of function in the telomerase complex may influence the turnover and healing of alveolar epithelial cells after an initial damaging stimulus, thereby triggering fibrosis.[100] In support of this, mice with defective telomere homeostasis develop spontaneous pulmonary fibrosis or are more susceptible to injury.[100 101] Telomere dysfunction in type II alveolar epithelial cells (mediated by deletion of the telomere shelterin protein TRF1) is also sufficient to cause lung fibrosis in mice.[102] Conversely, vector-induced telomerase expression has shown therapeutic effects in a mouse model of pulmonary fibrosis, indicating that telomerase activation may represent an effective treatment for pulmonary fibrosis provoked by or associated with short telomeres.[103] Telomerase activators have also shown activity in preclinical models of fibrosis.[104] In patients with ILDs, significantly shortened telomeres have been found, and these have been linked to defective immunity[105–107] (the shortest telomeres are found in patients with

IPF).[108] However, it is important to note that not all individuals with mutations in telomere-related genes will necessarily have short telomeres or develop ILD.[97] In RA-ILD, coding region mutations in the genes *RTEL1* and *TERT* lead to telomere shortening and onset of RA-ILD at a younger age.[99] In hypersensitivity pneumonitis, short telomere length has been associated with extent of fibrosis, histopathological features of UIP, and reduced survival, suggesting shared pathobiology with IPF.[109] Beyond these associations, however, no studies to our knowledge have exposed a direct link between specific telomere-related genotypes and progressive (or non-progressive) fibrosis.

Another gene implicated in some forms of ILD is the mucin 5B gene (MUC5B). A common variant in the promoter region of this gene (rs35705950) has been associated with an increase in IPF susceptibility and overall mortality.[110–113] Similar associations have also been observed in patients with RA-ILD,[65 110] as well as in hypersensitivity pneumonitis[109] and IPAF,[114] but not in SSc-ILD,[115] myositis-associated ILD[116] or sarcoidosis,[117] again highlighting not only the similarities but also the differences between ILDs.

Most of the available genetic data come from studies in IPF, but risk alleles in other genes have also been identified for a range of non-IPF ILDs, primarily in RA-ILD, and chronic hypersensitivity pneumonitis.[118] Currently, it is not clear whether specific genetic risk factors predispose certain individuals to develop a progressive fibrosing phenotype. If confirmed through longitudinal studies, genetic markers might help to identify those most at risk of progression.

Furthermore, epigenetic mechanisms play a key role in biological processes at the level of chromatin structure and organisation, including DNA methylation, post-translational modifications of histone tails and non-coding RNA. Under physiological conditions, the epigenome ultimately determines the silencing or activation of gene expression in a temporally coordinated way, and its dysregulation contributes to a variety of human diseases, including IPF.[119] Epigenetics may explain the profibrotic effect of ageing as a condition, or environmental factors such as tobacco smoke or inhaled air pollution in IPF, and other fibrotic conditions such as RA-ILD.[120]

SUMMARY

In recent years, phase III clinical trials have demonstrated the efficacy and safety of new classes of drugs in slowing disease progression in patients with IPF (nintedanib and pirfenidone), and SSc-ILD (nintedanib). Results from recent phase III clinical trials have now shown that nintedanib can slow the progression of ILD (as measured by FVC decline) in patients with a broad range of fibrosing ILDs with a progressive phenotype, including those associated with CTDs. Available data for pirfenidone in the treatment of clinically distinct ILDs with a progressive phenotype come from phase II trials in which, despite some positive endpoints, the primary endpoints were not met. Though not powered to detect efficacy by disease subgroup, these trials add weight to the hypothesis that in a number of clinically distinct ILDs, a progressive fibrosing phenotype may arise from common, underlying mechanisms of fibrosis, irrespective of the original clinical trigger or association. However, to date, this hypothesis has only been proven for the targets of nintedanib and partially for the targets of pirfenidone. This review found little evidence for other common pathways in progressive fibrosing ILDs, mostly because of the lack of appropriate studies. Thus, there is currently insufficient preclinical support for other treatment studies using the progressive phenotype as a target population. To identify common and distinct pathways, high-throughput genomics, proteomics and metabolomics studies using adequate lung tissue from patients with the progressive phenotype of different aetiologies are urgently needed. These analyses may then provide the preclinical rationale for additional, specific targeted therapies to support the novel and important concept of using the progressive fibrosing phenotype as a common target population in clinical studies.

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Tackling osteoarthritis during COVID-19 pandemic

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ABSTRACT

In this opinion article, we would like to draw attention to the fact that COVID-19 has a significant impact not only on immune-mediated arthritis but also on osteoarthritis (OA), the most common rheumatic disease. We suggest herein strategies for pain relief and symptom prevention in patients with OA during COVID-19 pandemic.

The recently characterised SARS_CoV-2 is the cause of COVID-19, a serious illness responsible for the current pandemic, as declared by WHO.[1 2] The number of deaths associated with COVID-19 has been partially linked to the incapacity of health systems to provide care to infected patients.[3] As of 15 July 2020, WHO reported 13 119 239 confirmed cases of COVID-19 globally, with a 573 752 death toll. There is no curative treatment and a vaccine will most probably not be available, at least to everybody, by the end of this year. Although the Food and Drug Administration in the USA has issued a statement allowing for remdesivir to be used as a treatment for COVID-19,[4] a defined therapy is yet to be made. Given the huge number of patients affected by COVID-19, health authorities have established rules for 'physical distancing' and a 'stay at home'' strong advice. In some situations, a strict lockdown norm has been issued in order to limit the number of people exposed in order not to overwhelm the health systems ability to provide assistance for those with more severe disease.[5]

GUIDELINES ON RHEUMATIC DISEASES

Most immune-mediated rheumatic disease patients are subjected to some sort of immunosuppressive therapy, rendering them more susceptible to infections. Characteristics associated with hospitalisation for COVID-19 in people with rheumatic disease based on the data from the COVID-19 Global Rheumatology Alliance physician-reported registry have been recently published[6] . It provides original and important information concerning the links between chronic inflammatory arthritis and COVID-19. Several organisations including the American College of Rheumatology,[7] the European League Against Rheumatism[8] and the Brazilian Society of Rheumatology[9] issued guidances for managing such patients during this pandemic. However, recommendations on dealing with patients affected by highly prevalent musculoskeletal diseases that are not considered to be immune-mediated are lacking. Indeed, neck pain, low back pain, 'other musculoskeletal disorders' and falls account for 4 out of the top 10 causes of years lost with disability worldwide.[10]

We would like to draw attention to the fact that COVID-19 have also had a significant impact on the most common rheumatic disease, osteoarthritis (OA). Mendy *et al* looked among the 689 COVID19 patients treated in 4 hospitals in the Cincinnati area for factors associated with severity and/or with hospitalisation.[11] One hundred and five patients had OA. After adjustment, patients with OA were more often hospitalised than patients without osteoarthritis (OR (95% CI) = 1.95 (1.19,3.19), p= 0.008), and had to use UCI more often (OR (95% CI) = 2.01 (0.98,4.11), p= 0.057). In an Austrian prospective study conducted on 63 patients who had to have a total knee or hip joint replacement for OA and who had to delay it because of the lockdown, there was a significant increase in pain, worsening of physical function and a decrease in physical activity when comparing the clinical condition at the beginning and end of the lockdown.[12]

OA MANAGEMENT IN COVID-19 DAYS

OA, the most prevalent chronic arthritis, is a major cause of musculoskeletal pain and years lost with disability. Usually, patients with OA are advised to avoid self-medication so that when severe pain ensues, it is not uncommon for them to seek help in emergency care. However, people are currently being strongly encouraged not to seek emergency treatment for fear of getting contaminated with SARS-Cov-2.[13 14] That is even more true for the elderly, which are exactly those most affected by musculoskeletal 'non-immune-mediated' diseases.[14] Some guidance to those patients would be helpful to decrease their demand for emergency care.

Besides a persistent inflammatory component,[15] OA is also related to mechanical derangement leading to joint failure affecting the cartilage, muscles, tendons, ligaments, menisci and the subchondral bone.[14] Although COVID-19 will virtually infect anybody, old people are more severely affected, particularly those displaying comorbidities including cardiovascular diseases, obesity, diabetes and chronic lung diseases.[16] Obesity is a well-defined risk factor in patients with OA, who usually suffer from frailty both secondary to reduced physical inactivity and ageing leading to sarcopenia which impacts respiratory capacity. Cardiovascular risk is also enhanced among patients with OA, being significantly associated with the use of non-steroidal anti-inflammatory drugs (NSAIDS).[17]

Patient education, information about the disease, stimulation of exercise programmes, weight control, nutritional orientation and mind-body exercises compose a core treatment for knee, hip

or polyarticular OA, regardless of comorbidities, in the recently updated Osteoarthritis Research Society International (OARSI) guidelines.[18] Actually, similar recommendations have been advocated as a general rule in the management of immune-mediated rheumatic diseases.[19] Restoration of daily life activities may not be fully implemented in the upcoming months, particularly for the elderly, which are the main target to be protected from getting COVID-19. Unfortunately, this group of people, which is heavily affected by OA, is less prone to physical activity.[20] Coincidentally, increased age, higher body mass index (BMI), reduced physical activity and cardiovascular diseases, which are more prevalent in the OA patient, have been associated with a worse prognosis among patients with COVID-19. We may then envision that prolonged periods of virtually complete physical inactivity will most likely worsen sarcopenia and frailty as well as cardiovascular risk in patients with OA.

A recent article has suggested home-based exercises rheumatic disease patients during this pandemic, as a strategy to reduce their disease burden.[21] An analysis of a meta-analysis on the effect of exercise in knee OA was so clearly positive that concluded that no further studies are needed to reinforce it.[22] Details on the type of exercises that can be performed by elderly people who are isolated because of COVID-19 have even been published by the Centre for Evidence-Based Medicine at Oxford University based on a systematic literature review (https://www.cebm.net/covid-19/maximising-mobility-in-the-older-people-when-isolated-with-covid-19/). Unfortunately, though commendable, such physical practices are probably easier said than done. Actually, being inactive throughout life carries a higher knee OA risk.[23] Adherence to self-exercise programmes are very low among those patients with OA, questioning the efficacy of such guidances.[24] Why would we believe patients will now adhere to home-based 'spontaneous' physical activity, especially experimenting a sort of segregation?

Usually, patients with OA rely on pain killers even without a medical prescription.[25] That theoretical perfect storm may lead elderly patients with OA with movement restrictions to increase NSAIDS use and the risk of a worse prognosis if they get infected by COVID-19. There are some questionable, though sometimes effective treatment options to mitigate joint pain in OA patients.[26]

STRATEGIES FOR PAIN RELIEF IN PATIENTS WITH OA DURING COVID-19 PANDEMIC (BOX 1)

Current OARSI guidelines[18] have disregarded paracetamol as an effective pain killer in OA. That was due to a very low effect size and safety issues with doses higher than 2 g per day. At least during COVID-19, stimulating on demand usage of paracetamol up to 1.5 g daily may provide partial pain relief. That could be to combined to other strategies including topical NSAIDS, which provide a better safety profile, although with undocumented adherence. A recommendation to avoid NSAIDs was disseminated in the media in the early weeks of the epidemy based on some experimental results.[27] This hypothesis has never been confirmed.[27] However, a warning to avoid systemic NSAIDS in patients with OA with cardiovascular comorbidities exists independently of the epidemy. Such medications can be obtained without a medical prescription, particularly in low/middle-income countries. Let us not forget that NSAID use may account for over 40% of the increased cardiovascular risk in patients with OA.[17] They should be used on demand, for the shortest period possible, restricting to naproxen given its less deleterious cardiovascular risk profile.[28]

Box 1

Points to emphasise to the osteoarthritis (OA) patient during COVID-19 pandemic

- Physical activity is a must need in OA patients, regardless of age.
- Rheumatology organisations should be stimulated to develop home-based supervised exercise programmes.
- Keep physical distancing as long as needed.
- Nutritional requirements should be adjusted to the degree of physical activity
- Non-steroidal anti-inflammatory drugs should be taken with higher scrutiny.
- Opioids should be not taken.
- If indicated, consider intra-articular steroids and hyaluronic acid in the appropriate setting.
- Psychological care includes medications and psychological/psychiatrical counselling.
- Information should be disseminated to healthcare professionals in primary care.

Psychological issues most commonly represented by depression carry a worse phenotype prognosis in OA.[29] Duloxetine has been recommended in patients with OA with depression and widespread pain. The psychological burden to those patients will probably increase in the isolated elderly. Hence, identifying the need for antidepressants might help them cope with the disease. Psychological and/or psychiatric counselling should be stressed as it can be provided using telehealth strategies.[30]

Although access to non-urgent hospital facilities is restricted, intra-articular injections of hyaluronic acid could be an interesting alternative, given the relatively long-term pain relief they provide. That could also be said of intra-articular corticosteroids. Despite the fear of the immunosuppressive effect of corticosteroids, usage in a non-infected patient may provide up to 3 weeks pain relief thus reducing the need for systemic NSAIDs without persistent immunosuppression.[31] Considering the current situation of social isolation, we believe opioids should not be used. Such drugs have been associated with fractures from falls[32] and that risk would probably increase under opioid use given the increased frailty due to persistent inactivity in COVID-19 days.

The elderly patient with OA must be seen in complete. Healthcare professionals (HCP) should try to establish more frequent, even short, online visits as well as encourage social

Box 2

Research agenda

- Determine the impact of confinement on osteoarthritis (OA) disability.
- Determine the loss of physical activity in patients with OA during and after the confinement.
- Determine the impact of confinement on frailty in the elderly patient with OA and its influence in mortality at the long term.
- Determine impact of confinement in OA according to phenotypes.
- Determine impact of confinement in spine OA pain and function.
- Determine safety of intra-articular injections in a home care setting.

'online gatherings' with family and friends. Adherence to healthy nutrition requirements, probably with further calorie restriction, with attention on protein requirements in those with no physical activity, should be emphasised. Although a high BMI is associated with a worse scenario in knee OA outcome[29] weight reduction will be harder in the current pandemic. Publications in social media conveying information that stigmatise weight gain as inevitable may disencourage attempts toward weight control.[33] That should not refrain HCP from being proactive in counselling on preventing weight gain as less activity calls for calorie restriction. As said above, psychological distress is expected to impact people. Physical activity can be a favourable double-edged sword helping with both mental and physical harms imposed by the 'stay at home' norms.[34] Stimulation of the practice of respiratory movements, avoiding being bedridden and long sitting periods is also a prophylactic measure in the event of a respiratory infection. Prophylactic nebulisers with no active drug help prevent mucus clot and strict adherence to cardiovascular and metabolic treatment is a must in order to improve chances if COVID-19 comes. It is noteworthy that some of these recommendations for patients with OA can also be applied to patients suffering from other types of RMDs, including immune-mediated ones.

CONCLUDING REMARKS

Not uncommonly, the rheumatologist is the physician most tightly linked to the elderly OA patient with musculoskeletal diseases. Being proactive, such specialists might improve our patient's opportunities to tackle this pandemic. Notwithstanding, spreading similar recommendations for HCP in the primary care setting would increase the number of patients reached. A worsening of symptoms in OA patients after this confinement period might be anticipated. Measures to mitigate this situation should not be overlooked, as they involve both non-pharmacological and pharmacological approaches (Box 2). In addition to patients affected by immune-mediated rheumatic diseases the burden posed by other musculoskeletal disorders cannot be disregarded.

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EPIDEMIOLOGICAL SCIENCE

Factors associated with progression to inflammatory arthritis in first-degree relatives of individuals with RA following autoantibody positive screening in a non-clinical setting

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ABSTRACT

Objectives Little is known about the likelihood of developing inflammatory arthritis (IA) in individuals who screen autoantibody positive (aAb+) in a non-clinical research setting.

Methods We screened for serum cyclic citrullinated peptide antibody (anti-CCP) and rheumatoid factor isotype aAbs in subjects who were at increased risk for rheumatoid arthritis (RA) because they are a first-degree relative of an individual with classified RA (n=1780). We evaluated combinations of aAbs and high titre aAbs, as defined by 2-times (2x) the standard cut-off and an optimal cut-off, as predictors of our two outcomes, aAb+ persistence and incident IA.

Results 304 subjects (17.1%) tested aAb+; of those, 131 were IA-free and had at least one follow-up visit. Sixty-four per cent of these tested aAb+ again on their next visit. Anti-CCP+ at levels ≥2x the standard cut-off was associated with 13-fold higher likelihood of aAb +persistence. During a median of 4.4 years (IQR: 2.2–7.2), 20 subjects (15.3%) developed IA. Among subjects that screened anti-CCP+ at ≥ 2x or ≥an optimal cut-off, 32% and 26% had developed IA within 5 years, respectively. Both anti-CCP cut-offs conferred an approximate fourfold increased risk of future IA (HR 4.09 and HR 3.95, p<0.01).

Conclusions These findings support that aAb screening in a non-clinical setting can identify RA-related aAb+ individuals, as well as levels and combinations of aAbs that are associated with higher risk for future IA. Monitoring for the development of IA in aAb+ individuals and similar aAb testing approaches in at-risk populations may identify candidates for prevention studies in RA.

Key messages

What is already known about this subject?

- The presence of anticyclic citrullinated peptide (CCP) and/or rheumatoid factor antibodies are associated with the development of rheumatoid arthritis (RA) in banked blood studies and in clinic settings where subjects present with joint symptoms.

What does this study add?

- High-risk subjects can be identified, recruited and followed for development of autoantibody positive+ persistence and incident inflammatory arthritis (IA) outside of clinical settings.
- In a prospective study, subjects without RA-like synovitis and with higher levels of anti-CCP are more likely to remain anti-CCP positive and develop IA within 5 years of screening.
- A prediction model including factors easy to assess at a study visit may be an efficient means to assemble an at-risk cohort ideal for future prevention, epidemiological and mechanistic studies.

How might this impact on clinical practice or future developments?

- Identification of individuals at-risk for future RA in a non-clinic setting will improve our capability to study preclinical RA at earlier stages and help to find and verify factors related to disease development.

INTRODUCTION

Seropositive rheumatoid arthritis (RA) is characterised by immune system dysregulation prior to any signs of inflammatory arthritis (IA) and classifiable disease.[1 2] The development of RA is thought to occur in several phases, with the earliest phase including genetic and environmental relationships that trigger autoimmunity and may initially occur in the absence of clinically apparent IA.[3–7] Individuals may be identified in this preclinical phase by the presence of circulating autoantibodies (aAbs), specifically antibodies to citrullinated protein/peptide antigens (ACPAs) and rheumatoid factor (RF).[1 2 7 8] ACPA and RF have been shown to appear an average of 3–5 years before clinically apparent IA and classifiable RA in a period termed pre-RA.[1 2] Many epidemiological factors associated with systemic autoimmunity and RA have been identified, including older age, female sex, race/ethnicity, smoking and lower omega-3 fatty acids.[6]

Our current understanding of RA-related aAbs and the timing of IA/RA development comes largely from the study of subjects with banked blood samples prior to RA diagnosis,[1 2 9 10] subjects

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with undifferentiated arthritis who presented to the healthcare system in rheumatology clinics,[11–16] or subjects who were tested for aAbs due to a clinical indication of arthralgia.[17] From these studies, the reported positive predictive value (PPV) for the future development of classified RA in the presence of aAbs has varied, depending on the specific cohort design, aAbs assessed and analytical approach. In a study using pre-RA diagnosis blood bank samples from patients with established RA and controls, the PPV for future RA was 82% among those positive for anticyclic citrullinated peptide (CCP) and 83%–87% when combined with an RF isotype.[1] In a study among patients with arthralgia, the risk of developing RA was nearly ninefold among those positive for both RF IgM and anti-CCP and 3–5-fold for those only positive for anti-CCP (depending on anti-CCP level) compared with those RF IgM positive only.[18] While informative, these studies do not represent the likelihood of developing RA in those who screen aAb+ outside of the clinical setting. Of the two studies that screened first-degree relatives (FDRs) of patients with RA for aAbs, both showed the risk of developing IA/RA was highest for subjects positive for both anti-CCP and RF (64%[19] and 38%[20] after 5 years of follow-up), although the latter study found that aAb reversion was equally as likely.[20]

The ability to identify individuals with elevated aAbs in absence of clinically apparent IA has led to an increased focus on preventing the development of future IA.[21–23] Determining factors associated with persistent aAb positivity and the development of IA is a crucial component in the design of RA prevention strategies. Importantly, screening for aAbs in populations at-risk for the development of future IA outside of clinical care settings, is a means to assemble cohorts for epidemiological, mechanistic and interventional studies. Our goal is to identify factors related to persistent aAb+ and future development of IA/RA in a prospective non-clinical population that is at-risk based on family history of disease.

METHODS

Study population

The multicenter Studies of the Etiology of Rheumatoid Arthritis (SERA) is a prospective study designed to examine the environmental and genetic factors leading to the development of RA among those who are at increased risk for developing RA. The SERA population of FDRs of RA probands has been described previously.[24]

At study entry, a 68-count joint examination was performed to confirm subjects do not have RA by 1987 American College of Rheumatology (ACR) criteria.[25] At each follow-up visit, a joint examination was performed to assess IA/RA status, and questionnaires capturing socioeconomic status, self-reported joint symptoms and environmental exposures, were collected. And, blood samples for biomarker and genetic studies were obtained.

As of March 2019, SERA has enrolled 1780 FDR participants. Subjects were included in current analyses based on having at least one visit where they were aAb+ for one or more of the aAbs listed below, had at least one additional follow-up visit after their first aAb+ visit, underwent joint examinations during the study, and did not have IA at their baseline visit (figure 1).

Measurement of RA-related biomarkers

Autoantibody assays were performed in the clinical and research lab at the University of Colorado.

Standard cut-offs for aAb positivity: Anti-CCP2 (IgG) was measured in serum using ELISA kits according to manufacturer's instructions (Diastat; Axis-Shield, Dundee, UK: cut-off >5 U/mL, reported specificity of 99.0%). Anti-CCP3.1 (IgG/IgA) was measured using ELISA kits (Inova Diagnostics, San Diego, California USA: cut-off ≥20 U/mL, reported specificity of 97.8%). RF isotypes IgM and IgA were measured using ELISA assays (QUANTA Lite: IgM positive cut-off >13.6 IU/mL, IgA positive cut-off >10.5 IU/mL). Based on recommendations included in the 1987 ACR RA Classification Criteria, positivity for RF isotypes was established using a cut-off level higher than that observed in 95% of 491 randomly selected blood donor controls.[25] In our analyses, we examined type of aAb+ (anti-CCP+ and RF isotype+/anti-CCP+ only/RF isotype+ only based on the standard cut-off), as well as whether the subject tested positive for the aAb at >2 x the standard cut-off (anti-CCP ≥2 x cut-off and RF isotype ≥2 x cut-off).

Optimal cut-offs for anti-CCP positivity: In addition to analysing standard cut-offs, we assessed optimal cut-offs for anti-CCP2 and anti-CCP3.1 positivity with the IA outcome using methods developed by Contal and O'Quigley for time-to-event outcomes with the SAS macro %findcut.[26] The optimal cut-offs were defined by the value of anti-CCP whose split is most significantly associated with incident IA using the log-rank test with a False Discovery Rate correction for multiple comparisons. The optimal cut-off for anti-CCP2 was ≥5 U/mL and for anti-CCP3.1 was ≥30 U/mL. Combining these two cut-offs we analysed anti-CCP+≥optimal cut-off (yes/no).

The 98th percentile cut-offs for aAb positivity: Positivity for anti-CCP2, anti-CCP3.1, RF IgM and RF IgA was established using a cut-off level higher than that observed in 98% of 200 randomly selected blood donor controls from the Denver, CO area. Results using this cut-off are presented in the online supplemental materials.

C reactive protein (CRP): Serum was tested for high sensitivity CRP by nephelometric assay (BN II Nephelometer, Dade Behring, Deerfield, Illinois, USA) and dichotomised using an elevated cut-off of >3 mg/L.[27]

Shared epitope

The presence of shared epitope (SE) alleles, HLA-DR4 and HLA-DR1, was tested and described previously.[24] A subject was considered SE positive if one or more alleles contained the following SE subtypes: DRB1*0401, *0404, *0405, *0408, *0409, *0410, and *0413; DRB1*0101, *0102; DRB1*1001.

Assessment of risk factors

Risk factors, which were assessed at the screened aAb+ visit, are listed in table 1. We dichotomised the following factors based either on small sample size or previously published associations with IA/RA[6 28 29]: race (non-Hispanic white (NHW)/other), SE (present/absent), >10 smoking pack-years as of the screening visit (yes/no: calculated as years of smoking multiplied by packs of cigarettes per day and dichotomised at >10 years), CRP (elevated/normal), tender joint on examination (yes/no), self-reported joint symptoms of pain, stiffness or swelling within the past week (yes/no).

Assessment of outcomes

We classified subjects as aAb persistent, that is, those who were aAb+ (for any aAb) at the next visit and aAb non-persistent, that is, those who tested aAb- (for all aAbs) at the next visit. We defined IA as the presence of at least one swollen joint consistent with RA-like synovitis. This was assessed using the 68-count joint examination performed by a study rheumatologist or trained study nurse, or through medical record review if

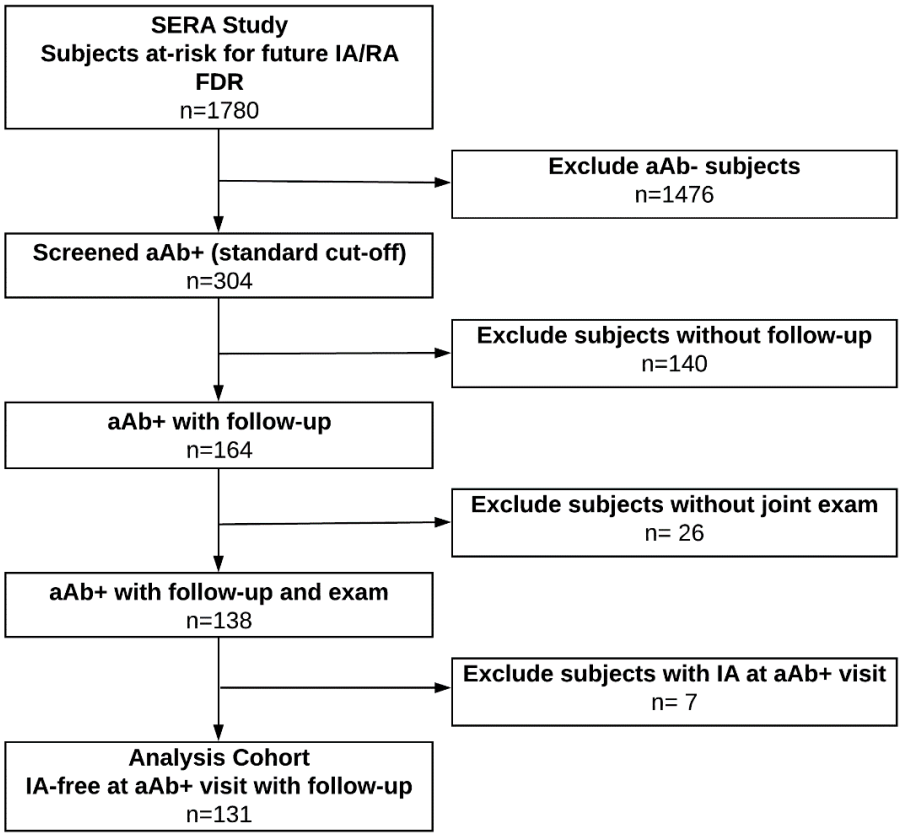

Figure 1 Flow chart of study subject inclusion. aAb, autoantibody; FDR, first-degree relative; IA/RA, inflammatory arthritis/rheumatoid arthritis; SERA, Studies of the Etiology of Rheumatoid Arthritis.

IA was identified outside of our study. All research study records and relevant medical records were reviewed by a single board-certified rheumatologist applying similar criteria to those classified from within and outside of the study.

Statistical analysis

To examine factors associated with aAb+ persistence, we conducted logistic regression analysis for the likelihood of aAb+ persistence.

To examine what predicts progression to IA, we first created Kaplan-Meier curves to assess IA-free survival after the screened aAb+visit by aAb classifications determined at the screened aAb+ visit. Univariable and multivariable Cox proportional hazards models were then used to determine risk factors associated with the risk of progression to IA. Years from first aAb+ visit to IA or last study visit was used as the time scale. The proportional hazards assumption was checked using methods developed by Lin *et al*.[30] Both the additive and multiplicative interaction between SE and pack-years >10 was assessed, given associations found in prior literature.[31 32]

The predictive ability of the risk models was compared by generating time-dependent area under the curve (AUC) statistics and Uno's concordance statistic (C-statistic) using the inverse probability of censoring weighting method.[33] Pairwise differences in the C-statistic were made between each consecutive model to determine whether one model was a better predictor of incident IA. A p value for the difference in Uno's C-statistic >0.05 indicates no difference in the predictive ability of the models. The time-dependent AUC was calculated and represents the average of the AUC statistics over time. Analyses were conducted in SAS (SAS V.9.4).

RESULTS

Study population

Of the 1780 subjects that were screened for aAbs, 304 (17.1%) subjects were aAb+. Of those, 164 subjects had at least one follow-up research visit. Twenty-six subjects did not have an exam and were removed from analyses. Of the 138 subjects remaining, 131 were IA-free at the aAb+ screening visit (figure 1). There were no significant differences between the screened aAb+ population (n=304) and the study population (n=131) (online supplemental table S1). The study population was 78% female and 79% NHW with a mean age of 48 years at the screened aAb+ visit (table 1).

Analysis of persistent aAb+

Three subjects developed IA/RA prior to the follow-up visit and were removed from analyses assessing predictors of aAb+persistence. Of the 128 subjects analysed for persistent aAb+, 82 subjects (64.1%) tested aAb+again with a median of 2.0 (IQR: 1.1–3.1) years between visits. Most subjects (96.3 %) were positive for the same aAb at follow-up. All subjects positive for both anti-CCP and RF isotype(s) at screening remained aAb+ at the follow-up visit. In addition, subjects who tested anti-CCP+ at levels ≥2x the standard cut-off at screening were significantly

Table 1 Characteristics of study population

	n=131
At screened aAb+ visit	
Age (year): mean±SD	47.7±15.0
Sex: % female	77.9
Race: % NHW	79.4
Shared epitope: % positive	54.2
Cigarette pack-years: % >10	13.7
CRP: % elevated (≥3 mg/L)*	34.6
Type of CCP	
CCP2: % positive (>5 U/mL)†	3.1
CCP3.1: % positive (≥20 U/mL)‡	41.5
CCP2 and CCP3.1: % positive	9.9
Type of RF isotype+	
RF IgM: % positive (>13.6 IU/mL)	35.9
RF IgA: % positive (>10.5 IU/mL)	13.0
RF IgM and RF IgA: % positive	5.3
Type of aAb+	
Anti-CCP– and RF isotype+	45.8
Anti-CCP +and RF isotype-	45.8
Anti-CCP +and RF isotype+	8.4
RF+ isotype ≥2x cut-off: % yes§	26.7
Anti-CCP+ ≥2x cut-off: % yes¶	27.5
Anti-CCP+ ≥optimal cut-off: % yes**	32.8
Tender joint on examination: % yes	28.8
Self-reported joint symptoms (pain, stiffness, swelling): % yes††	63.6
At follow-up visit	
Years from aAb+ screening visit to next follow-up visit: median (IQR)‡‡	1.6 (1.1–2.2)
Follow-up aAb+ status: % Persistent‡‡	64.1
No of visits (screening visit to IA or last visit): median (IQR)	3 (2–5)
Years from aAb+ screening visit to last study visit or IA: median (IQR)	4.4 (2.2–7.2)
Incident IA: % yes	15.3

*1 subject missing CRP.
†1 subject missing anti-CCP2.
‡1 subject missing anti-CCP3.1.
§RF IgM or RF IgA.
¶Anti-CCP2 or anti-CCP3.1.
**The optimal cut-off was calculated as ≥5 U/mL for anti-CCP2, and ≥30 U/mL for anti-CCP3.1.
††Subjects report presence of symptoms within the past week: 3 subjects missing data.
‡‡3 subjects developed IA/RA prior to the follow-up visit and are removed from analyses assessing predictors of aAb+ persistence.
aAb+, autoantibody positive; CCP, cyclic citrullinated peptide; CRP, C reactive protein; IA, inflammatory arthritis; NHW, non-Hispanic white; RA, rheumatoid arthritis; RF, rheumatoid factor.

more likely to remain aAb+ at the next follow-up visit compared with subjects who were negative for anti-CCP or tested positive at levels <2x the standard cut-off (OR 13.37, 95% CI 3.03 to 59.06, p=0.001). Testing positive for an RF isotype at ≥2x the standard cut-off was not associated with testing positive again (OR 1.70 95% CI 0.71 to 4.06, p=0.23).

Analysis of Incident IA

Over a median follow-up time of 4.4 years (IQR 2.2–7.2), 20 subjects (15.3%) developed IA. Of these, 12 IA subjects were identified as a result of a study visit exam, and in the remaining 8 subjects, IA was determined by a physician outside of the study, which was confirmed through medical record review. Overall sixteen (80.0%) of the 20 IA subjects met 2010 ACR/EULAR RA criteria[34] at some point during the study; 12 subjects met criteria at the time of incident IA and 4 subjects met criteria by the next study visit.

Kaplan-Meier curves illustrating IA-free survival after screening aAb+ by different aAb levels and aAb type are presented in figure 2; a summary of absolute risks of IA and 95% CI is presented in table 2. Of subjects who screened aAb+ for both anti-CCP and at least 1 RF isotype, 38.0% developed IA within 5 years, whereas 15.0% of anti-CCP+ only subjects and 9.0% of RF isotype+ only subjects developed IA within 5 years of follow-up (figure 2A). Among subjects with anti-CCP ≥2x the standard cut-off at screening, 32.0% developed IA within 5 years and had a shorter time to IA (p<0.01) (figure 2B). The absolute risk using the optimal cut-off for anti-CCP+ at screening produces a slightly lower absolute risk for IA (26.0%) (figure 2C). The absolute risk for IA at 5 years was highest among subjects with anti-CCP2+ or both CCP2+ and CCP3.1+ at the standard cut-off regardless of RF isotype status (figure 2D). Additional Kaplan-Meier curves by individual aAb are presented in online supplemental figure S1.

Subjects who report NHW race were less likely to develop incident IA (table 3). There was no evidence of a multiplicative or additive interaction between cigarette pack-years (>10 years) and SE (interaction p=0.37 and p=0.45, respectively). Anti-CCP ≥2x the standard and optimal cut-off at screening were associated with about a four-fold increased risk of developing incident IA. Anti-CCP2+ and anti-CCP3.1≥ the optimal cut-off are associated with increased risk of IA (table 3). We note that levels of anti-CCP2 are higher among subjects anti-CCP3.1+ at ≥ the optimal and 2x the standard cut-off (table 4).

The use of standard cut-offs for anti-CCP and RF isotypes may be problematic because they are set to different specificities, that is, the 98%ile for the former and 95%ile for the latter. To determine whether the stronger association of anti-CCP with IA than the RF isotypes was due to the higher specificity of the anti-CCP cut-off, we re-set all aAb+ cut offs to be the 98% ile of a control population, in this situation, anti-CCP was associated with IA risk and RF isotypes were not (see online supplemental tables S2 and S3).

We compared risk models to investigate combinations of factors that predict future development of IA using data available at the screened aAb+ visit. Anti-CCP2+ at the standard cut-off was the strongest predictor of incident IA. However, the presence of either anti-CCP2 or anti-CCP3.1 above the optimal cut-off or ≥2x the standard cut-off was also predictive of incident IA, and this combination identified a larger at-risk population. Therefore, we used the combined anti-CCP for the risk models. We first compared the AUCs of any anti-CCP+ at the standard cut-off and at the optimal cut-off and selected anti-CCP+ ≥optimal cut-off as a better descriptor of aAb status based on the higher AUC. The addition of NHW Race to model 2 resulted in marginal improvement in the predictive ability over model 1b, although this was not significant (difference in C-statistics p=0.21). Adding tender joint signs on examination to model 3 did not improve prediction of IA in this cohort (AUC=0.73, difference in C-statistics p=0.45) (table 5).

DISCUSSION

While screening an RA-free at-risk FDR population in a non-clinical setting, 17% tested positive for an RA-related autoantibody, and 15% of these aAb+ individuals developed incident IA over a median of 5 years of follow-up. The

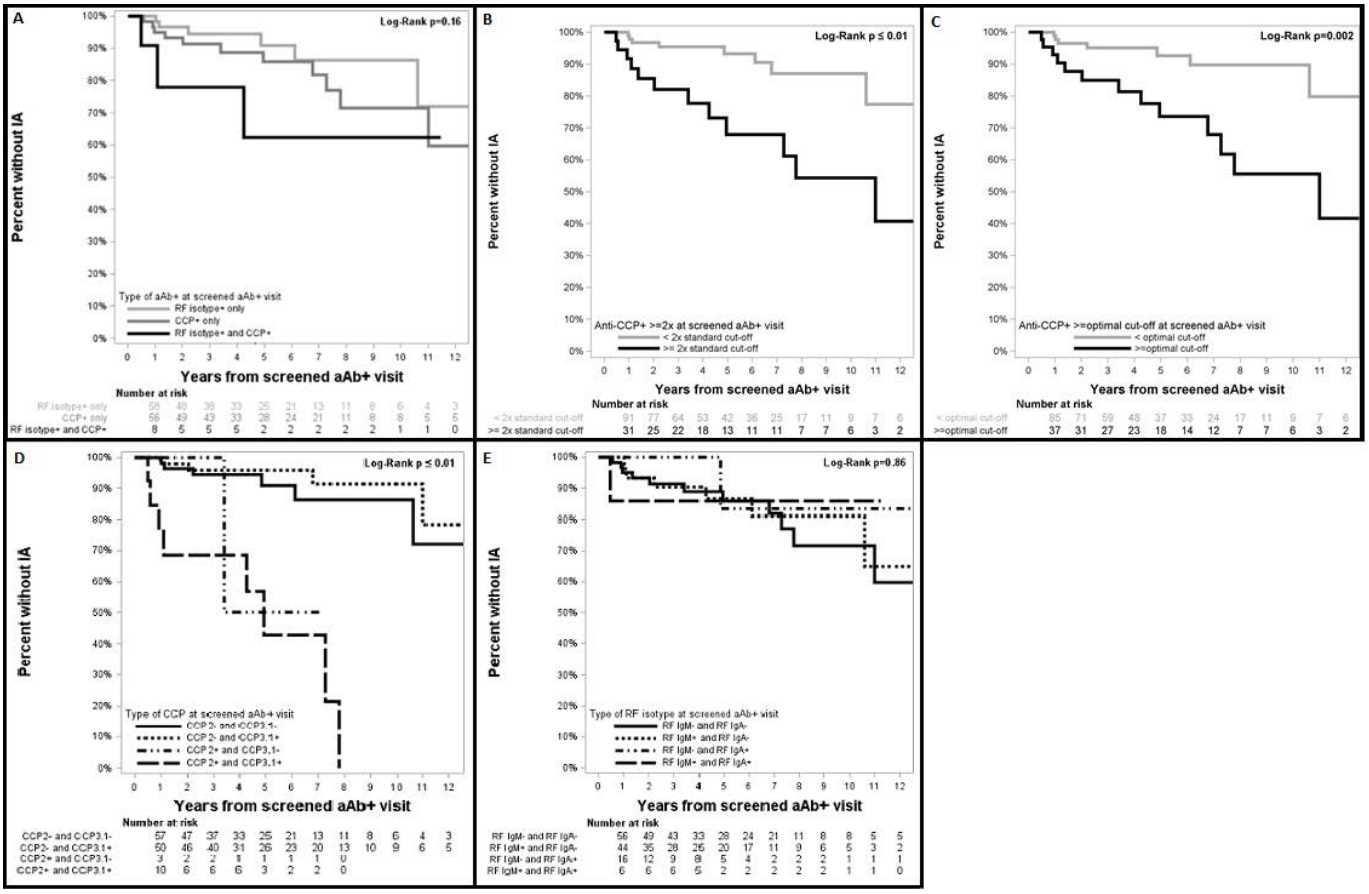

Figure 2 Probability of IA-free survival over 12 years of follow-up by aAb levels and type of aAb at screening visit. (A) Progression to IA by type of aAb+ at the screening visit; (B) Progression to IA by whether the anti-CCP at the screening visit was above or below 2 x the standard cut-off for positivity (note that the latter group includes those that were anti-CCP negative by the standard cut-off at the screening visit); (C) Progression to IA by whether the anti-CCP at the screening visit was above or below the optimal cut-off for positivity at the screening visit. The optimal cut-off was calculated as ≥5 U/mL for anti-CCP2, and ≥30 U/mL for anti-CCP3.1; (D) Progression to IA by type of anti-CCP+ (anti-CCP2 or anti-CCP3.1) at the screening visit regardless of RF isotype status; (E) Progression to IA by type of RF isotype+ (RF IgA or RF IgM) at the screening visit regardless of anti-CCP status. aAb, autoantibody; CCP, cyclic citrullinated peptide; IA, inflammatory arthritis; RF, rheumatoid factor.

strongest predictor of incident IA was screening anti-CCP2+ at or above the standard cut-off and was present in 13% of the study population. Levels of anti-CCP3.1 above an 'optimal' cut-off calculated from our data were also predictive of incident IA likely because anti-CCP2 levels were also high or because this indicated epitope spreading.

Table 2 Absolute risk of progression to IA among aAb+ subjects (n=131)

Characteristic	1-year risk (95% CI)	3-year risk (95% CI)	5-year risk (95% CI)
All Subjects	3% (2% to 8%)	8% (5% to 15%)	14% (8% to 23%)
Type of aAb+			
Anti-CCP– and RF isotype+	2% (1% to 12%)	6% (2% to 16%)	9% (4% to 24%)
Anti-CCP+ and RF isotype-	5% (2% to 15%)	9% (4% to 20%)	15% (5% to 28%)
Anti-CCP+ and RF isotype+	9% (1% to 49%)	22% (6% to 65%)	38% (13% to 79%)
Anti-CCP+ ≥2 x cut-off: Yes	8% (3% to 24%)	18% (8% to 36%)	32% (18% to 54%)
*Anti-CCP+ ≥optimal cut-off	7% (2% to 20%)	15% (7% to 31%)	26% (14% to 46%)
†Type of anti-CCP+			
Anti-CCP2+	0% (0% to 0%)	50% (9% to 99%)	50% (9% to 99%)
Anti-CCP3.1+	2% (13% to 30%)	4% (1% to 15%)	4% (1% to 15%)
Anti-CCP2+ and Anti-CCP3.1+	23% (8% to 56%)	32% (13% to 64%)	57% (29% to 88%)
‡Type of RF isotype+			
RF IgA+	0% (0% to 0%)	0% (0% to 0%)	17% (2% to 73%)
RF IgM+	2% (0.3% to 15%)	10% (4% to 24%)	14% (6% to 30%)
RF IgA+ and RF IgM+	14% (2% to 67%)	14% (2% to 67%)	14% (2% to 67%)

*The optimal cut-off was calculated as ≥5 U/mL for anti-CCP2, and ≥30 U/mL for anti-CCP3.1.
†Type of anti-CCP+ regardless of RF isotype status, using standard cut-off.
‡Type of RF isotype+ regardless of anti-CCP status.
aAb+, autoantibody positive; CCP, cyclic citrullinated peptide; IA, inflammatory arthritis; RF, rheumatoid factor.

Table 3 Factors associated with progression to inflammatory arthritis since screened aAb+ visit (n=131)

Characteristic*	Incident IA: no n=111	Incident IA: yes n=20	HR (95% CI)	P value
Age: years			0.98 (0.95 to 1.01)	0.24
Sex: female	87 (73.4%)	15 (75.0%)	0.69 (0.25 to 1.90)	0.47
Race: NHW	91 (82.0%)	13 (65.0%)	0.39 (0.16 to 0.98)	**0.04**
Shared epitope: present	58 (52.3%)	13 (65.0%)	1.24 (0.49 to 3.14)	0.65
BMI: ≥25	60 (54.1%)	11 (55.0%)	1.31 (0.54 to 3.17)	0.56
Cigarette Pack-years >10: yes	17 (15.3%)	1 (5.0%)	0.32 (0.04 to 2.43)	0.27
Tender joint on exam: yes	26 (23.4%)	8 (40.0%)	2.05 (0.79 to 5.35)	0.14
Self-report joint symptoms: yes†	67 (60.9%)	15 (79.0%)	1.76 (0.58 to 5.34)	0.32
CRP: elevated‡	38 (34.2%)	7 (36.8%)	1.05 (0.41 to 2.67)	0.92
Type of aAb+				
Anti-CCP– and RF isotype+	54 (48.7%)	6 (30.0%)	ref	ref
Anti-CCP+ and RF isotype-	49 (44.1%)	11 (55.0%)	1.67 (0.62 to 4.52)	0.31
Anti-CCP+ and RF isotype+	8 (7.2%)	3 (15.0%)	3.69 (0.92 to 14.83)	0.07
RF+ isotype (IgM and/or IgA) ≥2 x cut-off: yes	29 (26.1%)	6 (30.0%)	1.69 (0.64 to 4.48)	0.29
Anti-CCP+ ≥2 x cut-off: yes	24 (21.6%)	12 (60.0%)	4.09 (1.67 to 10.04)	**0.002**
Anti-CCP+ ≥optimal cut-off: yes§	30 (27.0%)	13 (65.0%)	3.95 (1.57 to 9.91)	**0.003**
aAb+ Persistence: yes¶	42 (37.8%)	4 (23.5%)	1.92 (0.63 to 5.91)	0.25
Anti-CCP Persistence: yes¶	37 (33.3%)	9 (52.9%)	1.95 (0.75 to 5.05)	0.17
Individual aAb+				
Anti-CCP2: % positive standard cut-off	8 (7.2%)	9 (47.4%)	11.51 (4.39 to 30.18)	**<0.001**
Anti-CCP2: % ≥2x standard cut-off	6 (5.4%)	8 (40.0%)	8.88 (3.48 to 22.64)	**<0.001**
Anti-CCP2: % positive optimal cut-off (same as standard cut-off)			11.51 (4.39 to 30.18)	**<0.001**
Anti-CCP3.1: % positive standard cut-off	54 (49.1%)	13 (65.0%)	1.61 (0.64 to 4.04)	0.31
Anti-CCP3.1: % ≥2 x standard cut-off	21 (18.9%)	10 (50.0%)	3.04 (1.26 to 7.33)	**0.01**
Anti-CCP3.1: % ≥optimal cut-off	27 (24.6%)	11 (55.0%)	2.84 (1.17 to 6.86)	**0.02**
RF IgM: % positive standard cut-off	46 (41.4%)	8 (40.0%)	1.00 (0.41 to 2.44)	0.99
RF IgM: % ≥2x standard cut-off	22 (19.8%)	6 (30.0%)	2.34 (0.88 to 6.21)	0.09
RF IgA: % positive standard cut-off	22 (19.8%)	2 (10.0%)	0.57 (0.13 to 2.45)	0.45
RF IgA: % ≥2x standard cut-off	7 (6.3%)	1 (5.0%)	1.05 (0.14 to 7.86)	0.97

Significant p values (<0.05) are shown in bold type.
*Separate unadjusted models for each risk factor were run.
†2 subjects did not complete self-reported joint symptom questionnaire.
‡1 subject missing CRP.
§The optimal cut-off was calculated as ≥5 U/mL for anti-CCP2, and ≥30 U/mL for anti-CCP3.1.
¶3 subjects removed from the analysis of aAb+ persistence because they developed IA prior to the follow-up visit at which aAb+ persistence would have been assessed.
aAb+, autoantibody positive; BMI, body mass index; CCP, cyclic citrullinated peptide; CRP, C reactive protein; IA, inflammatory arthritis; NHW, non-Hispanic white; RF, rheumatoid factor.

Prevention trials in preclinical RA[21 23 35 36] rely on the ability to identify at-risk individuals. Two trials use anti-CCP at either >2 x or >3 x the standard cut-off as inclusion criteria,[35 36] and our study confirms that anti-CCP ≥2 x cut-off is predictive of future IA. However, we also found that an optimal cut-off for anti-CCP, which included the standard anti-CCP2 cut-off (≥5 U/mL) or 1.5 x the standard cut-off for anti-CCP3.1 (≥30 U/mL) was just as predictive of IA. This may inform prevention studies to expand their recruited populations by lowering their anti-CCP inclusion cut-offs.

Table 4 Comparing levels of anti-CCP2 by anti-CCP3.1+ status

≥standard cut-off			
	Anti-CCP3.1 ≥20 U/mL	**Anti-CCP3.1 <20 U/mL**	**P value**
N	66	63	
Anti-CCP2: median (IQR)	0.28 (0.14–0.68)	0.32 (0.14–0.53)	0.87
≥optimal cut-off			
	Anti-CCP3.1 ≥30 U/mL	**Anti-CCP3.1 <30 U/mL**	**P value**
N	37	92	
Anti-CCP2: median (IQR)	0.37 (0.20–9.93)	0.30 (0.12–0.48)	**0.04**
≥2x standard cut-off			
	Anti-CCP3.1 ≥40 U/mL	**Anti-CCP3.1 <40 U/mL**	**P value**
N	30	99	
Anti-CCP2: median (IQR)	0.47 (0.20–41.91)	0.30 (0.12–0.47)	**0.02**

CCP, cyclic citrullinated peptide.

We found that 64% of our aAb+ study population maintained at least one of their aAbs from the screened positive visit to the follow-up visit. However, we did not find that aAb+ persistence was predictive of incident IA. This is in some contrast to findings in indigenous North American people who have a high prevalence of RA where conversion from aAb+ to aAb− status was associated with decreased risk of IA/RA.[20] We hypothesise that we may not have seen an association between aAb persistence and IA because of the speed by which some of the aAb+ subjects progress. For example, we excluded three subjects from the analysis examining aAb persistence as a risk factor for IA because they developed IA before the follow-up visit when they would have been tested for aAb again.

The high risk of progression to classifiable RA among those who are anti-CCP positive with joint signs in a clinic setting has previously been reported.[11 15 16] Of RA-free subjects who tested anti-CCP+ in tertiary care clinics, 46% with the highest levels of anti-CCP progressed to RA within 5 years.[17] The lower proportion of our study subjects with high anti-CCP levels that developed IA (29.0%) may be because our subjects were potentially

Table 5 Progression to inflammatory arthritis since aAb+ screening visit (n=131)

Model	Variable	HR (95% CI)	P value	Integrated Time-Dependent AUC
Model 1a	Anti-CCP+ ≥2 x standard cut-off at screening	4.09 (1.67 to 10.04)	0.002	0.66
Model 1b*	Anti-CCP+ ≥optimal cut-off at screening	3.95 (1.57 to 9.91)	0.003	0.67
Model 2	Anti-CCP+ ≥optimal cut-off at screening	3.52 (1.37 to 9.03)	0.01	0.70
	Race (NHW)	0.53 (0.21 to 1.36)	0.18	
Model 3	Anti-CCP+ ≥optimal cut-off at screening	3.23 (1.13 to 9.23)	0.03	0.73
	Race (NHW)	0.48 (0.16 to 1.40)	0.18	
	Tender joint on exam (yes)	1.61 (0.59 to 4.42)	0.37	

Differences in Uno's C-statistic.
Model 1b v. Model 2: p=0.21 Model 2 v. Model 3: p=0.45.
*The optimal cut-off was calculated as ≥5 U/mL for anti-CCP2 (the same as the standard cut-off), and ≥30 U/mL for anti-CCP3.1.
aAb+, autoantibody positive; AUC, area under the curve; CCP, cyclic citrullinated peptide; NHW, non-Hispanic white.

earlier in the development of RA, perhaps because they were tested outside of routine clinical care.

Our study adds to the literature on the importance of RA-related aAbs on the timing and progression to IA in a non-clinical research setting. Factors thought to be associated with risk of established disease, including older age, smoking, presence of the SE allele(s), the smoking*SE interaction, high levels of CRP and higher body mass index were not associated with IA, perhaps due to their primary influence at other stages of disease, including the initial development of RA-related aAbs. Alternatively, sample size may have limited our ability to detect some of these associations. Even though our population was ascertained outside of a clinical setting, 64.0% of subjects still report some joint symptoms including pain, stiffness, and swelling and 29.0% of subjects were found to have joint tenderness on examination. However, these joint signs and symptoms were not associated with either aAb+ persistence or incident IA. This finding is not unexpected because joint symptoms are common.[37] Because we could only ascertain self-reported joint symptoms within the past week or joint tenderness on the same day we may be missing the importance of these subtle fluctuations in the prediction of IA.

We have assembled and followed a large cohort of at-risk aAb+ subjects outside of the clinic setting, allowing us the best opportunity to identify factors specifically related to the evolution of systemic autoimmunity and later phases of RA development. However, follow-up studies are needed to validate the results of this study and usefulness of screening for subjects likely to have persistent aAb+ for selecting a targeted high-risk population for targeted epidemiological, mechanistic and intervention studies.

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Contributors All authors contributed to drafting or critically revising the article for intellectual content and approved the final version of the article. EAB had access to all data and analyses. Study conception and design: JMN and VMH. Acquisition of data: VMH, JMN, KDD, MKD, MHW, JRO, TRM, JAS, JHB, PKG and RK. Analysis and interpretation of data: EAB, JMN, KDD, KP and VMH.

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

Patient consent for publication Not required.

Ethics approval The study protocol was approved by the following institutional review boards (IRBs) at each SERA site: Colorado Multiple IRB, University of Nebraska Medical Center IRB, Benaroya Research Institute at Virginia Mason IRB, Cedars-Sinai Medical Center's IRB, North Shore-LIJ IRB and the Chicago Biomedicine IRB.

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CLINICAL SCIENCE

Dysbiosis in the oral microbiomes of anti-CCP positive individuals at risk of developing rheumatoid arthritis

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ABSTRACT

Objectives An increased prevalence of periodontitis and perturbation of the oral microbiome has been identified in patients with rheumatoid arthritis (RA). The periodontal pathogen *Porphyromonas gingivalis* may cause local citrullination of proteins, potentially triggering anti-citrullinated protein antibody production. However, it is not known if oral dysbiosis precedes the onset of clinical arthritis. This study comprehensively characterised the oral microbiome in anti-cyclic citrullinated peptide (anti-CCP) positive at-risk individuals without clinical synovitis (CCP+at risk).

Methods Subgingival plaque was collected from periodontally healthy and diseased sites in 48 CCP+at risk, 26 early RA and 32 asymptomatic healthy control (HC) individuals. DNA libraries were sequenced on the Illumina HiSeq 3000 platform. Taxonomic profile and functional capability of the subgingival microbiome were compared between groups.

Results At periodontally healthy sites, CCP+at risk individuals had significantly lower microbial richness compared with HC and early RA groups (p=0.004 and 0.021). Microbial community alterations were found at phylum, genus and species levels. A large proportion of the community differed significantly in membership (523 species; 35.6%) and structure (575 species; 39.1%) comparing CCP+at risk and HC groups. Certain core species, including *P. gingivalis*, had higher relative abundance in the CCP+at risk group. Seventeen clusters of orthologous gene functional units were significantly over-represented in the CCP+at risk group compared with HC (adjusted p value <0.05).

Conclusion Anti-CCP positive at-risk individuals have dysbiotic subgingival microbiomes and increased abundance of *P. gingivalis* compared with controls. This supports the hypothesis that the oral microbiome and specifically *P. gingivalis* are important in RA initiation.

Key messages

What is already known about this subject?

- Patients with rheumatoid arthritis have increased periodontal disease and a perturbed oral microbiome. The periodontal pathogen *Porphyromonas gingivalis* is able to citrullinate proteins via its peptidylarginine deiminase enzyme and can generate citrullinated antigens that may drive the autoimmune response in RA.
- Periodontitis and *P. gingivalis* were increased before joint inflammation in individuals at risk of RA, supporting the concept of periodontal inflammation and *P. gingivalis* as important risk factors in RA initiation.

What does this study add?

- This is the first study to demonstrate dysbiosis, including an increase of *P. gingivalis*, in the periodontally healthy microbiome (and altered diseased subgingival microbiomes) of individuals at risk of developing RA compared with healthy controls.

How might this impact on clinical practice or future developments?

- Our results indicate that dysbiosis in the subgingival microbiome precedes the onset of joint inflammation in at-risk individuals. This dysbiosis, together with the increase of *P. gingivalis*, may play an important role in the initiation of RA.
- Taken together with our previous findings, periodontal disease and the observed oral dysbiosis could be targets for future preventive interventions in individuals at risk of RA. Investigation of the overall metabolic capability of the subgingival microbiome may provide novel insights into the pathogenesis of RA.

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INTRODUCTION

Individuals at-risk of rheumatoid arthritis (RA) often have anti-citrullinated protein antibodies (ACPA) well before the development of joint inflammation.[1 2] Where the initiation of RA autoimmunity occurs is a critical question with significant implications for future preventative strategies. Recent data have implicated mucosal sites and the local microbiome and there has been considerable focus on the role of the oral mucosa and periodontium.[3 4]

There is an increased prevalence of periodontitis in patients with both early and established RA.[5] The subgingival microbiota in periodontitis, in particular the periodontal pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, may play a critical role in RA pathogenesis; *P. gingivalis* by contributing to ACPA production through citrullination of proteins

via its peptidylarginine deiminase enzyme (PAD) and *A. actinomycetemcomitans* by inducing leukotoxic hypercitrullination.[6–8] We recently reported an increased prevalence of periodontal inflammation and *P. gingivalis* in anti-cyclic citrullinated peptide (anti-CCP) positive at-risk individuals without arthritis (CCP+at risk), supporting the concept that periodontal inflammation and *P. gingivalis* precede joint inflammation, as important risk factors in RA initiation.[9] *A. actinomycetemcomitans* did not emerge as similarly significantly associated with at-risk individuals; *A. actinomycetemcomitans* is particularly important in severe generalised periodontitis,[10] which we did not see in our cohort.

Periodontitis is a complex disease, mediated by consortia of co-operating bacteria and the host responses to them. While *P. gingivalis* is a keystone pathogen that increases the risk of periodontitis, it depends on the activities of other members of the subgingival microbiome to establish within the community and express full virulence. Thus, to fully understand the role of periodontitis in RA pathogenesis, it is important to study the entire bacterial community. Although certain taxa and compositional and functional alterations were identified in RA-associated oral microbiomes,[11–13] it is difficult to clarify the cause and effect of these findings once clinical arthritis has developed. Furthermore, RA treatment is also likely to influence the oral microbiome.[12]

We therefore sought to comprehensively characterise the oral microbiome in CCP+at risk individuals without clinical arthritis; we aimed to report differences in the metagenomes, characterised by a shotgun metagenomic approach, sampled from periodontally healthy and diseased subgingival sites of CCP+at risk individuals, patients with early RA and healthy controls (HCs).

MATERIALS AND METHODS

HCs, CCP+at risk individuals with musculoskeletal symptoms but no clinical synovitis and patients with anti-CCP positive early RA (within the first 3 months of disease-modifying antirheumatic drug (DMARD) therapy) were recruited. The three groups were balanced for age, sex and smoking status (online supplemental table S1).[9] Periodontal assessments and subgingival plaque sampling were performed by three experienced dentists.[9] According to the latest Classification of Periodontal Diseases and Conditions, periodontally healthy sites were defined as sites with ≤3 mm probing depth and no bleeding on probing.[14] Diseased sites were those with ≥4 mm probing depth and ≥2 mm clinical attachment loss.[15] Subgingival plaque samples from a maximum of three healthy and three diseased sites were analysed for each participant using shotgun metagenomics sequencing (Illumina HiSeq 3000). Microbial diversity and community composition were compared between three groups. Periodontitis is a dysbiotic disease, with significant differences comparing microbiomes from healthy and diseased subgingival sites. The term dysbiosis is also used here to describe microbiomes from healthy sites that are distinct in composition from those of healthy sites from the HC group. Further details are given in the online supplemental material.

RESULTS

Microbial diversity

Within periodontally healthy sites, the CCP+at risk group showed a significantly lower Abundance Coverage Estimator value compared with the HC group (p=0.004) and the early RA group (p=0.021), indicating decreased estimated microbial richness of the subgingival microbiome (figure 1).

Bacterial community composition

Overall, 28 bacterial phyla, 593 genera and 1472 species were identified. Significantly altered community composition was found in the CCP+at risk group at different taxonomic levels. In periodontally healthy sites, phylum *Synergistetes* was found with significantly higher relative abundance in the CCP+at risk group compared with other groups (online supplemental figure S1a).

Among the top 20 most predominant genera in periodontally healthy sites (figure 2A), *Bifidobacterium* and *Porphyromonas* were present with significantly increased relative abundance in the CCP+at risk group (p=0.027, 0.033). In pairwise comparison, 523 species (35.6% of the community) differed significantly in membership and 575 species (39.1%) differed significantly in structure, comparing the CCP+at risk and HC groups. Less difference was found in the community membership (62 species, 4.2%) and structure (42 species, 2.9%) comparing the early RA and HC groups (figure 3A). Certain significant differences were also found between groups in periodontally diseased sites, for example, the abundance of phylum *Chlorobi* was increased in the HC group compared with other groups (online supplemental figure S1b) (corrected p<0.05). The genus *Porphyromonas* was significantly higher in the CCP+at risk group compared with other groups (p=0.015), and *Capnocytophaga*, *Cardiobacterium*, *Neisseria* and *Streptococcus* were significantly more abundant in the early RA group (p=0.009, 0.003, 0.024, 0.003) (figure 2B). At species level, only 1.4% and 5.7% of the microbial community differed significantly in membership and structure between the CCP+at risk and HC groups (figure 3B).

Core microbiome

The core microbiome, of which the species were present in at least 80% of the samples in each group, was used to compare stable associations between groups. Within periodontally healthy sites (figure 4A), 81 species were identified in the core microbiome of all study participants. The core microbiome from the CCP+at risk group was much less diverse than that of the HC or early RA group. There was no core species exclusively belonging to the CCP+at risk group, unlike the HC and early RA groups, which had 35 and 79 exclusive core species, respectively. In the periodontally diseased sites (figure 4B), 42 species

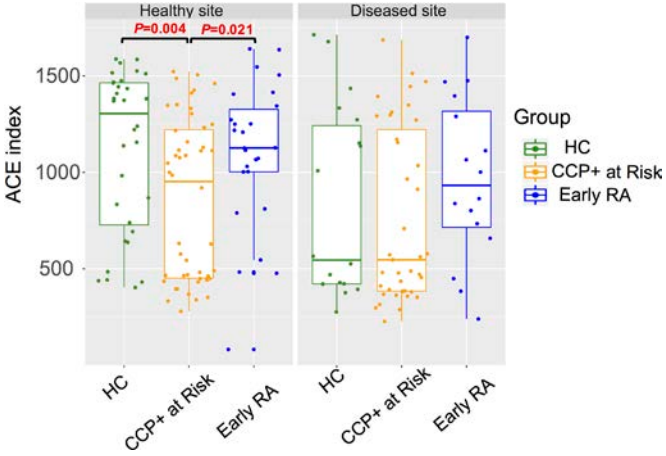

Figure 1 Comparison of α-diversity in healthy control (HC), CCP+at risk and early RA groups using samples from periodontally healthy sites and diseased sites. Abundance Coverage Estimator (ACE) index was significantly decreased in the CCP+at risk group compared with the HC group in periodontally healthy sites (Kruskal-Wallis test). CCP, cycliccitrullinated peptide; RA, rheumatoid arthritis.

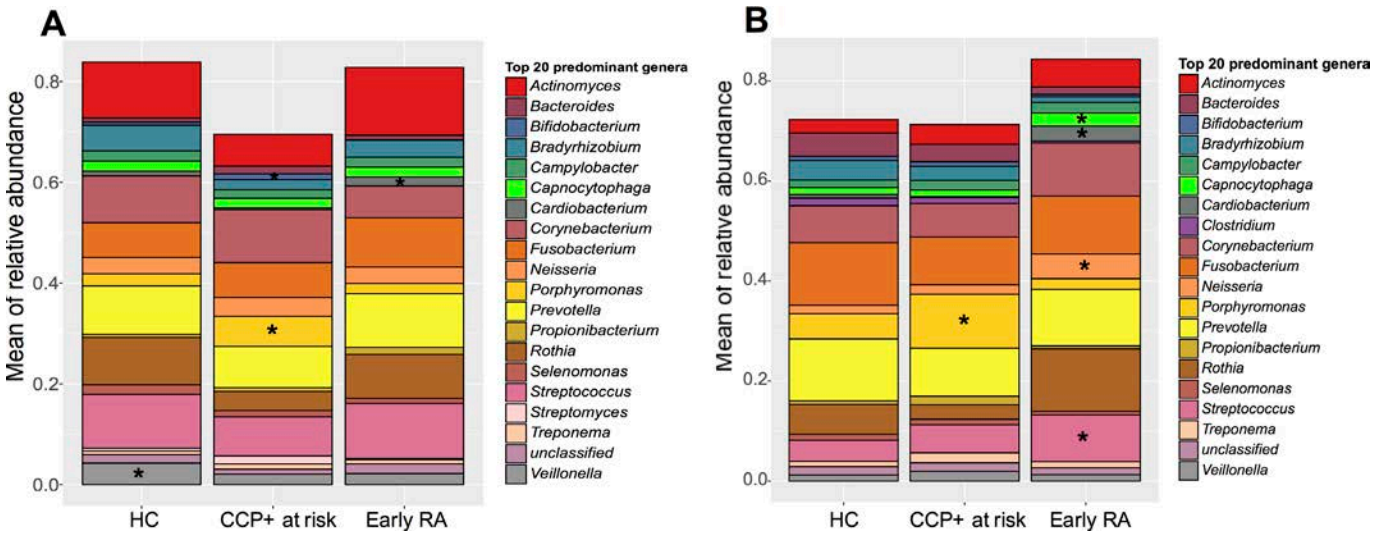

Figure 2 Taxonomic profiles for the 20 most abundant genera in subgingival plaque from periodontally healthy and diseased sites in healthy control (HC), CCP+at risk and early RA groups. Relative abundance of the 20 most abundant genera within (A) periodontally healthy sites and (B) diseased sites was plotted for each group. The permutation test (one-sided signassoc function, indicspecies R-package) was used to find the genera with significantly different relative abundances between groups. * corrected p<0.05 (Sidak's correction). CCP,cyclic citrullinated peptide; RA, rheumatoid arthritis.

were found in the core microbiome of all groups. Importantly, 6, 2 and 190 species were identified as uniquely belonging to the HC, CCP+at risk and early RA core microbiomes, respectively (online supplemental tables S2-S3). Certain species were significantly more abundant in each group compared with the other groups within periodontally healthy or diseased sites (online supplemental table S4). In particular, within both periodontally healthy and diseased sites, *Arthrobacter chlorophenolicus* and *P. gingivalis* were significantly more abundant in CCP+at risk individuals.

Bacterial co-occurrence networks in subgingival microbiomes

In periodontally healthy sites, Spearman's correlation analysis identified 347, 83 and 1024 edges as strong ($q<-0.7$ or >0.7) and significant (corrected $p<0.01$) pairwise correlations between nodes (species) in each the HC, CCP+at risk and early RA groups, respectively (online supplemental figure S2). In periodontally diseased sites, there were 49, 139 and 365 edges identified in HC, CCP+at risk and early RA groups, respectively (online supplemental figure S3). The edge/node ratio (density) of the network represents the number of co-occurrence instances in a microbial community; in the early RA group, this was higher than that of other groups in both periodontally healthy and diseased sites, reflecting a dysbiosis of the subgingival microbiome in early RA patients (online supplemental table S5).

To gain deeper insights into the differences between groups, the hubs in each network were identified by ranking the top 20 nodes with the maximal clique centrality (MCC) algorithm. In the periodontally healthy sites (figure 5A), the cluster of *Neisseria* spp. by which the network of HC group was dominated was not found in the hubs of other groups. Species including *Filifactor alocis*, *Campylobacter rectus*, *Porphyromonas endodontalis* and *Treponema vincentii* formed the network hubs for both HC and CCP+at risk groups, while the early RA group showed entirely different network hubs. Within the periodontally diseased sites (figure 5B), *Actinomyces viscosus* and *Actinomyces urogenitalis* were identified in the network hubs of all groups, indicating an implication in the development of periodontal disease irrespective of RA status. Intriguingly, the periodontal pathogen *A. actinomycetemcomitans*, which may also initiate protein citrullination in RA, was one of the hubs of the early RA group.

Functional capabilities of subgingival plaque microbiomes

Abundances of 3034 clusters of orthologous genes (COGs) functional units were normalised and compared between groups. Within periodontally healthy sites, 17 functional units were significantly over-represented in the CCP+at risk group compared with the HC group and 5 functional units were significantly over-represented in the early RA group compared with the HC group (online supplemental table S6) (corrected $p<0.05$). In periodontally diseased sites, significant differences were found comparing the early RA group with the HC and CCP+at risk groups (online supplemental table S7). The functional unit of 'PAD and related enzymes' were detected in 65.6%, 68.8% and 69.2% of samples in the HC, CCP+at risk and early RA groups from periodontally healthy sites and in 55.6%, 69.2% and 56.3% of each group from diseased sites. No significant difference was found in the normalised counts between groups either in periodontally healthy or in diseased sites (figure 6).

DISCUSSION

Although intensively studied, the mechanisms of disease initiation and development of autoimmunity in RA are still unclear.[16] ACPA are highly specific for RA and can be detected years before joint inflammation, suggesting a preclinical phase of RA, which could be a window of opportunity for disease prevention.[17] We previously showed that periodontitis and *P. gingivalis* were increased before clinical or subclinical joint inflammation in individuals at risk of RA.[9] Other studies have identified increased periodontitis in the first-degree relatives of patients with RA.[18 19] Compared with HCs, the alterations in the subgingival microbial community of patients with RA has been reported in different studies,[11–13] suggesting a potential role of oral microbial dysbiosis in RA development. However, it is unknown if subgingival microbial dysbiosis precedes the onset of RA. The present study, to our knowledge, is the first comprehensive characterisation of the subgingival microbiome from both periodontally healthy

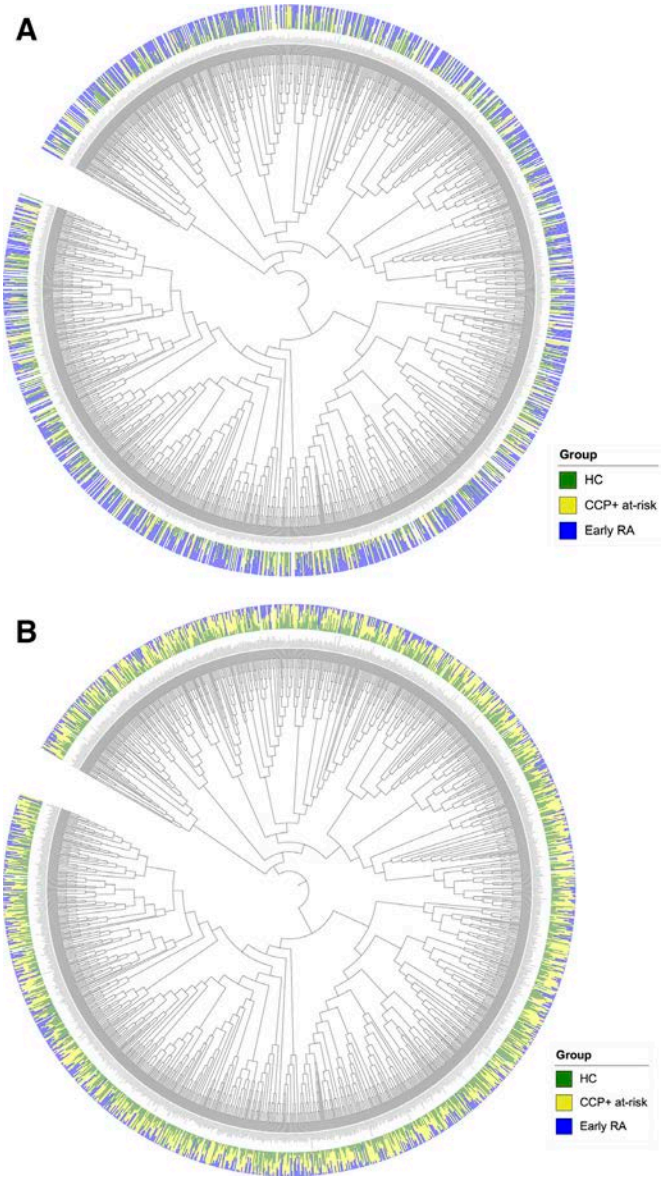

Figure 3 Phylogenetic tree representing normalised mean relative abundance of species (stacked bar chart) in the subgingival microbiome of (A) periodontally healthy and (B) periodontally diseased sites (phylogenetic tree constructed using the webserver iTOL.embl.de). CCP,cyclic citrullinated peptide; HC, healthy control; RA, rheumatoid arthritis.

and diseased sites in at-risk individuals. To preclude the effect of established periodontitis on the subgingival microbiome, analysis was performed on the samples from shallow gingival sulci (3 mm depth or less) with no bleeding on probing. This study comprised a relatively small sample size but participant groups were well balanced for age, sex and smoking status. Other variables currently being investigated for possible associations with periodontal disease (eg, body mass index, race, alcohol, education level) may also influence the subgingival microbiome. Larger sample size will be needed to more completely define the role of the subgingival microbiome in the development and progression of RA.

In CCP+at risk individuals, significant alterations were found in the composition of the periodontally healthy subgingival microbiome at different levels, which distinguished this group from matched controls and patients with early RA. In agreement with the present study, compositional change of salivary microbiota and decreased microbial diversity were found in individuals at high risk for RA in a recent study.[20]

Most previous studies utilised 16S rRNA gene sequencing to analyse the oral microbiome of RA patients.[11 13 20] However, a major limitation of this method is that only a single region of the bacterial genome can be sequenced and it is difficult to distinguish the species when their 16S rRNA gene sequences display high similarities.[21] The present study utilised shotgun metagenomics, which has several advantages including more confident identification of bacterial species, increased detection of diversity and prediction of genes.[22]

P. gingivalis may contribute to RA aetiology via the citrullination of local antigens by its PAD.[7 23] While some previous studies have examined the association between *P. gingivalis*, and established RA, few have looked at *P. gingivalis* in individuals at risk of RA. Studies determining levels of antibodies against *P. gingivalis*, or its virulence determinants, in HC, at-risk or established RA groups have been equivocal, possibly due to methodological and sampling differences.[7 24–28] A recent study demonstrated decreased levels of *P. gingivalis* in the saliva of high-risk individuals compared with HCs using 16S rRNA gene sequencing.[20] Analysis of the microbiome of saliva and supragingival dental plaque using shotgun sequencing revealed *P. gingivalis* to be enriched in HCs rather than patients with RA.[12] In another study, periodontitis, but not the subgingival presence of *P. gingivalis*, was more prevalent in patients who later progressed to classifiable RA.[29] de Smit *et al*[30] concluded that, while there was evidence that periodontitis may precede symptomatic RA, there was insufficient evidence to confirm a role specifically for *P. gingivalis* in disease progression. Thus, while the link between periodontitis and RA is established, the specific roles of *P. gingivalis* or its PAD have been less clear. Our data indicate that anti-CCP positive at-risk individuals have increased abundance of *P. gingivalis* compared with HCs.

A lower abundance of *P. gingivalis* as well as alterations in microbial composition and functional capability were found in the early RA group, which may be related to the inflammatory burden of RA. Lopez-Oliva *et al*[13] proposed RA may act as a condition shaping the subgingival microbiome, particularly promoting the growth of certain organisms. Moreover, these patients were receiving DMARDs, although for less than 3 months. It is likely that RA therapy, particularly drugs with additional antibacterial properties,[31 32]

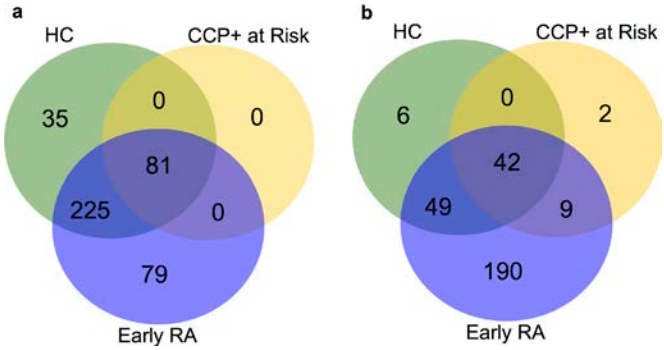

Figure 4 Overlap analysis of the group-specific and shared core species.Core species in each group of periodontally healthy and diseased site samples were identified, respectively (>80% prevalence). Number of group-specific and shared core species were visualised for (A) healthy sites and (B) diseased sites. CCP,cyclic citrullinated peptide; HC, healthy control; RA, rheumatoid arthritis.

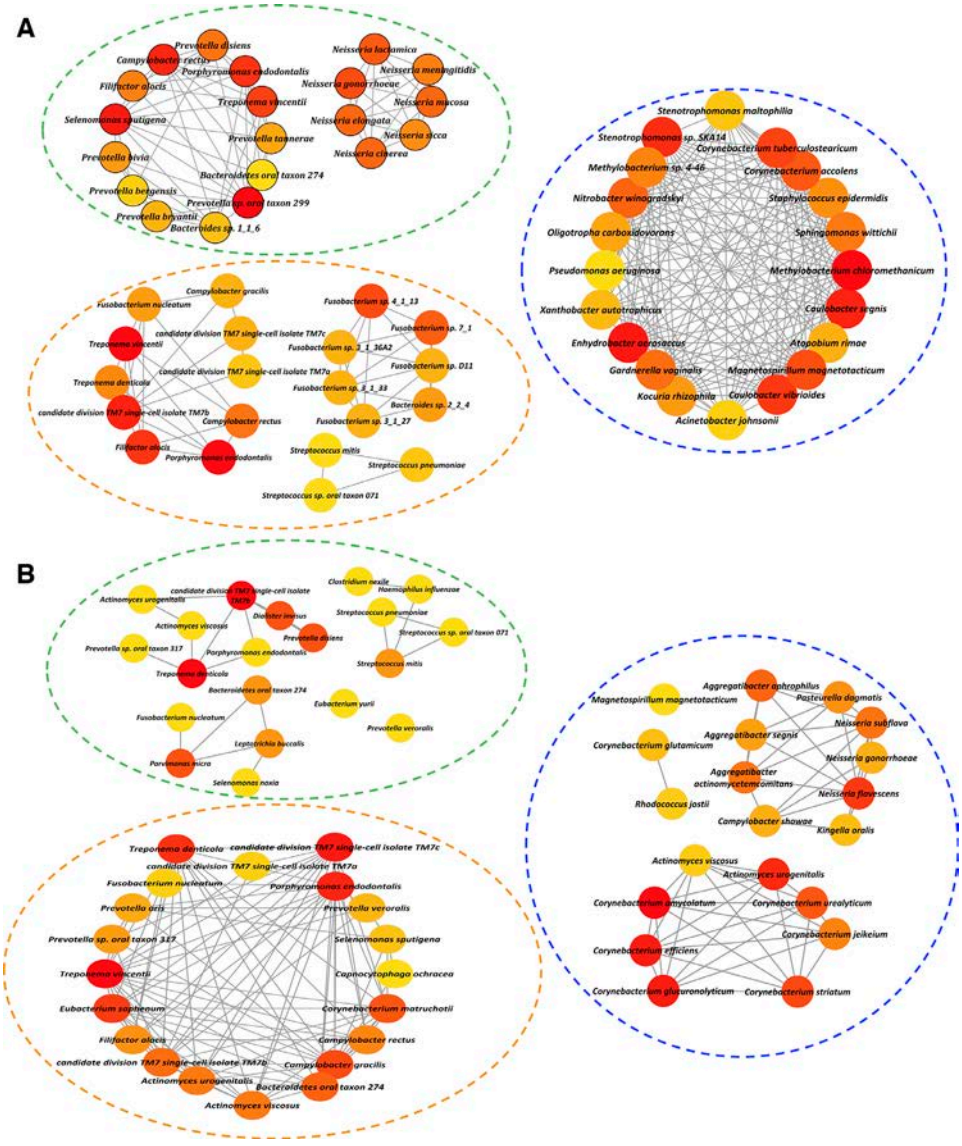

Figure 5 Identification in plaque from periodontally healthy and diseased sites of hubs in the networks of healthy control (HC), CCP+at risk and early RA groups. The top 20 nodes (species) ranked by maximal clique centrality were displayed in circular layout for each group from (A) periodontally healthy and (B) diseased site samples. Nodes are coloured based on rank; dark colour denotes high ranks. Green dashed line: HC; orange: CCP+at risk; blue: early RA. CCP,cyclic citrullinated peptide; RA, rheumatoid arthritis.

can influence the subgingival microbiome. RA regimes with immunomodulatory effects may influence both the development of the subgingival microbiome and the progression of periodontitis.[33 34] A recent shotgun sequencing study identified alterations in the oral microbiome in patients with RA, which were partially restored by DMARD treatment.[12]

The presence and abundance of PAD and related enzymes (the COG functional unit representing a family of orthologous protein-coding genes) were similar between groups. This is interesting given the differences that were observed between the groups in *P. gingivalis* abundance. Although *P. gingivalis* was once considered unique among prokaryotes in producing a PAD, PAD homologues were recently found in other *Porphyromonas* species.[35] Thus, the PAD in the subgingival microbiomes may arise from a range of species, not all of which may express PAD at the levels and with similar activity to the *P. gingivalis* PAD. A recent study also reported variations in the active site of PAD detected in clinical isolates of *P. gingivalis*, one of which was associated with increased *in vitro* activity.[36] Our data cannot reveal differences in the expression or activity of PADs, or *P. gingivalis* PAD specifically. Detailed comparison of the active *P. gingivalis* PAD site and potential enzyme activity in different groups related to RA status would be an important area for future work.

Other periodontal pathogens may also contribute to protein citrullination via routes different from *P. gingivalis*. The leukotoxin-A (LtxA) produced by *A. actinomycetemcomitans* has been implicated in inducing leukotoxic hypercitrullination, and exposure to *A. actinomycetemcomitans* was associated with ACPA.[6] This species was not dominant in the present study; considerable variations in isolation rates of *A. actinomycetemcomitans* have been reported in the literature, which may be the consequence of geographical differences in prevalence and methodological differences.[37] *P. intermedia* was recently reported to be associated with antibody responses to a novel citrullinated peptide related to RA,[38] but abundance of this organism did not emerge in our analyses as different in the groups sampled. It is clear

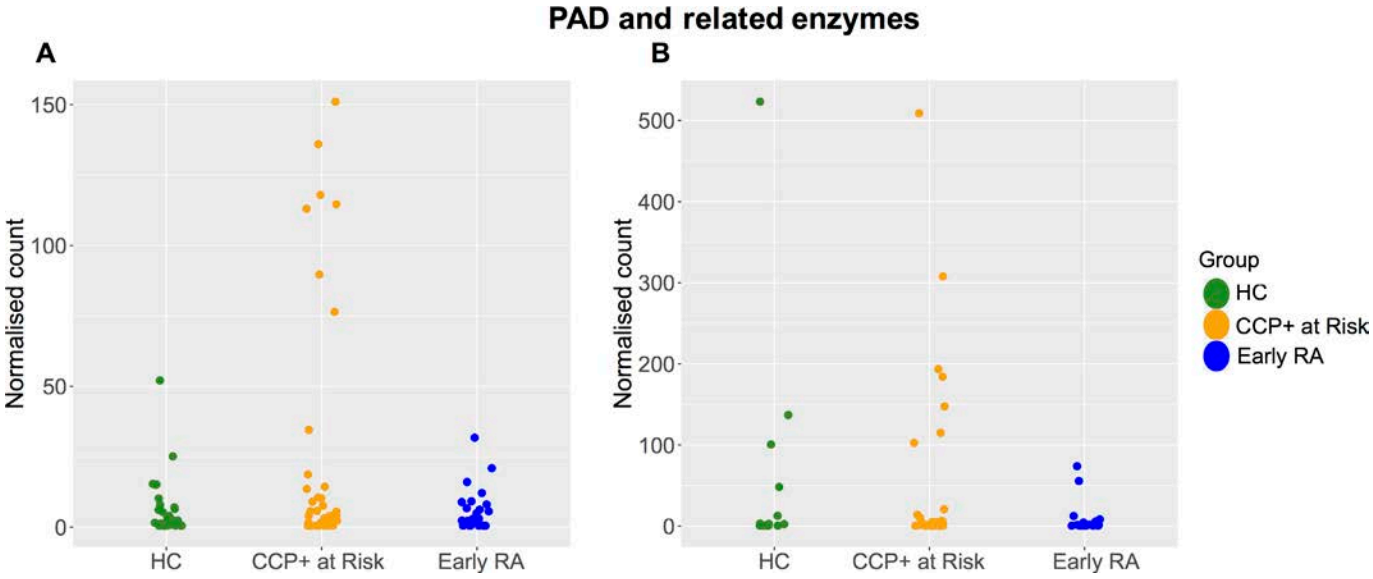

Figure 6 Normalised count of peptidylarginine deiminase enzyme (PAD) and related enzymes in healthy control (HC), CCP+at risk and early RA groups using samples from periodontally healthy sites and diseased sites. Abundance of PAD and related enzymes was normalised by sequencing depth and compared between groups using the Wald test in *DESeq2* R package. No significant difference was found between groups either in (A) periodontally healthy or in (B) diseased sites (corrected p>0.05). CCP,cyclic citrullinated peptide; RA, rheumatoid arthritis.

that the microbiome of these patients was highly perturbed compared with both HCs and CCP+at risk individuals and the influence of DMARDs and duration of therapy requires further consideration. Intriguingly, there were some species that have not previously been reported as abundant in the subgingival plaque of patients with early RA, for example, *Neisseria gonorrhoeae* (online supplemental table S4). This pathogen of the urogenital tract can adapt to display asymptomatic survival in the human nasopharynx and oropharynx, providing a potential reservoir for their further spread.[39 40] There is evidence of widespread horizontal gene transfer in the genus *Neisseria*[41] and of commensal species sharing many gene sequences with closely related pathogenic species,[42] and this may have impacted on our findings regarding the relative abundance of individual *Neisseria* species. *In vitro* culture and more in-depth analysis are necessary to clarify the presence of *N. gonorrhoea* and its potential contribution to oral microbial dysbiosis.

Several species were identified as hubs of the co-occurrence networks; those in the CCP+at risk group may be indirectly involved in the pathogenesis of RA via the interplay with *P. gingivalis* and possibly by supporting communities that promote citrullination by multiple routes. Among these hub species, *Streptococcus* spp are considered the principle early colonisers in dental plaque, and their colonisation influences the composition of maturing plaque.[43] *Fusobacteriumnucleatum*, which was demonstrated to accelerate collagen-induced arthritis in mice, functions in a bridging complex between early and late colonisers such as *P. gingivalis*.[44] A strong synergy was also observed between *Treponema denticola* and *P. gingivalis* in biofilm formation.[45] Therefore, it is logical to consider the overall capacity of the microbial community in future work.

In conclusion, this study has demonstrated dysbiosis in the subgingival microbiome alongside the specific increase of *P. gingivalis* in individuals at risk of RA. We propose that these may play an important role in the initiation of RA and that periodontitis and the observed oral dysbiosis may be attractive targets for future preventative interventions, such as periodontal therapy, in individuals at risk of RA.

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EPIDEMIOLOGICAL SCIENCE

Risk of venous thromboembolism in rheumatoid arthritis, and its association with disease activity: a nationwide cohort study from Sweden

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ABSTRACT

Objective To assess the incidence of venous thromboembolism (VTE) in rheumatoid arthritis (RA) relative to individuals without RA, and to investigate the relationship between aspects of clinical disease activity in RA and the risk of VTE.

Methods We conducted a nationwide register-based cohort study 2006 through 2018 using the Swedish Rheumatology Quality Register linked to other national patient registers to identify all patients with RA with at least one registered rheumatologist visit during the study period (n=46 316 patients, 322 601 visits). The Disease Activity Score 28 erythrocyte sedimentation rate (ESR) (DAS28 ESR) and its components served as the exposure, and a VTE event within the year following the visit was the main outcome. We also included general population referents (1:5) matched on age, sex and residential area.

Results Based on 2241 incident VTE events within 1 year of each included visit, and 5301 VTE events in the general population cohort, the risk ratio for VTE in RA was 1.88 (95% CI 1.65 to 2.15). Among patients with RA, the risk (and risk ratio) increased with increasing RA disease activity, from 0.52% following visits in remission to 1.08% following visits with DAS28 ESR high disease activity, RR compared with remission=2.03, 95% CI 1.73 to 2.38. Compared with the general population, also patients with RA in DAS28 ESR remission were at elevated VTE risk.

Conclusions This study demonstrates a strong association between clinical RA disease activity measured by DAS28 ESR and the risk of VTE. RA disease activity can be used as an additional tool for VTE risk stratification in patients with RA.

Key messages

What is already known about this subject?

- Patients with rheumatoid arthritis (RA) may be at increased risk of venous thromboembolism (VTE), but how any risk increase varies with RA disease activity is not known.

What does this study add?

- In this nationwide study, there was a close to doubled risk of VTE in patients with RA compared with the general population.
- We noted strong associations between measures of RA disease activity and risk of VTE events, for example, a twofold increase in risk from Disease Activity Score 28 (DAS28) remission to DAS28 high disease activity.

How might this impact on clinical practice or future developments?

- These results may be used as a basis for clinical VTE risk stratification in patients with RA.

INTRODUCTION

Venous thromboembolism (VTE), including deep vein thrombosis (DVT) and pulmonary embolism (PE), are common medical concerns associated with significant morbidity and mortality.[1] Established VTE risk factors include age, immobilisation, surgery, specific drugs and comorbid conditions such as malignancy, ischaemic heart disease, chronic obstructive pulmonary disease and hospitalised infection.[2]

Several studies have demonstrated that patients with rheumatoid arthritis (RA) are, on average, at increased risk for VTE.[3–10] By contrast, few if any studies have investigated the underlying reasons for this risk increase, let alone how it varies across patient subsets. Many established VTE risk factors occur more often in patients with RA. In vitro and in vivo studies have shown that aspects of inflammation might increase VTE risk by upregulation of procoagulatory factors and through endothelial damage.[11 12] Whether and how much clinical RA disease activity is linked to VTE risk remains to be understood. Such information would be important for a better understanding of the nature of the observed overall risk increase, and might constitute an important means for risk stratification in clinical practice, and in clinical trials.

The need for a better understanding of the association between the RA phenotype and VTE risk has radically increased with the recent safety signals arising from trials of Janus kinase inhibitors (JAKi). In 2019, and based on an increased number of VTE events in patients treated with the higher dose (10 mg) in an ongoing postmarketing safety trial, the European Medicines Agency and the US Food and Drug Administration issued caution for VTE events in patients treated with tofacitinib.[13 14] It remains unclear, however, if the purported VTE risk increase with JAKi is mainly explained by the drug itself, by the underlying disease, or by other factors.

The aims of this study were therefore to (1) investigate the relationship, if any, between clinical RA disease activity and the incidence of VTE, and

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(2) for contextualisation, to assess the incidence of VTE in RA patients relative to individuals without RA.

METHODS

Study design

We performed a nationwide cohort study of the association between clinical RA disease activity and VTE risk. We also compared VTE incidence in the RA population relative to matched general population referents.

Setting

Sweden has a population of around 10 million. Healthcare is publicly funded for all residents. Drug prescriptions are free of charge after an annual threshold of €220. Most patients with RA are treated by rheumatologists based at public hospitals.

Data sources

We identified a cohort of patients with RA from the Swedish Rheumatology Quality Register (SRQ), a large longitudinal clinical RA register operated by the Swedish Society for Rheumatology since the mid-1990s. SRQ contains longitudinal information on RA disease activity and treatment. Typically, at each outpatient visit to a rheumatologist, data are entered into SRQ by the rheumatologist, data on patient-reported outcome measures are entered by the patient. Cumulatively, SRQ holds information on around 52 000 patients with RA. We linked this RA cohort to a series of other national and population-based Swedish registers: The Swedish National Patient Register, the Prescribed Drug Register, the Cause of Death Register and the Swedish Population Register. The National Patient Register contains information, including all registered International Classification of Diseases (ICD) codes, on hospital discharges from inpatient care since 1969, and visit data from non-primary outpatient care since 2001. Since 1987, the inpatient coverage is >99%.[15] The Prescribed Drug Register contains information on all dispensations of prescribed drugs since 2005. The Cause of Death Register contains information about all deaths and causes (main and contributory ICD codes) since 1961. The Swedish Population Register contains information on residence and domicile, civil status and migration data for all inhabitants of Sweden. Individual-level data from these registers can be linked together using a unique personal identification number issued to all Swedish residents.

Study population and exposure

We identified all patients ≥18 years of age with a rheumatologist-based diagnosis of RA, and who had at least one visit registered in the SRQ during 1 January 2006 to 31 December 2017 (46 316 patients with RA who contributed data from 322 601 visits). For each visit, we obtained data on Disease Activity Score 28 ESR (hereafter DAS28) and its components (main outcome), Health Assessment Questionnaire (HAQ) and other clinical RA-variables, where available. We categorised each visit by its recorded DAS28 category; remission (0–2.6), low (2.7–3.2), intermediate (3.3–5.0) and high (>5.0) disease activity. For each unique patient, we randomly selected five referents from the Swedish Population Register (n=215 843), individually matched by sex, year of birth and residential area. Online supplemental table S1 describes the creation of the datasets.

Outcome

Through linkage to the Patient Register, the Prescribed Drug Register and the Cause of Death Register we identified incident VTE events occurring during the 365 days after each registered visit. One individual could thus contribute to more than one visit (and with more than one VTE event); each visit had its own baseline covariate status. In the main analysis, incident VTE was defined as a registration of any VTE diagnosis within the 365-day period in the Patient Register (inpatient and specialised outpatient care) or PE listed as underlying cause of death in the Cause of Death Register (online supplemental table S2 and online supplemental figure S1). We excluded visits for which the patient had a registered VTE event within the prior year, since VTE during this time was considered a prevalent VTE. Patients with a more distant history of VTE were included. If death (other than from PE) or emigration occurred during the 1-year follow-up that visit was excluded. For the general population referents, the 1-year follow-up period for assessment of incident VTE events started at the same date as the rheumatologist visit for their corresponding patient with RA.

Statistical analyses

We calculated the cumulative 1-year incidence for VTE in the RA population, by each DAS28 category, and in the general population referents. Risk ratios for the association between each DAS28 category and VTE risk, and for the RA population versus the general population referents, were calculated using log-binomial regression. We used robust cluster SEs to account for the correlated data structure in which one individual could contribute more than one visit. Models were adjusted for age at visit, via a restricted cubic spline with 3 df, sex and calendar year of visit (categorised 2006–2009, 2010–2013, 2014–2017). To test the robustness of our findings in relation to the definition of VTE, the length of the time window and the selection of rheumatologist visits, we performed a series of sensitivity analyses, as summarised in online supplemental table S2. We also performed multiple imputation (MI) using chained equations with 30 imputations to impute missing DAS28 category (17% of all visits); multinomial regression was used for MI which included the VTE outcome, indicators for PE/DVT, age, sex and year. All analyses were performed using Stata V.16.[16]

RESULTS

Table 1 displays characteristics at each rheumatologist-visit for the entire RA population, overall and separately according to the DAS28 category at the visit (percentage missing for each variable presented in online supplemental table S3). Comparing patient characteristics by DAS28 category, we noted even distribution regarding RA treatment and socioeconomic characteristics, but a slightly increased prevalence of comorbidities at visits with high DAS28 disease activity (vs remission). Online supplemental table S3 displays characteristics at the first and last visit.

Table 2 presents the number of VTE events and cumulative incidences of VTE for the entire RA population, by each DAS28 category, and for their general population referents, overall and by VTE subtype, sex and age. In the RA population, 2241 visits in 1360 unique individuals were followed by a VTE within 1 year. Of these, 1408 were DVT events and 833 were PE events.

The overall cumulative 1-year incidence of VTE was 0.71% in the RA population (ie, across all DAS28 categories) and 0.36% among their general population referents, corresponding to an adjusted risk ratio of VTE in RA of 1.88 (95% CI 1.65 to 2.15). In both populations, the incidence of DVT was about twice as that of PE. The cumulative incidence of VTE was higher in males, and increased with increasing age.

Table 1 Characteristics at each rheumatologist visit for entire study population and stratified by DAS28 category, in Swedish patients with RA registered in the Swedish Rheumatology Quality register from 2006 until 2017

		DAS28 category			
	RA population	Remission	Low	Intermediate	High
Observations (n)	322 601	97 347	43 756	94 611	33 217
Individuals (n)	46 316	29 264	22 637	31 611	17 385
Age at visit, median (IQR)	63 (52–71)	62 (50–70)	64 (54–72)	63 (53–71)	63 (53–71)
Females (%)	74	67	75	78	78
RA duration, median (IQR)	8.7 (3.2–17.6)	8.1 (3.2–15.9)	9.8 (3.8–19.1)	9.3 (3.2–18.7)	7.0 (1.5–16.2)
Clinical RA data					
DAS28ESR, median (IQR)	3.1 (2.2–4.3)	1.9 (1.5–2.3)	2.9 (2.8–3.1)	4.0 (3.6–4.5)	5.8 (5.4–6.3)
DAS28CRP, median (IQR)	2.9 (2.0–3.9)	1.9 (1.6–2.2)	2.6 (2.3–3.0)	3.7 (3.2–4.2)	5.3 (4.9–5.9)
CRP, median (IQR)	5.0 (2.0–10.0)	3.0 (1.0–5.0)	4.9 (2.0–8.0)	5.4 (3.0–12.0)	16.0 (7.0–36.0)
ESR, median (IQR)	14.0 (8.0–26.0)	8.0 (4.0–13.0)	14.0 (8.0–24.0)	20.0 (12.0–31.0)	36.0 (23.0–55.0)
HAQ, median (IQR)	0.8 (0.3–1.3)	0.3 (0.0–0.8)	0.8 (0.3–1.1)	1.0 (0.6–1.4)	1.4 (1.0–1.9)
Swollen joint count, median (IQR)	1.0 (0.0–3.0)	0.0 (0.0–0.0)	0.0 (0.0–2.0)	2.0 (1.0–4.0)	8.0 (5.0–11.0)
Tender joint count, median (IQR)	1.0 (0.0–4.0)	0.0 (0.0–0.0)	1.0 (0.0–2.0)	3.0 (1.0–5.0)	10.0 (6.0–14.0)
VAS global, median (IQR)	33.0 (14.0–57.0)	13.0 (4.0–28.0)	30.0 (15.0–48.0)	47.0 (29.0–64.0)	70.0 (54.0–81.0)
VAS pain, median (IQR)	32.0 (13.0–57.0)	13.0 (4.0–28.0)	28.0 (14.0–47.0)	45.0 (28.0–64.0)	69.0 (52.0–80.0)
Seropositive, (%)	77	75	78	78	77
Seronegative, unknown (%)	24	25	23	22	23
Smoker (%)	56	55	58	58	58
Comorbidities*					
ACS (%)	2	2	3	3	3
Other cardiac disease (%)	26	22	27	28	30
VTE (%)	1	1	1	1	2
Chronic kidney disease (%)	1	1	1	1	2
Cancer (in past 10 years) (%)	4	3	4	4	5
COPD (%)	15	12	15	16	17
Diabetes (%)	9	7	9	10	12
Surgery (%)†	3	3	4	4	4
No of hospitalisations, median (IQR)	6 (3–12)	5 (2–9)	6 (3–12)	7 (4–14)	8 (4–15)
No of specialist care visits, median (IQR)	33 (18–60)	29 (16–51)	34 (19–60)	35 (18–63)	32 (15–61)
Treatments‡					
Methotrexate (%)	69	74	71	68	64
Other csDMARD (%)	18	16	18	20	19
TNFi (%)	33	33	35	33	31
Other b/tsDMARD (%)	12	10	10	13	17
No previous biologics, median (IQR)	1 (1–3)	1 (1–2)	1 (1–3)	1 (1–3)	1 (1–3)
NSAID/ASA (%)	59	54	58	63	69
Anticoagulant§ (%)	8	6	8	8	9
Oral oestrogen¶ (%)	14	13	15	15	14
Socioeconomic charcateristics					
Married/cohabiting partner (%)	53	55	53	52	51
Disability pension in previous year (%)	2	2	2	2	2
Sick leave in previous year (%)	12	12	12	13	12

*Registered within the last 5 years unless otherwise stated.
†Surgery (musculoskeletal, gynaecological, gastrointestinal or cardiovascular) within 90 days before visit.
‡RA treatments: at time of visit. Other treatments: Registered within the last year.
§Collected anticoagulant drug from pharmacy within 1 year before VTE event.
¶Oral contraceptive w oestrogen *or* hormone replacement therapy.
ACS, acute coronary syndrome; b/tsDMARD, biological or targeted synthetic disease-modifying antirheumatic drug; COPD, chronic obstructive pulmonary disease; CRP, C reactive protein; DAS28, Disease Activity Score 28; HAQ, Health Assessment Questionnaire; HAQ, Health Assessment Questionnaire; NSAID/ASA, non-steroidal anti-inflammatory drug/acetylsalicylic acid; RA, rheumatoid arthritis; TNFi, tumour necrosis factor inhabitor; VAS, visual analogue scale; VTE, venous thromboembolism.

Within the RA population, we noted a strong association between RA disease activity and the 1-year cumulative incidence of VTE, table 2 and figure 1. The 1-year incidence of VTE events increased from 0.52% following visits in DAS28 remission, via 0.63% and 0.80% for low and moderate DAS28, respectively, to 1.08% following visit with a DAS28 above 5.1, which corresponded to an adjusted risk ratio of VTE with high DAS28 RA disease activity (vs remission) of 2.03 (95% CI 1.73 to 2.38).

When contrasting the DAS28 categories to the general population referents, the adjusted risk ratio of VTE was 1.34 (95% CI 1.13 to 1.58) for patients in DAS28 remission, and 2.74 (95% CI 2.31 to 3.25) for those with high DAS28 (table 2).

Figure 2 displays the association between DAS28 and VTE stratified by VTE type (DVT vs PE), by personal history of VTE (yes vs no), by smoking status (smoker vs non-smoker) and by C reactive protein (CRP) level (<5 vs ≥5) at the visit. For each

Table 2 Cumulative incidence and risk ratio of VTE during 1-year follow-up after rheumatologist visit in Swedish patients with RA versus matched general population referents (1:5), and in DAS28 categories

	No of VTE events (cumulative incidence, %)					
	Within RA, between DAS28 categories					
	Remission	Low	Intermediate	High	Entire RA pop*	Gen pop
Type						
Any	496 (0.52)	271 (0.63)	743 (0.80)	350 (1.08)	2241 (0.71)	5301 (0.36)
DVT	346 (0.36)	170 (0.40)	467 (0.50)	196 (0.61)	1408 (0.45)	3303 (0.22)
PE	150 (0.16)	101 (0.24)	276 (0.30)	154 (0.48)	833 (0.26)	1998 (0.14)
Sex						
Male	188 (0.60)	71 (0.66)	190 (0.94)	78 (1.10)	664 (0.80)	1631 (0.43)
Female	308 (0.47)	200 (0.62)	553 (0.76)	272 (1.08)	1577 (0.68)	3670 (0.34)
Age						
18–49	37 (0.16)	23 (0.30)	54 (0.31)	24 (0.39)	174 (0.27)	419 (0.13)
50–74	332 (0.54)	174 (0.62)	450 (0.74)	215 (1.01)	1386 (0.68)	3541 (0.37)
75-	127 (1.06)	74 (1.03)	239 (1.65)	111 (2.27)	681 (1.44)	1341 (0.71)
	Risk ratios (95% CI)†					
	Within RA, between DAS28 categories					
	Remission	Low	Intermediate	High		
Unadjusted	1 (ref)	1.22 (1.05 to 1.43)	1.56 (1.37 to 1.76)	2.10 (1.79 to 2.46)		
Adjusted	1 (ref)	1.12 (0.96 to 1.31)	1.48 (1.30 to 1.68)	2.03 (1.73 to 2.38)		
	RA population (including DAS28 categories) versus general population					
	Remission	Low	Intermediate	High	Entire RA pop	Gen pop
Unadjusted	1.43 (1.28 to 1.59)	1.75 (1.52 to 2.01)	2.23 (2.02 to 2.45)	3.00 (2.62 to 3.43)	1.96 (1.83 to 2.11)	1 (ref)
Adjusted	1.34 (1.13 to 1.58)	1.51 (1.25 to 1.82)	1.99 (1.72 to 2.31)	2.74 (2.31 to 3.25)	1.88 (1.65 to 2.15)	1 (ref)

Patients and referents followed from first visit registered after 2006 until 2018.
*Includes VTE events following a visit with missing DAS28 value (n=381, 17% of all VTE events).
†Risk ratios adjusted for age, sex and calendar year.
DAS28, Disease Activity Score 28; DVT, deep vein thrombosis; PE, pulmonary embolism; RA, rheumatoid arthritis; VTE, venous thromboembolism.

of these analyses, we noted a pattern of increasing risk and risk ratios for VTE with increasing DAS28 similar to that of the main analysis. For instance, the risk ratio for PE was 3.06 (CI 2.36 to 3.97) for high DAS28 (vs remission), and the corresponding risk ratio for DVT was 1.59 (1.30–1.95). Importantly, although the risk ratios were largely similar across subgroups, the corresponding absolute 1-year risks varied across these patient subsets.

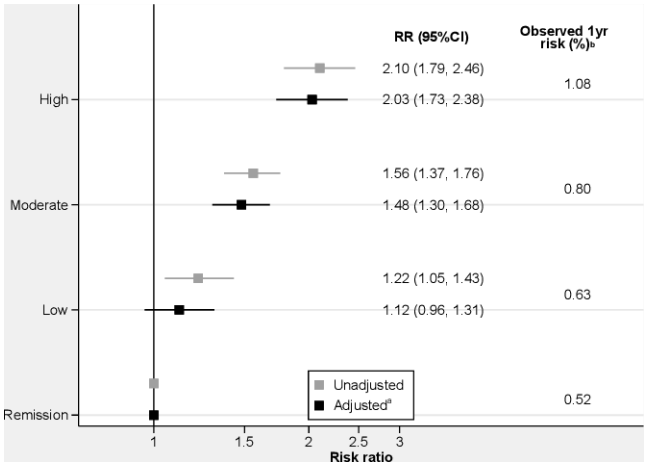

Figure 1 Risk ratios and absolute 1-year risks for the association between DAS28 and the risk of VTE within 1 year among Swedish patients with RA from 2006 until 2018. [a]Adjusted for age (restricted cubic spline), sex and calendar year of the visit year (categorised 2006–2009, 2010–2013, 2014–2017). [b]Absolute 1-year risks are calculated from observed data. DAS28, Disease Activity Score 28; RA, rheumatoid arthritis; VTE, venous thromboembolism.

For instance, among individuals with high DAS28 disease activity, the 1year risk for VTE was 7.8% for individuals with a history of VTE compared with 1.0% for individuals without previous VTE. Risk ratios and 1-year risks for VTE were relatively similar across strata as defined by RA serostatus, RA disease duration, sex and oral glucocorticoids (online supplemental figure S2 and online supplemental table S5).

Table 3 presents the association between individual DAS28 components and VTE risk, and the association between HAQ and VTE risk. The pattern for each DAS28 component, as well as HAQ, was largely similar to that of the main analysis.

Online supplemental table S6 presents the predicted 1-year risk of VTE in RA, from log binomial models, by age and gender. The risks varied from 0.2% to 0.4% (remission vs high DAS28) in the 40-year-old women, to the corresponding 1.1%–2.2% in the 80-year-old men.

In sensitivity analyses that altered the VTE definition, the width of the time windows, changed various definitions of our study population, and using imputed DAS28 values, results were similar to our main analysis (online supplemental figures S3, S4 and tables S7, S8).

DISCUSSION

Main findings

In this large nationwide study, to our knowledge the first to specifically investigate the association between measures of RA disease activity and VTE risk, we found a strong association between RA disease activity as measured by DAS28, as well as by its components and by HAQ, and the risk of VTE during the following year. The increase in risk with high DAS28 was twice as high for PE as for DVT. We also noted that compared with the

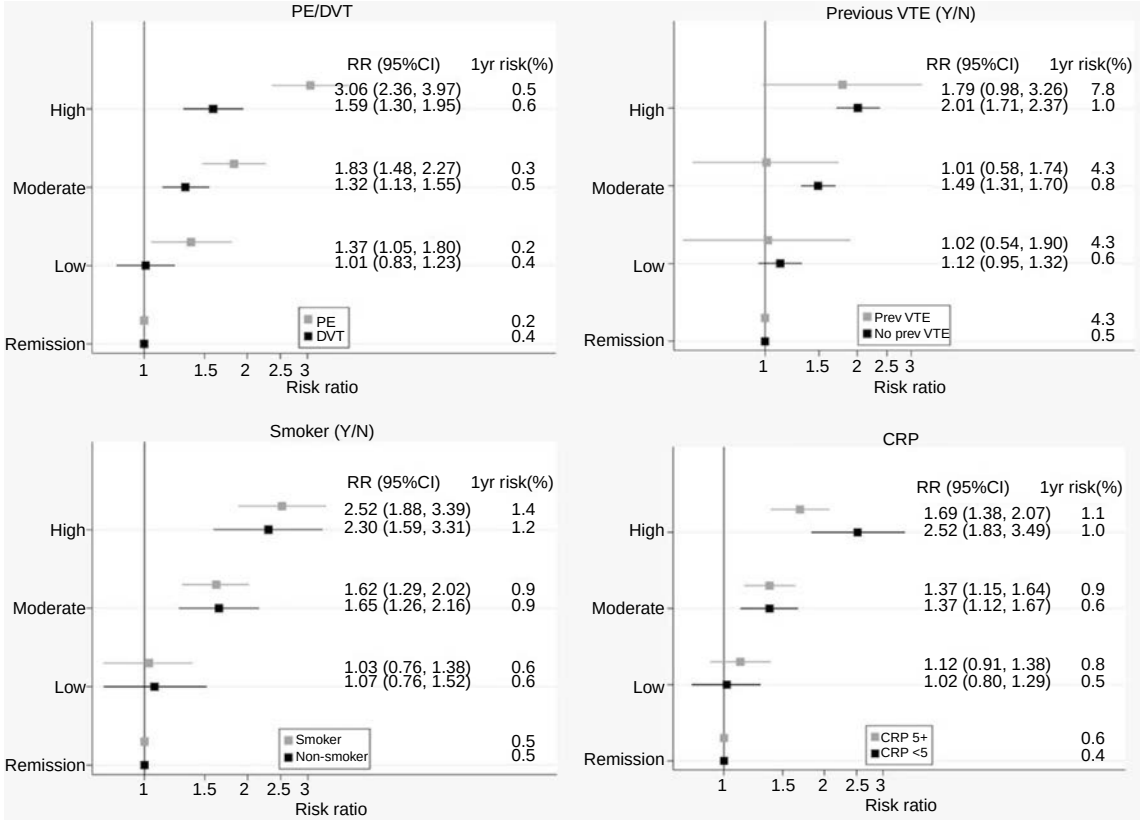

Figure 2 Risk ratios and absolute 1-year risks[a] for the association between DAS28 and VTE by specified stratification within 1 year among Swedish patients with RA. [a]Observed 1-year risk and adjusted risk ratios from log-binomial regression models adjusted for age (restricted cubic spline), sex and calendar year of the visit year (categorised 2006–2009, 2010–2013, 2014–2017). Absolute 1-year risks are calculated from observed data. CRP, C reactive protein; DAS28, Disease Activity Score 28; PE, pulmonary embolism; RA, rheumatoid arthritis; VTE, venous thromboembolism.

general population, even patients in DAS28 remission were at elevated VTE risk.

Few previous studies have investigated the relationship between characteristics in RA and VTE risk. One study on patients with RA (n=253 875) from a US claims database reported an increased incidence of inpatient VTE events in patients switching biologic or targeted synthetic disease-modifying antirheumatic drug (b/tsDMARD) treatment compared with patients remaining on a first b/tsDMARD or only conventional synthetic DMARD.[17] One might speculate that patients who switch b/tsDMARD on average have a higher disease activity than those who do not. If so, then the finding of an increased incidence of VTE among switchers of b/tsDMARD could be congruent with our findings, even if switching b/tsDMARD may occur for many other reasons than uncontrolled disease activity. Regarding RA disease activity and its role in coagulation, a cross-sectional study of 85 patients with RA reported an association between the rotational thromboelastometry (ROTEM) functional evaluation of the clotting cascade in whole blood, and DAS28.[18] However, in that study, the clinical correlation between the ROTEM analyses and incidence of clinical VTE was not studied.

Our results including stratifications and sensitivity analyses indicated a remarkable consistency in the pattern of association between RA disease activity and VTE risk. The risk ratios for the association between DAS28 and VTE were almost identical for patients with and without a history of VTE, demonstrating that the association between DAS28 and VTE risk is not confined to the patients with this history. At the same time, the absolute risk differences were much higher in patients with a history of VTE (8% vs 1% risk with high DAS28), indicating that the clinical significance of high RA disease activity is much larger in this patient subset, and that VTE risk stratification is especially important in this group of patients. By contrast, when stratified by CRP (<5 vs ≥5), the associations between increasing DAS28 and VTE risk were similar, as were the absolute 1-year risks in highly active RA (1.0% vs 1.1%), underscoring that the main result of this study is not necessarily driven by inflammation itself. Elevated CRP is known to cause pro-thrombotic activity and play a role in the pathogenesis of arterial thromboembolic events, although true causality with respect to VTE has not been established.[19] We also noted an association between HAQ and VTE risk, underscoring the known association between functional status/ mobility and VTE risk. Our stratification on smoking (yes vs no) showed no difference in either RR or absolute 1-year risk, but the limited access to smoking data in our population should be considered.

Important to keep in mind, this study investigated VTE risk in a 12 (and 6, respectively) month window after the visit, and therefore, does not claim that the VTE risk is particularly high at any specific time point in this window. Also, we set out to investigate the association between indices of RA disease activity and VTE risk, not risks with individual DMARDs or treatment strategies.

In keeping with our main aim of studying the association between DAS28 and VTE, we adjusted for age, sex and calendar period but not for other risk factors. Our study population mainly comprised prevalent RA, therefore, at the time point of each visit it is not possible to fully distinguish comorbidities and other covariates that might be true confounders from such that are consequential to the RA disease and potential mediators of

Table 3 Cumulative incidence and risk ratios (95% CI) for the components of DAS28 and HAQ

	No of VTE events (cumulative incidence, %)	Risk ratio* (95% CI) Unadjusted	Risk ratio (95% CI) Adjusted
Swollen joint count			
0	811 (0.59)	1 (ref)	1 (ref)
1–2	532 (0.72)	1.24 (1.10 to 1.39)	1.27 (1.13 to 1.43)
3–28	817 (0.87)	1.49 (1.33 to 1.67)	1.55 (1.38 to 1.74)
Tender joint count			
0	772 (0.61)	1 (ref)	1 (ref)
1–3	688 (0.74)	1.22 (1.10 to 1.36)	1.29 (1.16 to 1.44)
4–28	699 (0.82)	1.35 (1.19 to 1.52)	1.52 (1.35 to 1.72)
ESR			
0–10	589 (0.55)	1 (ref)	1 (ref)
11–21	595 (0.70)	1.28 (1.12 to 1.45)	1.11 (0.97 to 1.26)
22–488	833 (0.88)	1.61 (1.41 to 1.83)	1.24 (1.09 to 1.43)
Patient global health			
0–20	564 (0.55)	1 (ref)	1 (ref)
21–49	609 (0.65)	1.17 (1.03 to 1.32)	1.12 (0.99 to 1.27)
50–100	896 (0.92)	1.66 (1.47 to 1.89)	1.65 (1.46 to 1.87)
HAQ			
0–0.38	519 (0.53)	1 (ref)	1 (ref)
0.39–1.00	569 (0.63)	1.17 (1.01 to 1.35)	1.10 (0.95 to 1.27)
1.01–3.00	879 (0.95)	1.77 (1.55 to 2.04)	1.48 (1.29 to 1.71)

*Risk ratios estimated from separate unadjusted and adjusted (age, sex and calendar year of the visit year) log-binomial regression models where each of the separate exposures were categorised according to their tertiles.
DAS28, Disease Activity Score 28; HAQ, Health Assessment Questionnaire; VTE, venous thromboembolism.

the very association under study. Our results, therefore, accurately reflect clinical risks and relative risks for VTE in RA and how these vary across RA disease activity and patient subsets, but do not directly inform on the relative importance of different components of this risk, for example, the relative contribution of different established VTE risk factors on this association. While directly applicable to clinical practice and amenable for use for clinical risk stratification, the results of our study, therefore, do not necessarily reflect any direct causality between each aspect of RA disease activity and VTE risk.

Limitations

Using a clinical register for identifying VTE events has benefits but could potentially result in underdiagnosis or overdiagnosis, and patients with RA are at risk for musculoskeletal conditions that could be misdiagnosed as DVTs. We, therefore, investigated a series of alternative definitions of VTE, including or not also anticoagulant treatment, and also considered DVT and PE events separately. Since the results demonstrated a pattern remarkably consistent with our main analysis, we find it unlikely that misclassification of VTEs had any significant effect on our results. Using DAS28 as a measure of disease activity is also a potential source of misclassification of true RA disease activity, since any concomitant condition causing elevated ESR, such as malignancies, will contribute to the DAS28 score. However, since the risk ratios for ESR and VTE risk were, if anything, lower than those for other individual DAS28 components, such misclassification of ESR is unlikely to be a source of significant bias in our study. We had somewhat limited data on smoking, and for certain other variables of interest, such as body mass index and immobility, information was not available.

Strengths

Using the SRQ to identify our RA cohort, we were able to include around 90% of all Swedish patients with RA treated by rheumatologists, which reduces the potential for selection bias and increases the generalisability of our findings. We linked these data to other nationwide registers based on prospectively recorded data of high internal validity and coverage for information on other variables, thereby reducing selection and information bias, and enabling comparisons both within RA and vs the general population. Our study demonstrated risk ratios of around 2.0 for VTE (DVT as well as PE) in RA compared with the general population. These results are similar to previous reports. For instance, in a previous study from our group, from 1997 to 2006 (ie, before the start of our study period) we noted HRs of around 2.[3] A UK study of a prevalent RA population (n=9589) from 1986 to 2010, reported RRs of around 2.2 for VTE, DVT and PE.[8] This consistency further speaks to the generalisability of the results regarding the association between RA disease activity and VTE risk.

CONCLUSION

In conclusion, we found evidence of a strong association, with clinically relevant differences in absolute risks, between RA disease activity measured by DAS28 and the subsequent risk of VTE, which may be used for clinical risk stratification. Also, patients in remission are at increased risk vs the general population. The absolute risk increase with disease activity highlights the need for proper VTE risk assessment in patients with RA, especially for those with a history of VTE or other known risk factors. Our findings also suggest that patients with active RA, such as those typically recruited to phase III trials, are at particular elevated risk for VTE.

Correction notice This article has been corrected since it published Online First. Table 2 has been increased in size for clarity.

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Contributors All authors participated in the design of the study. HB conducted the statistical analyses. VM, HB, TF and JA contributed to interpretation of the results. VM and JA contributed to the drafting of the manuscript. All authors contributed to the critical revision of the manuscript for important intellectual content. The study was supervised by JA.

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Disclaimer Funders had no impact on the design or interpretation of the study or its results.

Competing interests Karolinska Institutet, with JA as principal investigator, has or has had research agreements with Abbvie, Astra-Zeneca, BMS, Eli Lilly, MSD, Pfizer, Roche, Samsung Bioepis, Sanofi, and UCB, mainly in the context of safety monitoring of biologics via ARTIS/Swedish Biologics Register.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Not required.

Ethics approval Regional Ethics Committee, Stockholm, Sweden. 2015/1844-31/2.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data may be obtained from a third party and are not publicly available. The study data forms part of a register linkage performed by Karolinska Institutet, and for which further sharing of the data is limited by legal restrictions.

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TRANSLATIONAL SCIENCE

In vitro elimination of autoreactive B cells from rheumatoid arthritis patients by universal chimeric antigen receptor T cells

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ABSTRACT

Objectives Autoreactive B cells play a crucial role in the pathogenesis of rheumatoid arthritis (RA), and B cell-depleting therapies using an antibodies, such as rituximab, have been suggested to be effective in RA treatment. However, transient B cell depletion with rituximab is associated with significant safety challenges related to global suppression of the immune system and thus increases the risks of infection and cancer development. To address selective and persistent issues associated with RA therapy, we developed a customised therapeutic strategy employing universal antifluorescein isothiocyanate (FITC) chimeric antigen receptor T cells (CAR-T cells) combined with FITC-labelled antigenic peptide epitopes to eliminate autoreactive B cell subsets recognising these antigens in RA.

Methods For a proof-of-concept study, four citrullinated peptide epitopes derived from citrullinated autoantigens, namely, citrullinated vimentin, citrullinated type II collagen, citrullinated fibrinogen and tenascin-C, and a cyclocitrulline peptide-1 were selected as ligands for targeting autoreactive B cells; Engineered T cells expressing a fixed anti-FITC CAR were constructed and applied as a universal CAR-T cell system to specifically eliminate these protein-specific autoreactive B cells via recognition of the aforementioned FITC-labelled autoantigenic peptide epitopes.

Results We demonstrated that anti-FITC CAR-T cells could be specifically redirected and kill hybridoma cells generated by immunisation with antigenic peptides, and autoreactive B cell subsets from RA patients via recognition of corresponding FITC-labelled citrullinated peptide epitopes. Additionally, the cytotoxicity of the CAR-T cells was dependent on the presence of the peptides and occurred in a dose-dependent manner.

Conclusions The approach described here provides a direction for precise, customised approaches to treat RA and can likely be applied to other systemic autoimmune diseases.

Key messages

- Chimeric antigen receptor T cells (CAR-T) cell therapy has shown promise in the targeted treatment of autoimmune diseases but is limited due to practical challenges regarding the heterogeneity and complexity of autoimmune diseases.
- Aiming to address the selective and persistent issues associated with systemic autoimmune disease therapies, exemplified by those for rheumatoid arthritis (RA), we developed a targeted and customised approach that employed universal anti-fluorescein isothiocyanate (FITC) CAR-T cells combined with FITC-labelled RA-immunodominant peptides to specifically eliminate various types of autoreactive B cell subsets.
- As a proof of concept, we demonstrated that anti-FITC CAR-T cells could be specifically redirected and kill hybridoma cells and RA patient-derived autoreactive B cell subsets from RA patients via recognition of corresponding FITC-labelled citrullinated peptide epitopes;.
- This system was shown to be highly specific to peptide epitope-positive autoreactive B cells, and the cytotoxicity of the CAR-T cells was strictly dependent on the presence of the peptides in a dose-dependent manner, underscoring the specificity and promising targeted effects of this approach.
- This study provides an appealing direction for the precise and customised treatment of RA according to individual patient autoantigen profiles and can likely be applied to other systemic autoimmune diseases. Further efficacy and safety studies warrant exploration.

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INTRODUCTION

Rheumatoid arthritis (RA) is a common systemic autoimmune disease characterised by autoantibodies against citrullinated antigens, which generally lead to chronic inflammation in the synovial joints and joint destruction.[1] Currently, the pathogenic mechanism and aetiology of RA are still unclear, but the citrullination of proteins has long been thought to trigger the immune reactions characteristic of RA.[1–3] The appearance of anticitrullinated protein antibodies (ACPAs) in the serum is one of the most specific serological markers of RA and is associated with disease development and the disease process. Several target proteins of ACPAs have been described.[4–8]

Traditional treatment for RA partly inhibits autoantibody production by systemically suppressing the immune system but may bring serious adverse

effects, such as an increased risk of infection.[9 10] Several targeted biologics have been developed to address this issue.[9] It has been proven that the application of rituximab, a human CD20-specific antibody with the capacity to deplete CD20-expressing B cells to an almost undetectable level, is therapeutically effective in RA.[11 12] A decrease in rheumatoid factor and serum ACPA levels has been reported in RA patients treated with rituximab, indicating that short-lived plasmablasts are the major source of autoantibodies in RA, and targeting $CD20^+$ B cell precursors could also reduce autoantibody-secreting $CD20^-$ plasmablasts indirectly.[13] The transient total absence of B cells, however, increases the infection risk of patients and sometimes induces failure to produce a recall response to a protective antigen.[14 15]

Chimeric antigen receptor T cells (CAR-T) therapy has shown promise in the treatment of autoimmune diseases, which is ascribed to its specificity and induction of durable autoimmunity remission.[9 16] Similar to tumour treatment, treatment of autoimmune disease with designed CAR-T cells relies on targeted elimination of autoantigenic B cells, which are defined by their reactivity against specific autoantigens, without the risk of general immunosuppression.[17] Several studies have been performed to investigate the application of CAR-T cell in autoimmune diseases.[16–19] For example, an engineered CAR-T cells that expressed the pemphigus vulgaris-specific autoantigen Dsg3 persisted and specifically eliminated autoreactive B cells expressing anti-Dsg3 B cell receptors (BCRs).[18] The extension of the application of this methodology to RA and other systemic autoimmune diseases, however, is limited since a CAAR or CAR targets a single cell type, which is not effective against the various types of autoreactive lymphocytes present in patients with RA.

Aiming to address the selective and persistent issues associated with RA therapy, we aimed to develop a customised therapeutic strategy using a universal CAR-T cell system that allows targeting of multiple BCRs by T cells expressing a single scFv combined with known autoantigen peptides to specifically eliminate various types of autoreactive B cell subsets with an increased level of BCR that recognises citrullinated protein epitopes. Our approach includes three key steps: (1) identify the kinds of autoreactive lymphocytes present by detecting the levels of autoantibodies against major epitope peptides of a given patient; (2) prepare universal CAR-T cells (CAR-Ts)[20] and mediators; and (3) specifically eliminate the corresponding autoreactive B cells by redirecting the CAR-Ts to the autoantigen-specific B cells tethered by the major epitope peptides. Mediators are generated by conjugation of an autoantibody-positive peptide(s) with bioorthogonal molecules, such as fluorescein isothiocyanate (FITC),[21 22] which can be recognised only by CAR-T and autoreactive B cells specific for this peptide.

As a proof of concept, we demonstrated that anti-FITC CAR-Ts could be specifically redirected by FITC-labelled citrullinated peptide epitopes to corresponding hybridoma cells or autoreactive B cells from RA patients with high levels of autoantibodies against the peptides. This approach makes it possible to eliminate various specific autoreactive cell subsets in a given RA patient according to his/her individual profile. More importantly, our work opens up a new field of targeted treatment for systemic autoimmune diseases.

RESULTS

Design and characterisation of RA-associated antigenic peptides and universal CAR-T cells

We first designed and synthesised RA-associated antigenic peptides for specific recognition by pathogenic RA B cells. We focused on citrullinated autoantigens, which generally appear long before RA symptoms onset and have been well identified as targets with therapeutic potential. Four citrullinated peptide epitopes derived from citrullinated autoantigens, namely, citrullinated vimentin (cVIM),[7] citrullinated type II collagen (cCOII),[4] citrullinated fibrinogen (cFib)[6] and citrullinated tenascin-C (cTNC-5),[8] were selected as ligands for targeting autoreactive B cells due to their well-identified and studied associations with RA. In addition, a cyclic derivative of a citrullinated filaggrin peptide, referred to as cyclocitrulline peptide (CCP-1),[1 23] was also selected due to its well-documented and wide application in the clinic for the detection of ACPAs (online supplemental table S1).

The universal anti-FITC CAR-Ts were applied as a universal CAR-T cell system in this study.[21] Engineered T cells expressing a fixed anti-FITC CAR could be redirected to various types of autoreactive B cells via recognition of FITC-labelled autoantigen peptides (figure 1A). To construct this CAR, the anti-FITC single-chain variable fragment (scFv) clone 4M5.3 fused with a second-generation CAR sequence was subcloned into a lentiviral vector (figure 1B) and then transduced into human T cells as described previously.[21 22] The transduction efficiency was generally ~40%–50%, as measured by flow cytometry (online supplemental figure S1). On incubation of transfected T cells either with the anti-mouse IgG antibody and FITC-peptide simultaneously or with an unrelated FITC-conjugated antibody followed by a secondary antibody, we found that nearly all T cells expressing the scFv bound FITC (figure 1B), indicating that the scFv was properly folded on the cell surface and accessible to the FITC-conjugated ligand.

Anti-FITC CAR-Ts recognised and killed hybridoma cells by binding with a corresponding antigenic FITC-conjugated peptide

To detect whether anti-FITC CAR-T cells can be redirected by an antigenic FITC-conjugated peptide, we first designed an in vitro model for detecting the cytotoxicity of anti-FITC CAR-Ts to hybridoma cells generated by immunisation with an antigenic peptide in the presence of the corresponding FITC-labelled peptide. Two out of five tested peptides, cFib and cCOII, were selected as antigens and were subcutaneously injected into BALB/c mice. After three rounds of screening for each peptide, two hybridoma cell strains producing the highest titres of a specific antibody were identified and cloned. The BCR expression for each hybridoma strain was verified using an anti-mouse IgG antibody, and the hybridoma strains for Fib (2-2) and COII (6-1) were found to have higher BCR expression than the other strains (figure 2A). The availability and specificity of antigenic FITC-peptides for these hybridoma cells were measured by incubating these hybridoma cells with either FITC-labelled antigenic peptide or mock peptide and then staining them with a secondary anti-FITC allophycocyanin (APC) -conjugated antibody. The hybridoma cells stained with corresponding antigenic FITC-peptides showed significantly increased APC staining compared with that of the cells treated with mock FITC-peptide and APC staining was positively correlated with BCR expression (figure 2B).

On validation of the bifunctional binding of antigenic FITC-peptides, we next evaluated the ability of these mediators to redirect CAR-T cells to kill hybridoma cells through recognition of the corresponding antigenic FITC-peptide in vitro. Anti-FITC CAR-Ts effectively lysed Fib- or COII-specific hybridoma cells at various effector-to-target (E:T) ratios in the presence of

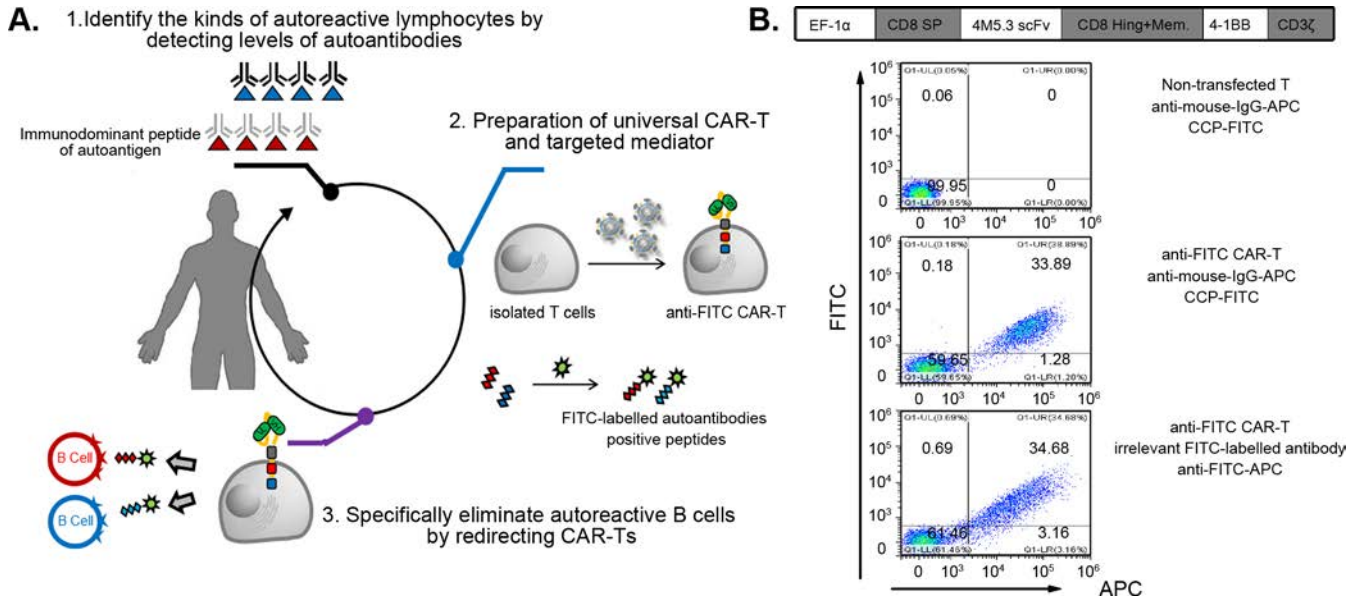

Figure 1 Schematic representation of universal CAR-T-mediated autoreactive B cell elimination. (A) Three key steps were included in this methodology: (1) identify the kinds of autoantibodies of a given patient by ELISA; (2) prepare universal anti-FITC CAR-Ts and FITC-labelled autoantibody-positive peptides and (3) eliminate corresponding autoreactive B cells through peptide-mediated CAR-T cytotoxicity. (B) generation and characterisation of anti-FITC CAR-Ts. The second generation of anti-FITC cars was constructed in a lentiviral vector. the expression and function of cars on human T cells was assessed by staining with APC-conjugated anti-mouse IgG and FITC-labelled peptide (CCP-1) simultaneously. The function of cars was detected by primary staining with an irrelevant FITC-labelled antibody and secondary APC-conjugated antibody. The percentage of double-positive cells was determined. Data are representative of at least three independent experiments. APC, allophycocyanin; CAR-T, chimeric antigen receptor T cells ; FITC, fluorescein isothiocyanate.

the corresponding antigenic FITC-peptide and acted in a dose-dependent manner. Optimal cytolytic effects were detected at an E:T ratio of 20:1 due to the high efficiency and specificity seen at this ratio (figure 3A). This effect was also verified by flow cytometry, in which the percentage of FITC-Fib-positive cells was decreased only in the presence of the FITC-Fib peptide, not in the presence of mock FITC-peptide or a nonpeptide control, indicating the high selectivity of this approach (figure 3B). Notably, when we incubated both Fib and COII hybridoma cells together with anti-FITC CAR-Ts, we found that compared with each single peptide alone, the FITC-Fib and FITC-COII peptides together induced significant increases in cytotoxicity and cytokine release (figure 3C), suggesting the potential of this universal CAR-T cell system to kill more than one antibody-secreting B cell strain. In addition, selective formation of aggregates was visible only in the cocultures of anti-FITC CAR-Ts and hybridoma cells with the corresponding antigenic FITC-peptide but not in those containing any of the controls (figure 3D). Collectively, these results indicated that hybridoma cells that secrete specific antibodies could be specifically and dose-dependently killed by CAR-T cells tethered by the corresponding antigenic peptide.

Anti-FITC CAR-T cells preferentially killed antibody-secreting target cells over FcγR-expressing cells in the presence of a specific antibody

Considering that FcγR-expressing cells anchoring specific antibody/peptide immune complexes may also become targets for these CAR-T cells, we next evaluated the potential off-target effects of this approach against macrophage-derived Raw264.7 cells in the presence of a specific antibody. The expression of CD64 (FcγRI) was first validated by flow cytometry (online supplemental figure S2A). It was found that the cytotoxicity against Fib(2-2) was not significantly reduced even in the presence of an equal amount of a monoclonal anti-cFib antibody. The toxicity induced by cFib/antibody complexes against Raw264.7 cells was undetectable except that an enormous amount of antibody (over one tenth of cFib) was present; despite the high amount of antibody, the toxicity against Raw264.7 cells was significantly weaker than that against the target cells (online supplemental figure S2B). Consistent with the observation from the cytotoxicity assay, when we incubated both Fib(2-2) and Raw264.7 cells with CAR-Ts, the reduction in Raw264.7 cells was observed only when the antibody concentration reached an extraordinarily high level (online supplemental figure S2C). Notably, these off-target effects could be further reduced by blocking FcγR with an irrelevant antibody (online supplemental figure S2B,C). Overall, these results suggested the specificity and promising targeted effects of this approach.

Anti-FITC CAR-T cells recognised and killed anti-COII antibody-secreting B cells from collagen-induced arthritis (CIA) mice in vitro by binding with an antigenic FITC-conjugated peptide

Having validated the effectiveness and specificity of this methodology in a hybridoma model, we next sought to determine whether this methodology could be used in eliminating specific antibody-secreting cell pools by applying appropriate antigenic FITC-conjugated peptides. CIA was employed due to its wide application in RA studies and ability to be triggered with a single COII protein.[24] Three identified peptide epitopes derived from the CNBr-fragment 11 region of the bovine COII protein, referred to as P1–P3, were selected as potential ligands[25–27] (online supplemental table S2). The capacity of these peptides to redirect anti-FITC CAR-Ts to anti-COII antibody-secreting B cells was first determined using hybridoma cells generated by immunisation of DBA/1 mice with the bovine COII protein. Flow cytometry binding analysis demonstrated that selected

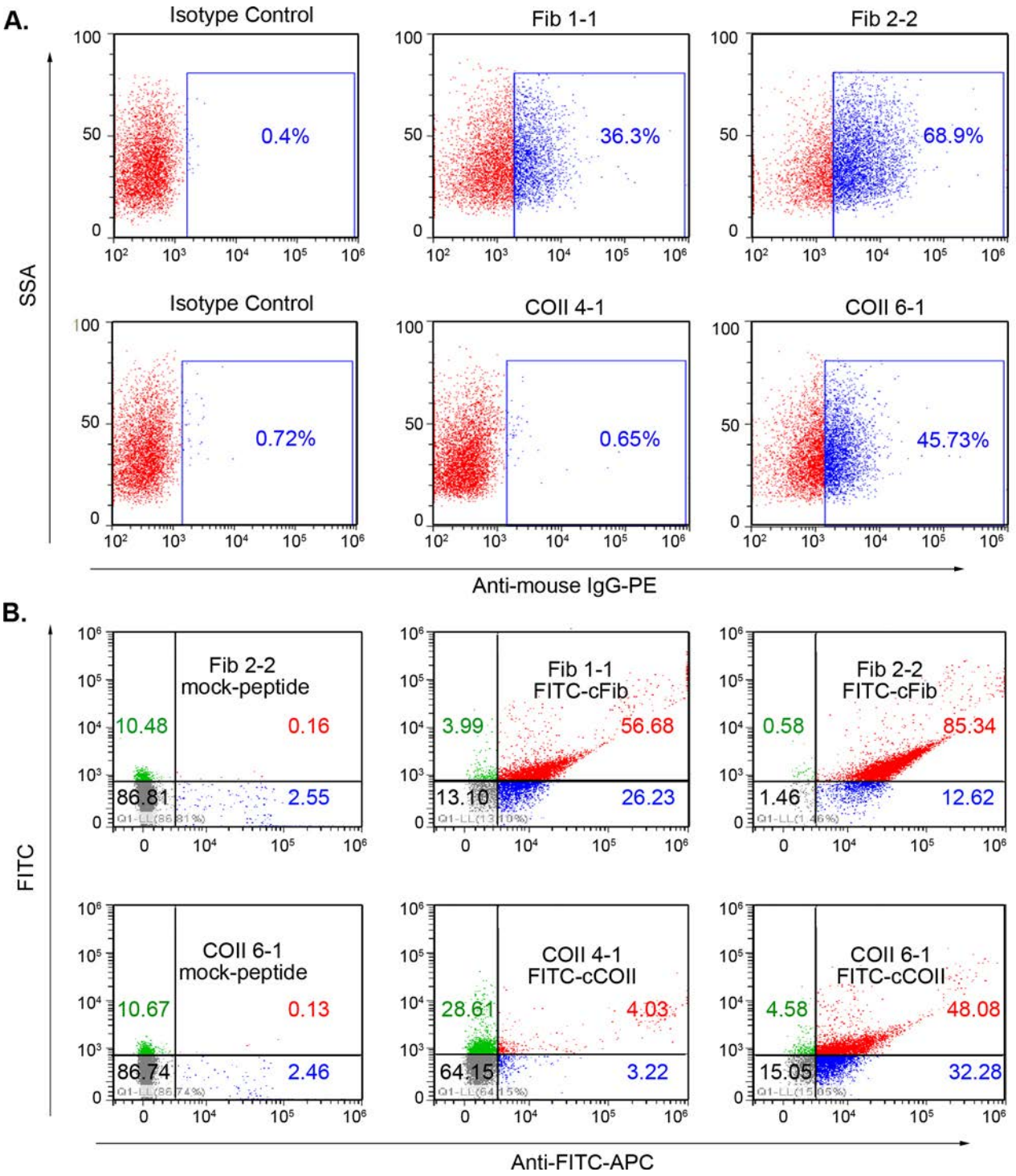

Figure 2 Assessment of the binding and specificity of the antigenic peptides for their hybridoma cells. (A) comparison of the expression levels of antigen-specific BCRs on different monoclonal hybridoma strains. The expression of BCRs on hybridoma cells was assessed by single staining with a PE-conjugated anti-mouse IgG antibody. (B) assessment of binding capacity of antigenic peptide to its hybridoma cells. Hybridoma cells were primary incubated with the corresponding FITC-labelled antigenic peptide and then stained with anti-FITC-APC antibodies. Cells treated with a FITC-labelled mock peptide with random sequence were used as a negative control. The results shown represent the findings from three experiments. APC, allophycocyanin; BCR, B cell receptors; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

peptides exhibited different binding affinities for each of three hybridoma cell strains, which accounted for the different levels of specific cytotoxicity induced by the CAR-T cells (figure 4A and online supplemental figure S2).

Specific killing of target COII-specific cells was measured by a COII-specific ELISPOT assay for antibody secretion. B cells were isolated from the splenocytes of COII-immunised DBA/1 mice with the highest anti-COII antibody titre (online supplemental figure S3A) and stimulated with COII. As before, the binding affinities of the FITC-conjugated antigenic peptides to stimulated B cells were verified (figure 4B and online supplemental figure S3B). The stimulated B cells were then cocultured with an anti-FITC CAR-Ts in the presence of FITC-conjugated antigenic peptides in COII-coated ELISPOT plates. COII-specific

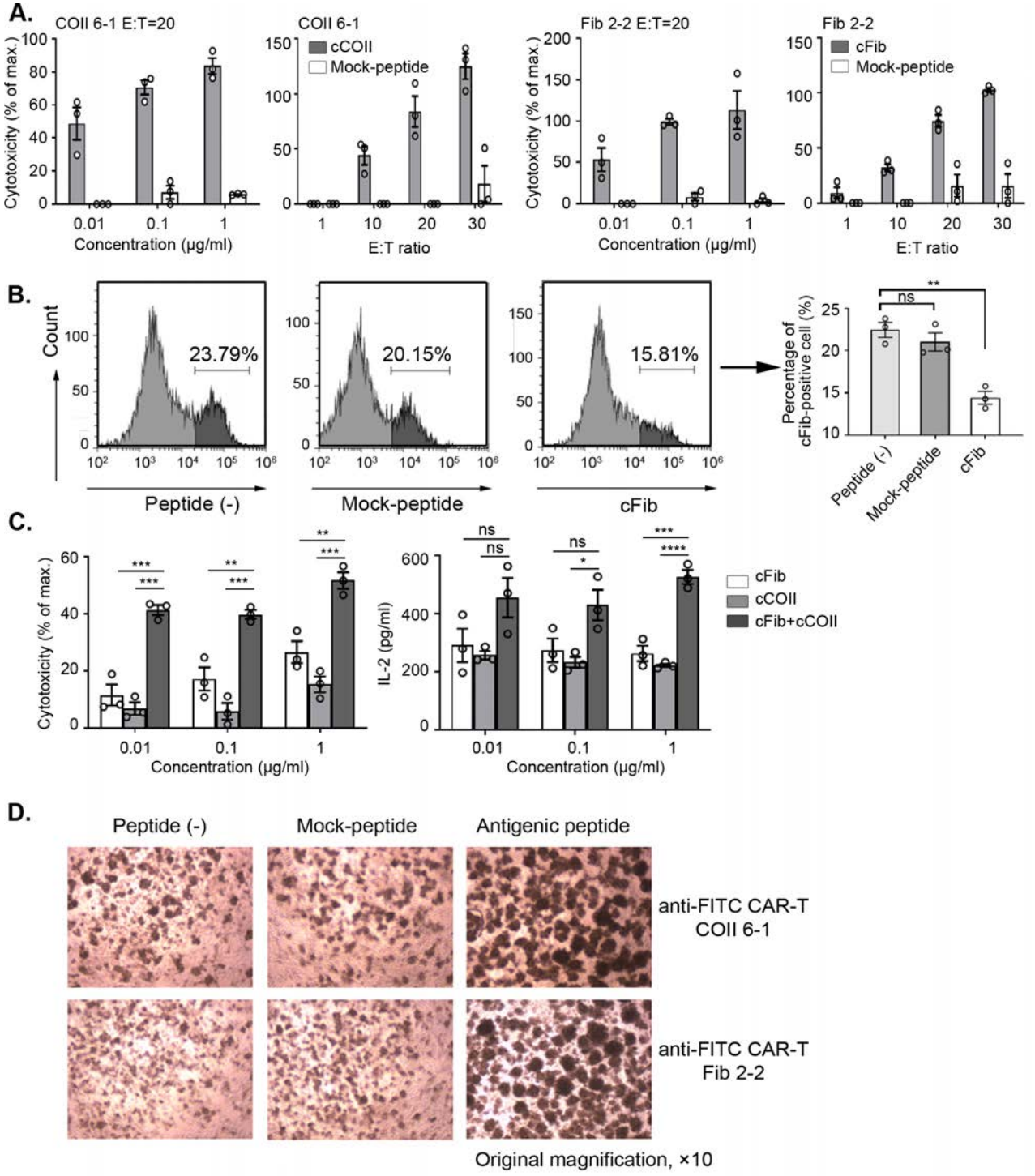

Figure 3 Characterisation of antigenic peptide-mediated cytotoxicity of anti-FITC CAR-Ts in vitro. (A) cytotoxic activity of anti-FITC CAR-Ts against hybridoma cells in the presence of FITC-labelled antigenic peptide or FITC-labelled mocked peptide at the indicated concentration and effector-to-target (E:T) ratios. Cytolytic activity was determined by measuring the amount of lactate dehydrogenase released into the culture medium. (B) assessment of the selective elimination of target hybridoma cells by flow cytometry. Fib (2-2) hybridoma cells were incubated with FITC-CAR-Ts in the presence of FITC-labelled cFib or mock peptide for 24 hours, and the cultured cells were washed and stained with a 100-fold excess of FITC-labelled cFib peptide to detect the remaining Fib (2-2) cells. (C) Assessment of simultaneous cytotoxicity and cytokine release of anti-FITC CAR-Ts against two types of hybridoma cells. Fib (2-2) and COII (6-1) hybridoma cells were incubated with ani-FITC CAR-Ts at a ratio of 1:1:20 in the presence of FITC-labelled cFib, CoII or both of them, respectively, for 24 hours. Cytotoxicity and IL-2 levels in the cultures were determined by the lactate dehydrogenase-based method and ELISA, respectively. For A–C, the data are presented as the mean±SEM derived from triplicate samples (n=3). Representative results from one of three experiments are shown. The p values shown were determined by one-way ANOVA (Dunnett's multiple comparison test) compared with those of non-peptide-treated group for B and two peptide-treated group for C. (D) morphological features of FITC-labelled antigenic peptide-dependent activation of CAR-Ts. The middle position of the well for each group was demonstrated, and the original magnification was 10-fold. The data are representative of three independent experiments. *P≤0.05, **p≤0.01, ***p≤0.001 and ****p≤0.0001. ANOVA, analysis of variance; CAR-T, chimeric antigen receptor T cells ; FITC, fluorescein isothiocyanate; SEM, SE of mean.

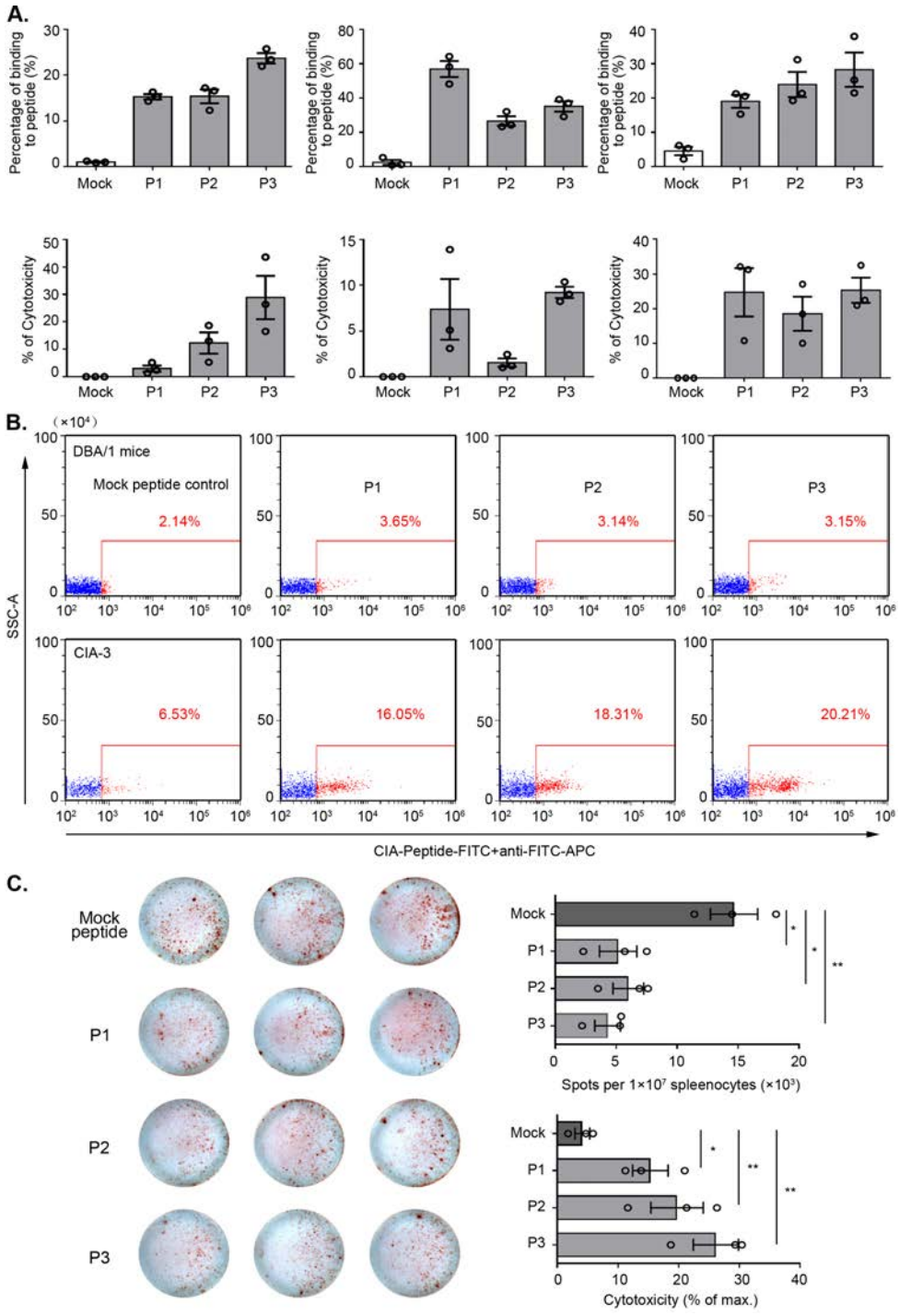

Figure 4 Assessment of immunodominant peptide-mediated CAR-T cytotoxicity against anti-COII antibodies secreting B cells from collagen-induced arthritis (CIA) mouse. (A) comparison of the binding affinity and peptide-mediated cytotoxicity of CAR-T for each COII-specific hybridoma cell subset. Hybridoma cells were stained with each FITC-labelled immunodominant peptide and anti-FITC-APC antibody. The binding affinities of each peptide to different hybridoma cells were determined by the percentage of peptide-positive cells. The cytotoxicity of CAR-Ts induced by immunodominant peptides were measured by incubation of hybridoma cells and anti-FITC CAR-Ts at a ratio of 1:20, and detected the LDH release as described above. The data are representative of three independent experiments and are shown as the mean±SEM derived from triplicate samples (n=3). See also online supplemental figure 3. (B–C) anti-FITC CAR-Ts-specific kille anti-COII antibodies secreting B cells from CIA mouse in vitro by binding with corresponding FITC-conjugated antigenic peptides. DBA/1 mice were immunised with emulsified bovine COII from CFA and were rechallenged with COII from IFA 21 days later. (B) Binding analysis of FITC-conjugated immunodominant peptide to B cells isolated from DBA/1 mice with the highest anti-COII antibody titre. The B cells were isolated and stimulated with COII for 5 days before analysis, and B cells isolated from non-treated mice were used as negative control. see also online supplemental figure 4B. (C) In vitro kill of antibody producing B cells by anti-FITC CAR-Ts. Left: the stimulated B cells from panel B were cocultured with CAR-Ts for 2 days, and anti-COII secreting cells were enumerated by ELISPOT assay; right: quantification of COII-specific spots formed by anti-COII secreting B cells after coculture with anti-FITC CAR-Ts and cytotoxicity of CAR-Ts. The data are shown as the mean±SEM derived from triplicate samples (n=3), and p value was determined by two-tailed unpaired Student's t-test. *P≤0.05, **p≤0.01. APC, allophycocyanin; CAR-T, chimeric antigen receptor T cells; FITC, fluorescein isothiocyanate; SEM, SE of mean.

antibody-producing B cells were detected after 2 days of coculture with CAR-Ts in the presence of mock FITC-peptide, while antibody production was significantly diminished in the presence of FITC-conjugated antigenic peptides. This observation was consistent with a cytotoxicity assay (figure 4C). Overall, these results indicate that these selected peptides have the potential to redirect CAR-Ts to kill COII-specific B cells.

In vitro elimination of specific autoreactive B cells from RA patients with autoantibody-specific peptides

We finally evaluated the therapeutic potential of this CAR-T cell system in vitro with samples from RA patients. Blood samples were taken from patients with established RA, and the serum and purified B cells were isolated for analysis. To determine which kinds of autoreactive B cells were present in certain patients, we first screened the autoantibodies in the serum by direct ELISA. We found different kinds of autoantigen-positive antibodies present in these patients: compared with healthy donors, the first RA patient (RA-1) had higher titres of autoantibodies against CCP-1 and cFib, and RA-2 showed only CCP-1-specific antibodies; the distinct high titres of autoantibodies against cVIM, CCP-1 or cTNC5 in RA-3 and RA-4 were found as indicated (figure 5A). On staining of the B cells purified from these patients, a significantly higher percentage of the peptide-positive population was observed in cells treated with FITC-peptide than in cells treated with mock FITC-peptide (figure 5B and online supplemental figure S3). Clearly, the presence of specific autoreactive B cells in patients was associated with the production of the corresponding autoantibodies, and these cells could be targeted by the corresponding immunodominant autoantigen peptide.

We then explored the cytotoxicity and specificity of CAR-Ts to these autoreactive B cells. Purified and stimulated B cells from RA-1 and RA-2 were incubated with CAR-Ts in the presence of corresponding antibody-specific peptides. Anti-FITC CAR-Ts efficiently and specifically lysed peptide-specific autoreactive B cells in the presence of FITC-labelled serum positive peptide but demonstrated minimal cytolytic activity with mock FITC-peptide. The peptide-specific autoreactive B cells were not lysed in the presence of irrelevant serum negative peptide (NP) and nontransduced T cells, and cytotoxicity could be blocked by the addition of excess free FITC molecules (figure 5C,D). The secreted levels of the cytokines were consistent with the cytolytic activity results (figure 5E). Collectively, these data showed that CAR-Ts efficiently and specifically killed autoreactive B cells from patients via antigenic epitope peptide-mediated CAR-T cell cytotoxicity.

DISCUSSION

CAR-T therapy has shown promise in the target treatment of autoimmune diseases because of its high efficiency, targeting and durability but is largely limited by practical challenges due to the heterogeneity and complexity of autoimmune diseases.[9] As an increasing number of autoantigens along with their antigenic epitopes have been identified, it is becoming possible to individually eliminate major autoreactive B cell populations with customised sets of immunodominant peptides according to personalised circumstances. As a proof of concept, here, we designed a targeted and customised scenario that employed universal anti-FITC CAR-Ts combined with FITC-labelled immunodominant peptides to eliminate autoantigen-specific B cell subsets recognising citrullinated antigens present in RA.

Studies initiated in a hybridoma cell model using corresponding FITC-labelled antigenic peptides showed that anti-FITC CAR-Ts could be redirected by the FITC-labelled antigenic peptides to the hybridoma cells and that they exhibited cytotoxicity effects in a dose-dependent manner, verifying the applicability and selectivity of this methodology. Furthermore, we found that more than one strain of hybridoma cells could be eliminated simultaneously by anti-FITC CAR-Ts in a manner dependent on the presence of the corresponding FITC-labelled antigenic peptide, potentially addressing the heterogeneity issue in RA therapy using CAR-Ts.

Whether this approach had a significant off-target effect was evaluated using an in vitro cell model. Cytotoxicity against FcγR+Raw264.7 cells was not detected except when an excessive amount of specific antibody was added. Nevertheless, anti-FITC CAR-Ts preferentially targeted hybridoma cells at a wide range of antibody concentrations, whereas they did not reduce FcγR-expressing cells even in the presence of tremendous amounts of specific antibodies. These results were consistent with a previous CAAR-T cell study,[18] implicating that the off-target toxicity seems insignificant and unlikely; this toxicity is ascribed to certain autoantibodies, exemplified by ACPA, which generally have a low avidity and comprise only a small fraction of total IgG.[28 29] Numerous approaches, including optimisation of FITC-peptide dosage and preblocking of FcγR using intravenous immunoglobulin, could be applied to further address this potential safety issue. The therapeutic potential of this approach for RA treatment was preliminarily confirmed in ex vivo tests using autoreactive B cells from RA patients. According to a serological study, we found that the four tested RA patients showed different types of specific autoantibodies against panels of peptides, highlighting the necessity for precise cell elimination adapted to local conditions. On primary characterisation of the autoreactive serum of given patients to immunodominant peptides, FITC-labelled antibody-positive peptides were observed to successfully induce lysis of corresponding autoreactive B cells in the presence of anti-FITC CAR-Ts, and no significant cytotoxic activity was observed for the mock- and serum NP-treated group, demonstrating the high selectivity of this approach and implying the potential to target elimination of pathogenic autoimmune cells without impact on protective immunity.

The major limitation of this pilot study is that it showed only in vitro specific elimination of autoreactive B cells and lacked substantial evidence to prove the therapeutic effects of this approach in vivo. Theoretically, this strategy could directly eliminate autoantigenic B cells that express high levels of a specific BCR and may indirectly affect short-ived or long-lived autoantibody-secreting plasma cells that are replenished from the memory B cell pool. The consequence of long-term application of CAR-Ts on plasma cell differentiation and antibody secretion remains to be explored. An additional issue is the in vivo stability of the peptide-derived mediator. Structural optimisation of the bifunctional antigen-specific targeting ligands,[30–32] such as the conjugated immunogenic domain comprising antigen peptides and antibody fragments for an increase in mediator molecular weight, might be feasible to address these issues. Other factors, such as a long-term administration regimen, a sufficient amount to reach a specific BCR and an injectable format to avoid proteolysis, should be considered for evaluation of the therapeutic effects in vivo. Moreover, given the abundant studies that have disclosed the consequential reduction in humoral responses on sequential vaccination and rituximab treatments,[33 34] it is also important to evaluate the safety of this approach in terms of the impact on host immune defence and vaccination recall responses.

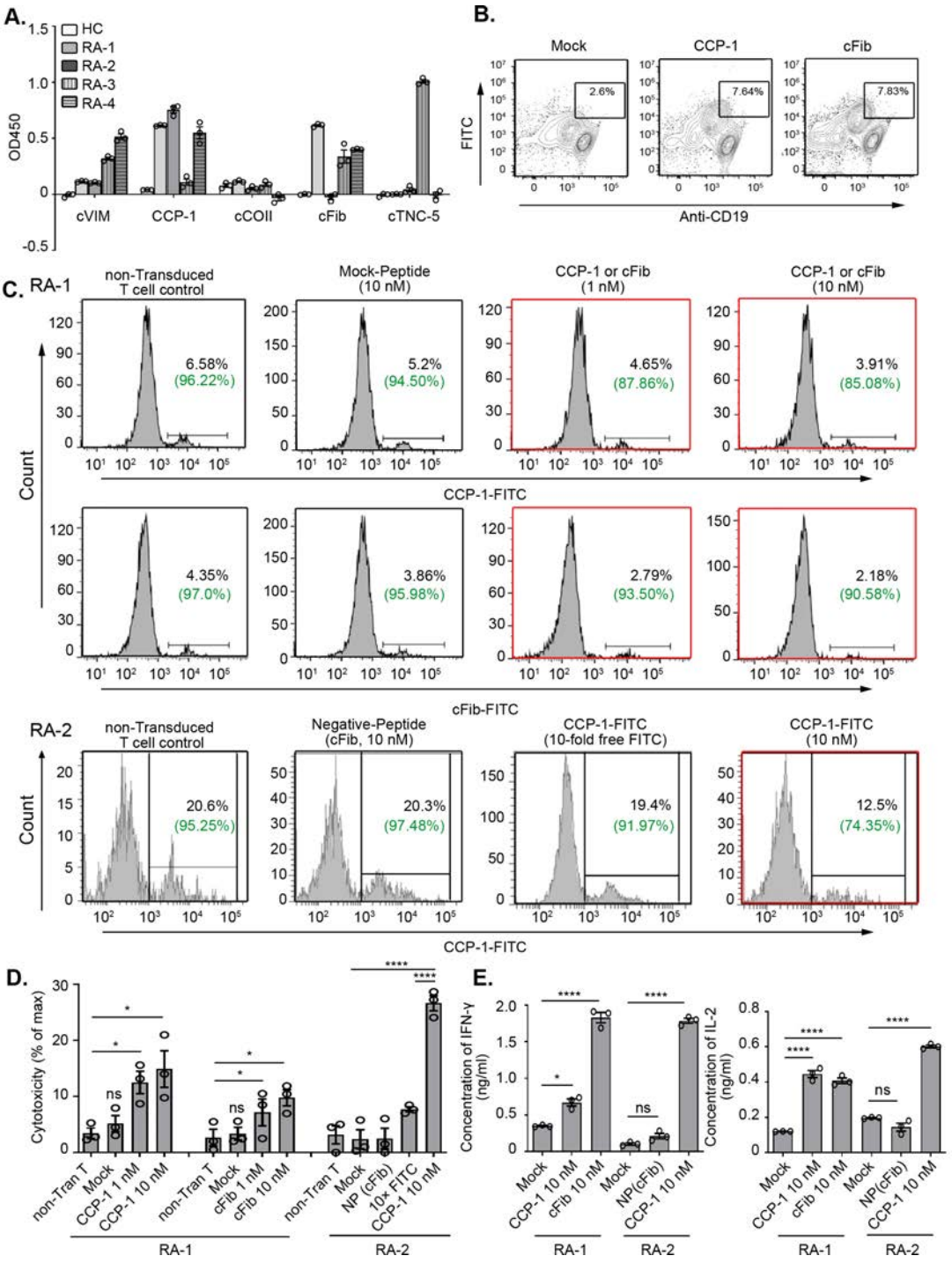

Figure 5 Targeted elimination of specific autoreactive B cells from RA patients by immunodominant peptide-mediated CAR-T cell cytotoxicity. (A) identification of autoantibodies recognising immunodominant peptides in the sera of given RA patients. Biotinylated peptides were immobilised on neutravidin-precoated plates, and the levels of autoantibodies recognising immobilised peptides were determined by TMB detection. Each symbol represents an individual donor, the data are shown as the mean±SEM derived from triplicate samples (n=3). (B) binding assessment of antibody-positive peptides to autoreactive B cells. B cells of RA-1 were isolated by magnetic separation and stimulated ex vivo for 5 days prior to assay. (C) Assessment of targeted elimination of specific autoreactive B cells by flow cytometry. Purified and stimulated B cells from RA-1 and RA-2 were incubated with anti-FITC CAR-Ts at a ratio of 1:20 in the presence of antibody-positive peptides at the indicated concentrations. The cultured cells were washed and stained with excess cultured peptides to detect the remaining corresponding autoreactive B cells. The diminished percentage of the peptide-positive cell population caused by CAR-T cell-mediated cytotoxicity is indicated as a red frame and compared with that of the negative control. The viability of cultured cells, which was calculated as 100 × (1 - cytotoxicity%) wherein cytotoxicity% was demonstrated in panel D, is indicated in parentheses. (D) Assessment of cytotoxicity and (E) cytokine secretion (IFN-γ and IL-2) of anti-FITC CAR-T cells against the specific autoreactive B cells in C. The serum negative peptide (NP) cFib-treated group for RA-2 is referred to as NP (cFib). The data are shown as the mean±SEM derived from triplicate samples (n=3), and p values were determined by one-way ANOVA (Dunnett's multiple comparison test) and compared with the non-ransduced T cell group (D) and mock group (panel E). *P≤0.05, **p≤0.01, ***p≤0.001 and ****p≤0.0001. ANOVA, analysis of variance; CAR-T, chimeric antigen receptor T cells; FITC, fluorescein isothiocyanate; RA, rheumatoid arthritis; SEM, SE of mean.

In conclusion, we report a novel versatile platform that provides a precise and customised approach for RA treatment. We preliminarily verified the feasibility of this approach, and further efficacy and safety studies remain to be performed. In light of the emerging identification of autoantigens and the development of genetic and proteomic analyses, various combinations of epitope peptides could be customised and applied to specifically eliminate pathogenic autoreactive lymphocytes according to serological and omic analyses. We believe that such a universal CAR-T cell system provides a direction for precise, customised approaches to treat RA and can likely be applied to other systemic autoimmune diseases.

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Contributors BZ, YW, LL and DH designed the study; BZ, YW, YY, JS, LL and DH performed the experiments and analysed the data; JH and MW contributed to the collection and assembly of data, data analysis and interpretation; WS, SL and HC gave comments; BZ, DZ and XZ wrote and reviewed the manuscript. All authors reviewed the manuscript.

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Competing interests None declared.

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

Patient consent for publication Not required.

Ethics approval All studies reported here were approved by the Ethics Review Board of PUMC Hospital, Chinese Academy of Medical Science (CAMS).

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CLINICAL SCIENCE

Brodalumab in psoriatic arthritis: results from the randomised phase III AMVISION-1 and AMVISION-2 trials

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ABSTRACT

Objective To compare the efficacy and safety of brodalumab, an interleukin-17 receptor subunit A inhibitor, with placebo, in patients with psoriatic arthritis (PsA).

Methods Adult patients with active PsA and inadequate response to, or intolerance to, conventional treatment were enrolled into two phase III studies (NCT02029495 and NCT02024646) and randomised 1:1:1 to receive subcutaneous brodalumab 140 mg or 210 mg or placebo at weeks 0, 1 and every 2 weeks up to 24 weeks. About 30% of patients had prior use of biologics. The primary endpoint for both studies was the American College of Rheumatology 20 (ACR20) response at week 16.

Results 962 patients were randomised across the studies prior to early termination due to sponsor decision. The primary endpoint was met in both studies. Based on comparable design and eligibility criteria, data from both studies were pooled. Significantly more patients achieved ACR20 at week 16 in both brodalumab treatment groups (45.8% and 47.9% for 140 mg and 210 mg, respectively) versus placebo (20.9%) (p<0.0001). Similar results were observed at week 24. Significantly higher proportions of patients receiving brodalumab achieved ACR50/70, Psoriasis Area and Severity Index 75/90/100 and resolution of dactylitis and enthesitis versus placebo (p<0.01). Adverse event rates were similar across treatments at week 16 (54.4%, 51.6% and 54.5% for placebo, brodalumab 140 mg and 210 mg, respectively). No new safety signals were reported.

Conclusion Brodalumab was associated with rapid and significant improvements in signs and symptoms of PsA versus placebo. Brodalumab was well tolerated, with a safety profile consistent with other interleukin-17 inhibitors.

Key messages

What is already known about this subject?

► Brodalumab has demonstrated efficacy in a phase II trial of patients with psoriatic arthritis (PsA).

What does this study add?

► These phase III trials summarise the efficacy and safety of brodalumab in a much larger population, namely 962 patients with PsA.

How might this impact on clinical practice or future developments?

► Receptor-level targeting of the interleukin-17 cytokine family involved in the pathogenesis of PsA by brodalumab results in clinically meaningful improvements in articular, enthesitis, dactylitis, skin and health-related domains. These trials provide important information for clinicians treating patients with PsA with brodalumab.

INTRODUCTION

Psoriatic arthritis (PsA) is a chronic inflammatory disorder that can affect the joints, tendon sheaths, entheses and axial skeleton.[1 2] PsA is a heterogeneous condition with different clinical phenotypes, varying in severity, disease course and numbers of affected joints.[3] Patients with PsA can experience substantial disability, with severe joint damage, digital deformation, functional impairment and impairment of quality of life (QoL).[4]

Current treatment guidelines recommend biologic disease-modifying antirheumatic drugs (DMARDs) as a treatment option on inadequate response following treatment with non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and conventional synthetic DMARDs.[5 6] Despite the advent of therapeutics targeting tumour necrosis factor (TNF), interleukin (IL)-17A and IL-12/23,[5–7] and, more recently, Janus kinase and phosphodiesterase type 4, an unmet need remains in PsA as a significant proportion of patients either do not respond or eventually lose response to currently available therapies.[5 6 8] Brodalumab is a fully human monoclonal antibody with a unique mechanism of action that binds to the IL-17 receptor subunit A (IL-17RA) with high affinity and, as a consequence, blocks the action of multiple proinflammatory cytokines of the IL-17 family, beyond that of IL-17A alone. Brodalumab 210 mg is currently approved for the treatment of moderate-to-severe plaque psoriasis[9 10] in the USA, EU, Canada and certain Asian countries and for PsA currently only in Japan.[11] The efficacy and safety of brodalumab in PsA were evaluated in a phase II, randomised, double-blind, placebo-controlled trial (NCT 01516957).[12] Brodalumab 140 mg and 280 mg once every 2 weeks (Q2W) were associated with significantly greater improvements in clinical response (American College of Rheumatology 20 (ACR20); primary

endpoint) versus placebo at 12 weeks. The safety profile of brodalumab in PsA was consistent with the safety profile established in the psoriasis clinical trial programme,[13 14] and clinical responses were sustained during an open-label extension up to week 52.[12]

To further evaluate the efficacy and safety of brodalumab in PsA, two double-blind, randomised, phase III trials, AMVISION-1 (NCT02029495) and AMVISION-2 (NCT02024646), were conducted. The primary objective of both trials was to compare the efficacy of brodalumab with placebo in patients with PsA. Both trials were placebo controlled through week 24. Data at week 16 from individual trials and week 24 from a pooled analysis are presented.

METHODS

Trial design and participants

AMVISION-1 and AMVISION-2 were multicentre, randomised, double-blind, placebo-controlled trials with planned long-term extensions. Both trials evaluated the efficacy and safety of subcutaneous brodalumab at doses of 140 mg and 210 mg Q2W in patients with active PsA who had an inadequate response or intolerance to conventional treatment with NSAIDs and/or DMARDs. The trial protocols were approved by an independent ethics committee or institutional review board at each trial site, and the trials were conducted in accordance with the International Conference on Harmonisation guideline on Good Clinical Practice. All patients provided written informed consent at trial procedure commencement.

Patients were aged ≥18 years with a diagnosis of PsA for ≥6 months at the time of enrolment and fulfilled the Classification for Psoriatic Arthritis criteria[2] at screening. Full inclusion and exclusion criteria are presented in online supplemental methods. Briefly, patients were included if they had ≥3 tender and ≥3 swollen joints (excluding the distal interphalangeal joints of the feet as part of a 66/68 joint count), an active psoriatic skin lesion and a history of intolerance or inadequate response to NSAIDs and/or DMARDs. Concomitant NSAIDs, DMARDs (methotrexate, sulfasalazine or leflunomide) and corticosteroids (≤10 mg/day prednisone or equivalent) were permitted, provided the dose was stable for ≥4 weeks prior to initiation of trial treatment. Patients in AMVISION-1 had ≥1 erosion of the hands or feet or C reactive protein (CRP) ≥1.0 mg/dL. Patients were excluded if they had an active or history of infection (including active tuberculosis), Crohn's disease, TNF inhibitor therapy within 2 months prior or other biologic therapy within 3 months prior to trial initiation or anti-IL-17 or anti-IL-12/IL-23 biologic therapy at any time. Patients with a prior history of suicidal ideation and behaviour (SIB) were excluded after the implementation of a protocol amendment. This amendment was implemented following the identification of SIB as a potential risk and after discussion with regulatory agencies. Specific tools were added to assess eligibility and monitor subject safety (ie, stopping rules). Three patients with a history of SIB were recruited into the studies prior to the amendment taking effect and continued in the trial after the amendment.

The first patient was enrolled in AMVISION-1 on 6 March 2014 and in AMVISION-2 on 24 March 2014. Both trials were terminated on 24 June 2015.

Treatment and randomisation

Following a 4-week screening period, patients in each trial were randomised 1:1:1 to receive subcutaneous brodalumab 140 mg or 210 mg or placebo on day 1 and weeks 1, 2, then Q2W through week 22 (in online supplemental figure 1), stratified by baseline body weight, prior biologic use and geographical region using a permuted block design within each stratum. Biologic-experienced

Table 1 Patient demographics and baseline disease characteristics (all randomised patients)

	AMVISION-1			AMVISION-2			Pooled		
Parameter	PBO (N=161)	BRO 140 mg Q2W (N=158)	BRO 210 mg Q2W (N=159)	PBO (N=161)	BRO 140 mg Q2W (N=160)	BRO 210 mg Q2W (N=163)	PBO (N=322)	BRO 140 mg Q2W (N=318)	BRO 210 mg Q2W (N=322)
Age, years	48.1 (11.8)	49.9 (12.8)	49.1 (12.2)	48.3 (13.0)	47.4 (12.8)	47.0 (12.6)	48.2 (12.4)	48.6 (12.8)	48.1 (12.4)
Female, n (%)	80 (49.7)	80 (50.6)	70 (44.0)	85 (52.8)	80 (50.0)	84 (51.5)	165 (51.2)	160 (50.3)	154 (47.8)
Race, n (%)									
White	152 (94.4)	152 (96.2)	155 (97.5)	154 (95.7)	150 (93.8)	159 (97.5)	306 (95.0)	302 (95.0)	314 (97.5)
Black or African American	0	0	0	0	1 (0.6)	0	0	1 (0.3)	0
Asian	4 (2.5)	0	0	1 (0.6)	0	0	5 (1.6)	0	0
Other	5 (3.1)	5 (3.2)	2 (1.3)	2 (1.2)	4 (2.5)	2 (1.2)	7 (2.2)	9 (2.8)	4 (1.2)
Duration of PsA, years	9.4 (9.3)	8.1 (8.1)	8.2 (8.2)	6.4 (7.7)	6.5 (7.4)	7.1 (7.5)	7.9 (8.6)	7.3 (7.8)	7.7 (7.9)
Psoriasis affecting ≥3% of BSA, n (%)	103 (64.0)	113 (71.5)	102 (64.2)	118 (73.3)	107 (66.9)	117 (71.8)	221 (68.6)	220 (69.2)	219 (68.0)
PASI score	6.4 (8.0)	8.2 (8.6)	7.7 (9.2)	8.9 (9.9)	9.0 (11.2)	7.9 (9.4)	7.7 (9.0)	8.6 (10.0)	7.8 (9.3)
Dactylitis score	2.3 (3.6)	2.6 (4.2)	2.2 (3.9)	2.4 (4.0)	1.7 (3.4)	1.9 (3.5)	2.4 (3.8)	2.1 (3.8)	2.0 (3.7)
Dactylitis score >0, n (%)	84 (52.2)	79 (50.0)	78 (49.1)	77 (47.8)	60 (37.5)	71 (43.6)	161 (50.0)	139 (43.7)	149 (46.3)
Enthesitis score	1.9 (1.9)	1.8 (2.0)	1.6 (1.9)	1.7 (1.8)	1.7 (2.0)	1.6 (1.8)	1.8 (1.9)	1.8 (2.0)	1.6 (1.8)
Enthesitis score >0, n (%)	107 (66.5)	93 (58.9)	93 (58.5)	101 (62.7)	92 (57.5)	100 (61.3)	208 (64.6)	185 (58.2)	193 (59.9)
Swollen joint count	12.3 (8.3)	13.3 (10.1)	12.4 (10.2)	11.0 (8.6)	11.4 (9.2)	11.1 (8.5)	11.7 (8.5)	12.4 (9.7)	11.7 (9.4)
Tender joint count	21.4 (14.8)	23.4 (15.5)	20.7 (14.4)	20.9 (14.3)	20.5 (15.7)	17.2 (12.5)	21.1 (14.5)	21.9 (15.6)	18.9 (13.6)
CRP, mg/dL	1.5 (2.2)	1.9 (3.0)	1.7 (2.7)	0.8 (1.4)	1.0 (1.8)	0.9 (1.3)	1.2 (1.9)	1.5 (2.5)	1.3 (2.1)
HAQ-DI score	1.2 (0.6)	1.3 (0.7)	1.2 (0.6)	1.1 (0.6)	1.1 (0.7)	1.1 (0.6)	1.1 (0.6)	1.2 (0.7)	1.1 (0.6)
Prior biologic use, n (%)	44 (27.3)	42 (26.6)	46 (28.9)	58 (36.0)	54 (33.8)	56 (34.4)	102 (31.7)	96 (30.2)	102 (31.7)

Data are mean (SD) unless otherwise specified.
BRO, brodalumab; BSA, body surface area; CRP, C reactive protein; HAQ-DI, Health Assessment Questionnaire-Disability Index; PASI, Psoriasis Area and Severity Index; PBO, placebo; PsA, psoriatic arthritis; Q2W, every 2 weeks.

Table 2 Comparison of brodalumab versus placebo at weeks 16 and 24 using generalised estimating equation

	AMVISION-1			AMVISION-2			Pooled		
Response, %	PBO	BRO 140 mg Q2W	BRO 210 mg Q2W	PBO	BRO 140 mg Q2W	BRO 210 mg Q2W	PBO	BRO 140 mg Q2W	BRO 210 mg Q2W
Week 16									
ACR20	16.0	39.5†	51.8†	24.8	50.9†	44.3***	20.9	45.8†	47.9†
ACR50	4.6	18.3***	28.8†	9.1	29.3†	23.2**	7.2	24.8†	26.1†
ACR70	2.7	7.8	12.5**	3.6	13.3**	10.2*	3.4	11.3***	12.2***
PASI75	10.9	55.0†	81.1†	9.3	51.5†	70.5†	10.4	52.4†	75.5†
PASI90	7.3	44.0†	69.0†	5.1	35.0†	49.3†	6.1	38.5†	58.8†
PASI100	4.3	21.2***	49.6†	3.0	18.3***	34.1†	3.9	20.7†	40.8†
Dactylitis resolution	17.3	31.3	48.8***	29.7	49.6*	53.4**	24.2	40.9**	50.8†
Enthesitis resolution	16.0	34.2*	37.8**	28.9	48.5*	39.6	23.7	42.3***	39.1**
HAQ-DI LS mean change from baseline‡	−0.136	−0.346***	−0.439†	−0.161	−0.299**	−0.325**	−0.154	−0.321†	−0.385†
Achievement of HAQ-DI MID§	25.0	42.6**	59.1†	34.6	51.6**	53.8**	30.3	47.5***	56.1†
DAS28 CRP LS mean change from baseline‡	−0.203	−1.075†	−1.299†	−0.324	−1.148†	−1.086†	−0.269	−1.115†	−1.189†
CDAI LS mean change from baseline‡	−1.981	−12.03†	−12.61†	−4.153	−12.05†	−11.53†	−3.325	−12.01†	−12.04†
DAPSA LS mean change from baseline‡	0.068	−18.73***	−18.96***	−5.142	−16.41***	−16.22***	−3.152	−17.51***	−17.61***
PASDAS LS mean change from baseline‡	−0.163	−1.578***	−2.120***	−0.434	−1.474***	−1.725***	−0.325	−1.526***	−1.913***
Week 24									
ACR20	18.9	52.2†	59.7†	27.8	49.5***	48.8***	23.8	51.0†	53.6†
ACR50	8.0	26.8***	37.5†	11.9	31.2***	35.4†	10.4	29.8†	36.4†
ACR70	2.5	11.2**	20.0†	6.1	15.7*	19.2**	4.7	14.4***	20.9†
PASI75	9.4	51.4†	80.7†	8.9	49.0†	62.3†	9.6	50.5†	70.5†
PASI90	3.8	42.2†	61.8†	3.8	32.3†	52.0†	3.8	36.6†	57.1†
PASI100	3.5	26.8***	52.0†	0.7	23.5**	45.5***	1.9	26.0†	48.6†
Dactylitis resolution	14.0	26.4	59.4†	22.9	59.5***	60.0***	19.8	43.0***	60.1†
Enthesitis resolution	15.7	33.8*	54.8†	27.3	47.2*	37.8	22.7	41.6**	43.8***
HAQ-DI LS mean change from baseline‡	−0.192	−0.411**	−0.526†	−0.226	−0.332	−0.398**	−0.216	−0.371**	−0.467†
Achievement of HAQ-DI MID§	23.5	44.4**	60.9†	28.9	47.5**	49.6**	26.3	46.1†	54.3†
DAS28 CRP LS mean change from baseline‡	−0.682	−1.348**	−1.734†	−0.714	−1.233**	−1.276**	−0.698	−1.275†	−1.495†
CDAI LS mean change from baseline‡	−7.086	−14.64†	−16.38†	−8.778	−12.67*	−13.36**	−8.153	−13.53†	−14.87†
DAPSA LS mean change from baseline‡	−6.797	−21.97***	−25.68***	−11.75	−17.28*	−19.25**	−10.12	−19.53***	−22.46***
PASDAS LS mean change from baseline‡	−0.636	−1.981***	−2.599***	−0.857	−1.810***	−2.155***	−0.778	−1.892***	−2.369***

Full analysis set. Response rates were calculated using a GEE model. NRI was applied following early withdrawal from trial for reasons other than premature trial termination, and to subjects who satisfied the inadequate response criteria prior to week 24. GEE analysis assumed missing data due to early trial termination and intermittent missing data were missing completely at random. Patient numbers are reported in online supplemental table 1.

ACR responses were modified based on 66/68 joint counts. PASI responses were calculated using the psoriasis efficacy full analysis set (patients with baseline BSA ≥3%). Dactylitis and enthesitis responses were evaluated in patients with these conditions at baseline. Dactylitis was assessed as present (yes/no) on 20 digits (fingers and toes). Enthesitis was assessed as present (yes/no) on six entheses (lateral epicondyle, medial femoral condyle and Achilles tendon insertion).

*p<0.05 versus placebo; **p<0.01 versus placebo; ***p<0.001 versus placebo; †p<0.0001 versus placebo.

‡Change in LS mean values from baseline. p values shown are calculated for the LS mean difference versus placebo. The estimates are calculated as active treatment minus placebo using a linear mixed-effects model for repeated measures. A reduction indicates a beneficial treatment effect. The model contains visit, treatment, treatment by visit and baseline by visit interaction, baseline and three randomisation strata: baseline weight (≤100 kg, >100 kg), prior biologic use (yes/no) and geographical region (North and Latin America, Central/Eastern Europe, Western Europe).

§The MID used for HAQ-DI in PsA is 0.35.[15]

ACR20/50/70, American College of Rheumatology 20/50/70% improvement criteria; BRO, brodalumab; BSA, body surface area; CDAI, Clinical Disease Activity Index; CRP, C reactive protein; DAPSA, Disease Activity in Psoriatic Arthritis; DAS28, Disease Activity Score with a 28-joint count; GEE, generalised estimating equation; HAQ-DI, Health Assessment Questionnaire-Disability Index; LS, least squares; MID, minimally important difference; NRI, non-responder imputation; PASDAS, Psoriasis Arthritic Disease Activity Score; PASI75/90/100, 75/90/100% improvement in Psoriasis Area and Severity Index; PBO, placebo; PsA, psoriatic arthritis; Q2W, every 2 weeks.

and biologic-naïve populations were each capped at no more than 60% of the global trial population; however, the final trial population included about 70% of biologic-naïve patients. This deviation is not considered to have an impact on the interpretation of the data. From week 14, patients were evaluated for inadequate response, defined as failure to achieve ≥10% improvement from baseline

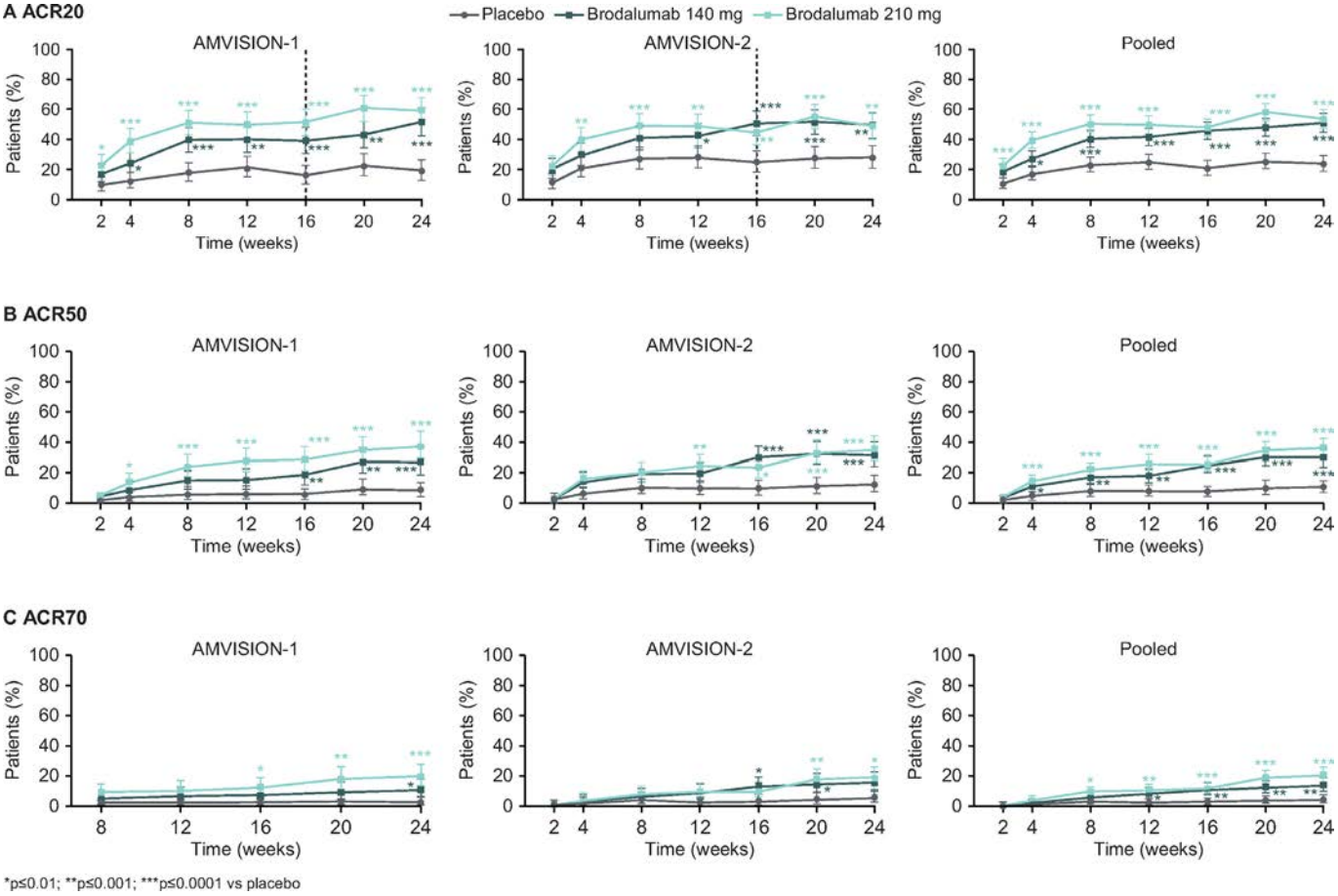

Figure 1 (A) ACR20, (B) ACR50 and (C) ACR70 response rates from baseline to week 24. Full analysis set. Dashed line represents the primary endpoint for each study. Response rates (95% CI) were calculated using a GEE model. NRI was applied following early withdrawal from trial for reasons other than premature study termination, and to subjects who satisfied the inadequate response criteria prior to week 24. GEE analysis assumed missing data due to early trial termination and intermittent missing data were MCAR. ACR responses were modified based on 66/68 joint counts. ACR20/50/70, American College of Rheumatology 20/50/70% improvement criteria; GEE, generalised estimating equation; MCAR, missing completely at random; NRI, non-responder imputation.

in tender and swollen joint counts at two consecutive scheduled visits where joint counts were assessed (eg, weeks 14 and 16). If the criteria for inadequate response were met, initiation and/or dose adjustments of non-biologic treatments were permitted. Patients on placebo with inadequate response were switched to brodalumab 210 mg, and from week 24, patients who were originally randomised to placebo and had not already met criteria for inadequate response received brodalumab 210 mg with an additional dose at week 25. From week 28 through week 34, patients who did not achieve ≥10% improvement from baseline in their tender and swollen joint counts at any visit, despite ≥12 weeks of continuous treatment after meeting inadequate response criteria, were considered non-responders and treatment was discontinued. Further information on inadequate response, rescue treatment and other criteria for permanent discontinuation is provided in the online supplemental appendix. Both trials were planned with a 52-week double-blind treatment phase, followed by a long-term open-label extension phase. After treatment assignments were unblinded, all patients subsequently received open-label brodalumab at their current Q2W dose.

Endpoint

The primary endpoint for both trials was ACR20 response at week 16. Secondary efficacy endpoints included ACR 50/70 and Psoriasis Area and Severity Index (PASI) 75/90/100 response rates; change from baseline in Health Assessment Questionnaire-Disability Index (HAQ-DI); Disease Activity Score with a 28-joint count and CRP; improvement in dactylitis assessed as present (yes or no) on 20 digits and enthesitis assessed as present (yes or no) on six entheses); Psoriatic Arthritis Disease Activity Score (PASDAS); and Clinical Disease Activity Index score (CDAI). A radiographic endpoint (evaluated through modified Total Sharp Score) was included in AMVISION-1. Due to the premature cessation of the trial and the subsequent smaller trial population recruitment, no definitive conclusions could be drawn with respect to the radiographic progression endpoint; as such, the data are not reported herein. Disease Activity in Psoriatic Arthritis (DAPSA) was included as a post hoc analysis. The primary and all secondary endpoints were also evaluated at week 24. The safety profile of brodalumab was evaluated in all patients who had received ≥1 dose of trial drug by recording adverse events (AEs), serious AEs (SAEs), laboratory assessments and vital signs. Safety at week 16 was reported as number and percentage of subjects who reported AEs, and at week 24 as number of AEs and AE rate/100 patient years, due to the early termination of the studies.

Statistical analyses

The trials were designed to detect significant treatment differences in ACR20 response between the brodalumab and placebo arms at week 16 with >90% power, assuming the underlying

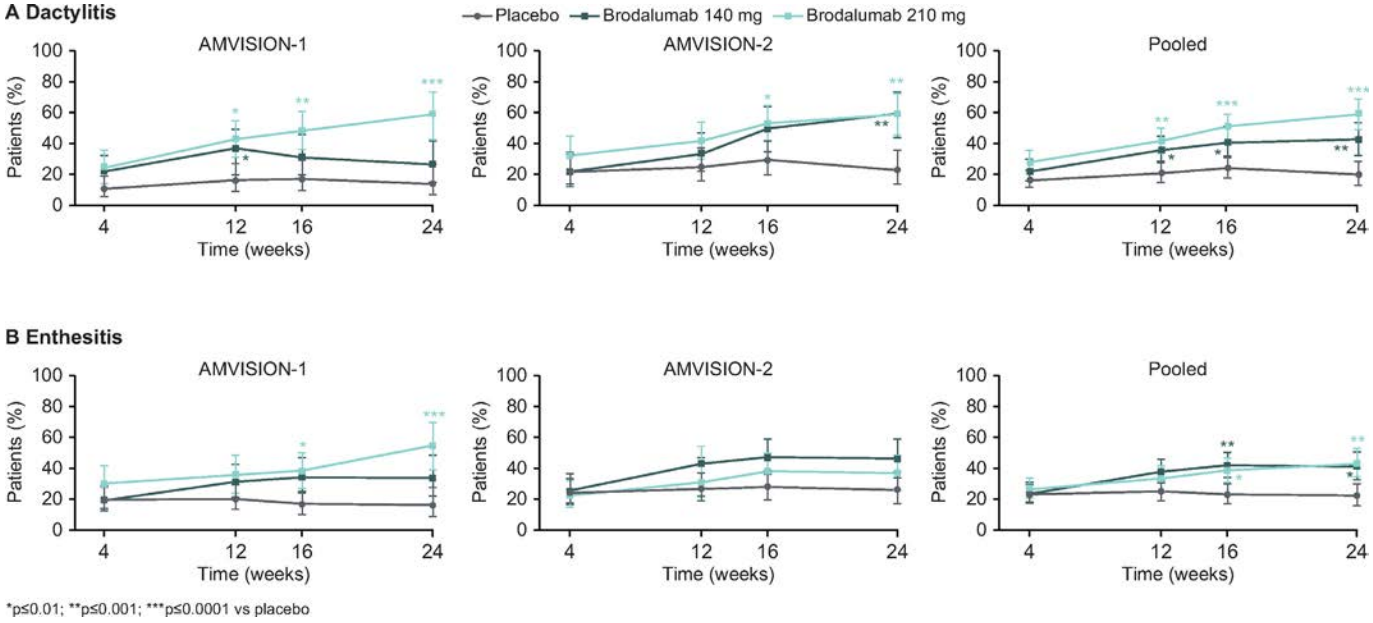

Figure 2 Resolution of dactylitis and enthesitis. Full analysis set. Response rates (95% CI) were calculated using a GEE model. NRI was applied following early withdrawal from trial for reasons other than premature trial termination, and to subjects who satisfied the inadequate response criteria prior to week 24. GEE analysis assumed missing data due to early trial termination and intermittent missing data were MCAR. Dactylitis and enthesitis responses were evaluated in patients with these conditions at baseline. Dactylitis was assessed as present (yes/no) on 20 digits (fingers and toes). Enthesitis was assessed as present (yes/no) on six entheses (lateral epicondyle, medial femoral condyle and Achilles' tendon insertion). GEE, generalised estimating equation; MCAR, missing completely at random; NRI, non-responder imputation.

rate of response was 45% and 18% in the brodalumab and placebo arms, respectively.

Both studies were terminated by the sponsor (Amgen) prior to reaching their recruitment targets. The power of the primary endpoint in both studies, although reduced, was still sufficient. To account for all randomised patients and those who did not have the opportunity to complete the trial, non-responder imputation (NRI) and a generalised estimating equation (GEE) model were implemented. The GEE model included prior biologic use, geographical region, baseline body weight and treatment by visit interaction terms as fixed effects. Under the assumption that missing data were missing completely at random (MCAR) the estimated treatment effects derived from the GEE model would be unbiased. For this analysis, patients qualifying for rescue treatment or withdrawing from the trial for any reason other than trial termination were treated as non-responders. Results of the GEE analysis are presented in this manuscript by trial and as a pooled analysis. Further information on trial termination, analysis of continuous endpoints and subsequent statistical analysis is provided in the online supplemental methods.

RESULTS

Patients

At the time of trial termination, in AMVISION-1 478 of the planned 630 patients, and in AMVISION-2, 484 of the planned 495 patients had been enrolled. The majority of these patients (693, 72%) completed 24 weeks of treatment. Patient disposition is summarised in online supplemental figure 2. The main reasons for discontinuation during the first 24 weeks were sponsor decision (19.8%) and withdrawal of consent (7.6%).

Demographics and baseline disease characteristics were balanced across treatment groups in both trials (table 1). The mean age across the trials was approximately 48 years, 50% of patients were women, and at baseline, one-third of patients had received prior biologic treatment in all treatment groups. Approximately two-thirds of patients had psoriasis covering ≥3% of their body surface area. Mean swollen and tender joint counts were 12 and 21, respectively. Fifty per cent of the patients had dactylitis (score >0) and nearly 67% of patients had enthesitis (score >0); 30% of patients had prior use of biologics.

Efficacy

The primary objective was met, with significantly greater proportions of patients achieving ACR20 with both brodalumab doses versus placebo at week 16 (table 2 and figure 1A). Pooled analysis of data from both trials showed ACR20 response rates of 45.8% and 47.9% for brodalumab 140 mg and 210 mg, respectively, versus 20.9% for placebo at week 16 (both $p<0.0001$ vs placebo; table 2 and figure 1A). The marginal response rate was maintained through 24 weeks (51.0%, 53.6% and 23.8% for brodalumab 140 mg, 210 mg and placebo, respectively; both $p<0.0001$ vs placebo).

Other endpoints were also significantly improved in brodalumab recipients. Higher ACR50 response rates were observed for brodalumab versus placebo at all time points from week 2 for brodalumab 210 mg and week 4 for brodalumab 140 mg (figure 1B), and higher ACR70 response rates were observed versus placebo from week 8 for brodalumab 210 mg and week 12 for brodalumab 140 mg (figure 1C). Generally, the proportions of patients achieving ACR responses were higher in the brodalumab 210 mg group versus the 140 mg group.

A significantly higher proportion of patients with dactylitis at baseline achieved resolution in both brodalumab groups at weeks 12, 16 and 24 versus placebo (table 2 and figure 2A), with higher observed rates of dactylitis resolution with brodalumab 210 mg versus 140 mg at these time points. Among patients with enthesitis at baseline, resolution was achieved by a significantly higher proportion in both brodalumab groups versus placebo at weeks 16 and 24 (table 2 and figure 2B). Furthermore, brodalumab treatment resulted in significantly greater mean changes

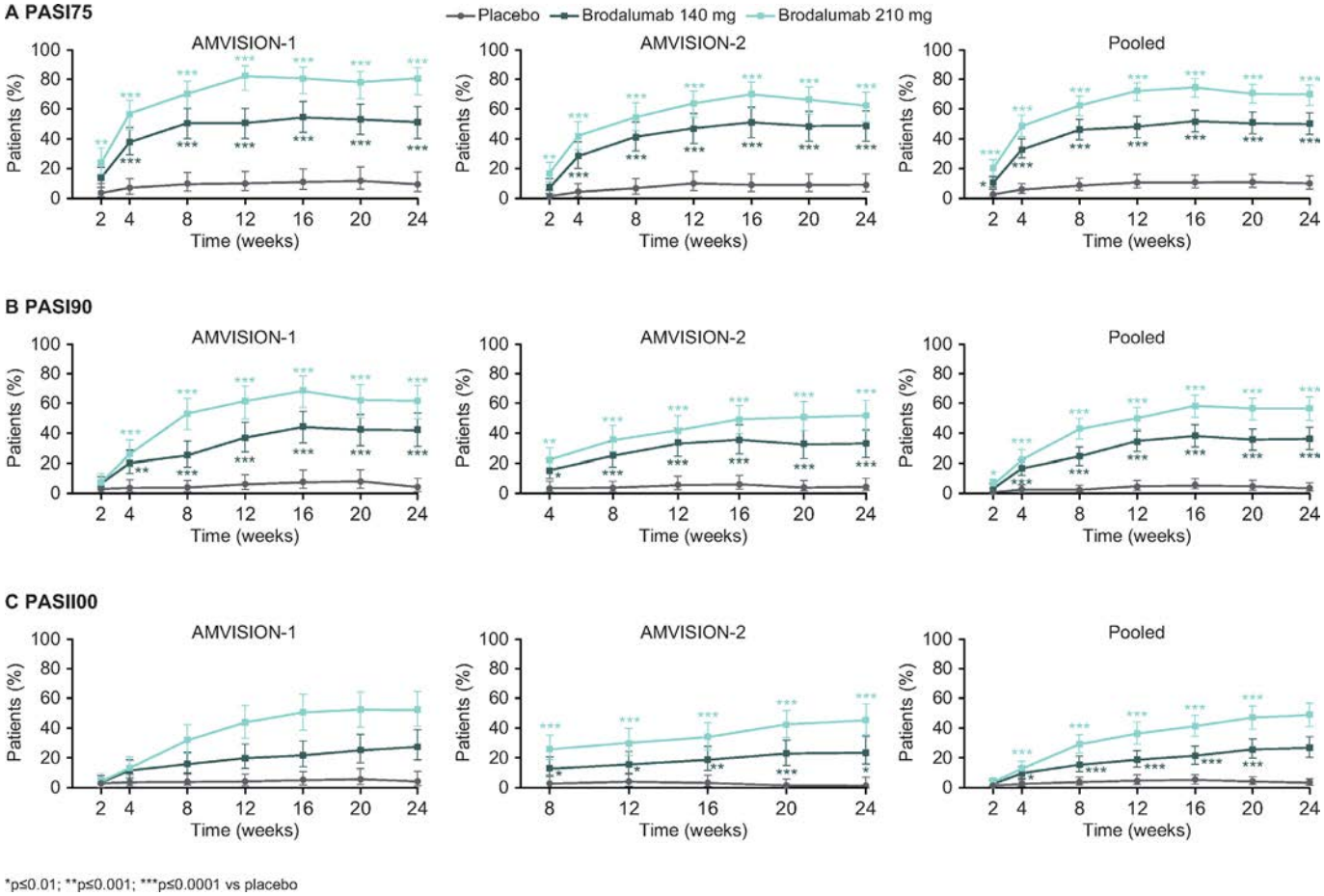

Figure 3 (A) PASI75, (B) PASI90 and (C) PASI100 response rates from baseline to week 24. PASI responses were calculated using the psoriasis efficacy full analysis set (patients with baseline BSA ≥3%). Response rates (95% CI) were calculated using a GEE model. NRI imputation was applied following early withdrawal from trial for reasons other than premature trial termination, and to subjects who satisfied the inadequate response criteria prior to week 24. GEE analysis assumed missing data due to early trial termination and intermittent missing data were MCAR.
BSA, body surface area; GEE, generalised estimating equation; MCAR, missing completely at random; NRI, non-responder imputation; PASI75/90/100, 75/90/100% improvement in Psoriasis Area and Severity Index.

in HAQ-DI, CDAI, DAPSA and PASDAS scores from baseline versus placebo at weeks 16 and 24. Finally, the proportions of patients achieving a minimally important difference in HAQ-DI scores from baseline (defined as 0.35[15]) were significantly higher with brodalumab versus placebo at weeks 16 and 24 (table 2).

Brodalumab also demonstrated efficacy in skin-related endpoints. A significantly higher proportion of patients in both brodalumab groups achieved PASI75 versus placebo at all time points from week 2 (table 2 and figure 3A). Similarly, significantly more patients achieved PASI90 (figure 3B) and PASI100 (figure 3C) at all time points from week 4 onwards. There was a clear dose-dependent difference over time in the proportion of patients achieving PASI75/90/100 in the brodalumab 140 mg and 210 mg groups, with greater proportions of patients achieving PASI75/90/100 at the higher brodalumab dose.

Safety

In the pooled analysis, the total duration of exposure to trial treatment was 107.8, 121.7 and 123.9 patient years in the placebo, brodalumab 140 mg and 210 mg groups, respectively. Overall, the percentage and type of AEs reported in the brodalumab 210 mg group at week 16 was similar to that of placebo and most events were mild to moderate in severity (table 3). The proportion of patients reporting AEs of interest was low (most commonly infection (24% to 30%)). No major imbalances or emergent safety signals were detected. Safety data at week 24 (event rates per 100 patient years) were similar to those at week 16 (online supplemental table 3). There were no deaths throughout the duration of the trials; the event rate of SAEs with brodalumab was low. Patients treated with brodalumab experienced a numerical increase in cases of neutropenia versus placebo, but none was related to infection. Serious infections were reported infrequently at week 24, with only one event recorded in the brodalumab 210 mg group in AMVISION-1 during the trial period (urosepsis reported during the first 16 weeks, which resolved). One patient with a history of SIB and depression in the brodalumab 140 mg group in AMVISION-2 was diagnosed with suicidal ideation following the first completed Columbia Suicide Severity Rating Scale 8 days after the first dose of brodalumab. The event was not considered related to trial medication by the investigator and resolved on the same day. The patient continued in the study for about another year without reporting other SIB events. Injection site reactions were generally mild and infrequent.

DISCUSSION

The heterogeneity of PsA requires treatment options that are active across all disease domains (including skin, arthritis, dactylitis and enthesitis). The results from AMVISION-1 and

Table 3 Summary of safety: adverse events up to week 16 (safety population, pooled analysis)

AEs, n (%)*	PBO (N=320)	BRO 140 mg Q2W (N=318)	BRO 210 mg Q2W (N=321)
Any AE	174 (54.4)	164 (51.6)	175 (54.5)
AEs causally related to treatment†	62 (19.4)	52 (16.4)	48 (15.0)
SAE	9 (2.8)	6 (1.9)	11 (3.4)
Death	0	0	0
AEs leading to treatment discontinuation	7 (2.2)	3 (0.9)	4 (1.2)
AEs leading to treatment interruption	41 (12.8)	30 (9.4)	38 (11.8)
Selected AEs of interest‡			
Infections and infestations	91 (28.4)	75 (23.6)	96 (29.9)
Crohn's disease	0	0	0
Neutropenia	0	3 (0.9)	3 (0.9)
Suicidal ideation and behaviour	0	1 (0.3)§	0
MACE	2 (0.6)	0	0
Hypersensitivity¶	2 (0.6)	1 (0.3)	7 (2.2)
Malignancy	0	1 (0.3)	1 (0.3)

*Subjects with multiple events in the same category are counted only once in that category. Subjects with events in more than one category are counted once in each of those categories.
†Causally related to treatment as assessed by investigator.
‡Adverse events of interest are important identified risks (eg, infections, neutropenia, worsening of Crohn's disease), important potential risks (eg, hypersensitivity, suicidal behaviour (including attempted/completed suicide attempt), suicidal ideation, MACE, malignancy) and other events of interest (injection site reactions) in response to the emerging safety profile of brodalumab.
§Patient (35-year-old female, history of suicidal ideation) diagnosed following the first completed electronic Columbia Suicide Severity Rating Scale assessment, 8 days after first dose; resolved on same day.
¶Adverse events occurring within 1 day of an injection and corresponding to the broad scope for the hypersensitivity SMQ have been included.
AE, adverse event; BRO, brodalumab; MACE, major adverse cardiovascular events; MedDRA, Medical Dictionary for Regulatory activities; PBO, placebo; Q2W, every 2 weeks; SAE, serious adverse event; SMQ, standardised MedDRA query.

AMVISION-2 demonstrate that brodalumab provided rapid and significant improvement compared with placebo in the signs and symptoms of PsA. The primary objective was met in both trials, and improvements were observed in articular, enthesitis, dactylitis, skin and QoL domains. These data suggest that brodalumab, with its unique mechanism of action, can offer clinical benefit to patients with PsA and thus reassure clinicians using brodalumab in people with psoriasis that musculoskeletal benefits can also accrue.

Brodalumab is a fully human monoclonal antibody that binds to the IL-17RA with high affinity. By blocking the IL-17RA, brodalumab inhibits the action of multiple proinflammatory IL-17 family cytokines (IL-17A, IL-17F, IL-17A/F, IL-17E (IL-25) and IL-17C).[16–18] These cytokines all play broad roles in the type 17 T cell pathway, including complex crosstalk and endogenous control of the inflammatory response, and their dysregulation can lead to the destruction of tissue and the pathogenesis of autoimmune diseases such as psoriasis and PsA.[19–31]

Overall, the trial populations were representative of patients with PsA. Significant differences versus placebo were observed for endpoints related to joint involvement (ACR20/50/70) as well as those associated with skin manifestations (PASI75/90/100) and were accompanied by an improvement in patient-reported physical function (HAQ-DI). Throughout the trials, response and improvement in musculoskeletal and psoriasis endpoints were generally greater among patients who received the higher brodalumab dose. This trend was more marked in the AMVISION-1 trial. In order to enrich the trial population for evaluation of radiographic progression, the trial population of AMVISION-1 consisted of patients who had a more severe disease at baseline, as compared with patients in AMVISION-2. Consequently, a dose–response may have been more easily observed in AMVISION-1 than in AMVISION-2. The onset of effect occurred as early as 2 weeks after initiation of treatment with the 210 mg brodalumab dose for some endpoints such as PASI75. Specifically for AMVISION-2, nominal and statistical improvement in both brodalumab groups was evident despite the response rate in the placebo group (ACR20, 24.8%) being in the upper range of placebo response rates previously reported in other studies of biologics treating similar PsA populations (11% to 24% for IL-17, IL-12/-23 and TNF inhibitors).[32–36]

The trials were terminated early (24 June 2015) following a decision from the sponsor (Amgen) to stop its participation in the codevelopment of brodalumab after events of SIB had been observed in the clinical programme and an anticipation that it would lead to restrictive labelling, (refer to online supplement for further information). Given that the assessments of treatment effects at weeks 16 and 24 were both clinically relevant, as well as the trajectory of the response over the duration of treatment, the GEE model was chosen as it provided a more succinct presentation of the clinical trial data. In this model, missing data due to trial termination and intermittent missing data were assumed to be MCAR. Monotone missing data due to reasons other than early trial closure were imputed using NRI, which assumes that patients who discontinued early, due to an AE, lack of efficacy and so on, would have been non-responders, had they remained in the trial. In essence, MCAR assumes that the decision to stop the trial/the reason for missing data is unrelated to the individual subject's ability to respond to treatment. The results of these analyses are robust regarding the statistical method and assumptions regarding missing data as the primary analysis for patients completing the week 16 visit prior to study termination closely matched the GEE analysis at week 16 (online supplemental table 4). In addition, the primary and GEE analyses at week 24 were also closely matched. Post hoc sensitivity analyses of ACR20 at week 24 were performed using different models and different methods for imputation of missing data. These analyses are consistent with the results of the primary analysis and confirm the robustness of the conclusions despite the change in model and imputation method from the original

statistical analysis plan and protocol. Sensitivity analysis II, using NRI for all missing data, corresponds to the analysis specified in the protocol (see online supplemental table 2).

The incidences of AEs and SAEs with brodalumab were consistent with the known safety profile of brodalumab previously reported in psoriasis and PsA,[12–14] with no increased rate of AEs related to brodalumab versus placebo, and no evidence of dose-dependence. Patients with a prior history of SIB were excluded from these trials after the implementation of a protocol amendment, allowing for an assessment of whether new instances of SIB were encountered in the brodalumab-treated population. The overall frequencies of depression and SIB in these trials were similar for brodalumab- and placebo-treated subjects during the double-blind period, suggesting that brodalumab treatment did not increase the risk for depression and SIB among patients with no prior history.

In summary, the AMVISION-1 and AMVISION-2 trials showed that brodalumab 140 mg and 210 mg Q2W are associated with substantial improvements in both joint-related and skin-related endpoints versus placebo in patients with PsA, and these improvements are maintained through 24 weeks. The safety profile of brodalumab was consistent with that reported in previous trials in psoriasis and the phase II PsA trial.[12] The favourable safety profile and efficacy data from these trials suggest that inhibition of IL-17RA with brodalumab, a unique mechanism of action, may represent an additional treatment strategy for patients with PsA.

Correction notice This article has been corrected since it published Online First. Table 2 has been corrected.

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Competing interests PJM reports, outside the submitted work, grants and personal fees from AbbVie, Amgen, Bristol Myers Squibb, Celgene, Janssen, Eli Lilly, Novartis, SUN Pharma, UCB Pharma; personal fees from Genentech, Boehringer Ingelheim, Galapagos, Gilead and GlaxoSmithKline. PSH reports, outside the submitted work, grants, personal fees and non-financial support from AbbVie; grants from Amgen, Celgene, Janssen, MSD, Pfizer and UCB; and personal fees from Galapagos. KFH was an employee of LEO Pharma at the time this study was conducted. KFH also reports, outside the submitted work, grants from AbbVie, Celgene, LEO Pharma and Novartis; personal fees from AbbVie, CSL Behring, Eli Lilly, Janssen, LEO Pharma, Novartis and Pfizer. KR is an employee of LEO Pharma. IBM reports personal fees from LEO Pharma during the conduct of the study. IBM also reports, outside the submitted work, grants and personal fees from Celgene, Compugen and UCB; grants from AstraZeneca, Novartis and Roche; personal fees from AbbVie, Galvani, Eli Lilly and Pfizer.

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

Patient consent for publication Not required.

Ethics approval The study was conducted in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonisation Guidance for Good Clinical Practice. Independent institutional review board approvals were obtained, and all patients provided written informed consent in accordance with local requirements.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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TRANSLATIONAL SCIENCE

Natural killer cells and type II interferon in Ro/SSA and La/SSB autoantibody-exposed newborns at risk of congenital heart block

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ABSTRACT

Objective Congenital heart block (CHB) with immune cell infiltration develops in the fetus after exposure to maternal Ro/La autoantibodies. CHB-related serology has been extensively studied, but reports on immune-cell profiles of anti-Ro/La-exposed neonates are lacking. In the current study, we characterised circulating immune-cell populations in anti-Ro/La+mothers and newborns, and explored potential downstream effects of skewed neonatal cell populations.

Methods In total, blood from mothers (n=43) and neonates (n=66) was sampled at birth from anti-Ro/La+ (n=36) and control (n=30) pregnancies with or without rheumatic disease and CHB. Flow cytometry, microarrays and ELISA were used for characterising cells and plasma.

Results Similar to non-pregnant systemic lupus erythematosus and Sjögren-patients, anti-Ro/La+mothers had altered B-cell subset frequencies, relative T-cell lymphopenia and lower natural killer (NK)-cell frequencies. Surprisingly, their anti-Ro/La exposed neonates presented higher frequencies of $CD56^{dim}CD16^{hi}$ NK cells ($p<0.01$), but no other cell frequency differences compared with controls. Type I and II interferon (IFN) gene-signatures were revealed in neonates of anti-Ro/La+ pregnancy, and exposure of fetal cardiomyocytes to type I IFN induced upregulation of several NK-cell chemoattractants and activating ligands. Intracellular flow cytometry revealed IFNγ production by NK cells, $CD8^+$ and $CD4^+$ T cells in anti-Ro/La exposed neonates. IFNγ was also detectable in their plasma.

Conclusion Our study demonstrates an increased frequency of NK cells in anti-Ro/La exposed neonates, footprints of type I and II IFN and an upregulation of ligands activating NK cells in fetal cardiac cells after type I IFN exposure. These novel observations demonstrate innate immune activation in neonates of anti-Ro/La+pregnancy, which could contribute to the risk of CHB.

Key messages

What is already known about this subject?

- The serology in congenital heart block (CHB) has been extensively studied, but reports on immune-cell profiles of anti-Ro/La autoantibody-exposed neonates are lacking.

What does this study add?

- We show that anti-Ro/La exposed newborns have increased frequencies of natural killer (NK) cells with intracellular pools of interferon-γ (IFN), and that NK-cell attracting and activating ligands are expressed in fetal cardiac cells after type I IFN exposure.

How might this impact on clinical practice or future developments?

- These observations indicate that innate immune mechanisms may contribute to the pathogenesis of CHB, which could form the basis of novel treatment strategies.

INTRODUCTION

Isolated congenital heart block (CHB) is defined as a third-degree atrioventricular (AV) block without major heart malformations observed in utero or within 28 days of birth.[1] Pathology reports of deceased subjects are rare, but have evidenced IgG deposition and mononuclear cells in the AV nodal area in the acute stage,[2 3] as well as infiltrating macrophages later during the disease course with fibrotic and calcified tissues.[4] Maternal Ro/SSA and La/SSB autoantibodies are found in >95% of these cases.[5 6] Some mothers whose children are affected are asymptomatic, while others are diagnosed with a rheumatic disease, most commonly Sjögren's syndrome (SS) or systemic lupus erythematosus (SLE).[5 7] Although the association of Ro/La autoantibodies with CHB is strong, only around 2%–3% of Ro/La autoantibody-exposed fetuses develop CHB,[8–11] leading to an incidence around 1:20 000 in the general population.[11 12] Timely administration of fluorinated steroids have been suggested to revert incomplete AV blocks and postpone the need for pacemaker treatment in the child,[11] although their efficacy in reducing mortality is currently a matter of debate.[12 13]

The low penetrance of CHB in Ro/La exposed fetuses suggests that the mere presence of autoantibodies is not sufficient for the condition to develop. Recent studies show that autoantibody exposed fetuses, like their mothers,[14] have a type I interferon (IFN) gene expression signature,[15–17] indicating a role for IFN and IFN-stimulated genes in the pathogenesis of CHB. The type I IFNs include IFNα and

Table 1 Demographic and clinical characteristics mothers and neonates

	Mothers*			Neonates*		
	Rheumatic disease		Controls	Of rheumatic mother		Controls
	Ro/La+**n=36**	Ro/La- **n=7**	**n=23**	Ro/La+**n= 36**	Ro/La- **n=7**	**n=23**
Age at delivery (years, mean, SD)	32.2±3.18	33.1±3.92	33.2±5.30	n.a.	n.a.	n.a.
Rheumatic diagnosis				n.a.	n.a.	n.a.
SS	11	0	0			
SLE	15	6	0			
Polyarthritis	*	0	0			
RA	*	0	0			
APS	*	*	0			
Myositis	*	0	0			
No diagnosis	6	0	0			
Medication				n.a.	n.a.	n.a.
HCQ	7	4				
Prednisolone	7	5				
Azathioprine	*	2				
Mode of delivery				n.a.	n.a.	n.a.
Vaginal	24 (67%)	4 (57%)	12 (52%)			
Sectio	12 (33%)	3 (43%)	11 (48%)			
GA at birth (mean weeks+days)	n.a.	n.a.	n.a.	38+6	39+2	39+6
Sex (male)	n.a.	n.a.	n.a.	19 (53%)	4 (57%)	12 (52%)
Weight at birth (g, mean±SD)	n.a.	n.a.	n.a.	3046±607	3914±491	3570±544
Length at birth (cm, mean±SD)	n.a.	n.a.	n.a.	48.0±4.5	52.6±3.0	50.63±1.9

*The table summarises the clinical data for mothers and their neonates if either contributed samples to the study. Specification of sample contribution and which figures derived data appear in is specified in online supplemental table S1.
APS, antiphospholipid syndrome; GA, gestational age; HCQ, hydroxychloroquine; n.a., not applicable; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome.

IFNβ which can be produced by most nucleated cells, while IFNγ is the sole type II IFN, the production of which is restricted to immune cells.[18]

While much effort has been invested in understanding the CHB-associated autoantibodies,[19] the circulating immune cells of Ro/La autoantibody-exposed neonates have not been characterised to date. Considering the potential role of immune cells in this lethal condition, we analysed peripheral immune populations in newborns of anti-Ro/La+ mothers and healthy donors, and explored potential downstream effects of skewed neonatal cell populations.

PATIENTS AND METHODS

Subjects of the study

Pregnant women with Ro/La autoantibodies in a clinical surveillance programme at the Department of Pediatric Cardiology,[11 12] pregnant women with systemic autoimmunity with or without Ro/La autoantibodies at the Department of Rheumatology and healthy pregnant women at the Labor and Delivery Unit, Karolinska University Hospital, Sweden, were offered to participate in the study. Mothers with a diagnosis of Sjögren's syndrome fulfilled criteria by Vitali *et al*[20] and mothers with SLE the revised 1982 ACR criteria.[21] At delivery, peripheral blood was sampled from the mothers and umbilical cord blood was sampled from the neonates to generate PBMC and CBMC, respectively, as well as plasma. Demographic and clinical information on mothers and newborns are summarised in table 1, with additional detail in online supplemental table S1. All mothers gave written informed consent.

Blood sample processing

PBMC/CBMC was isolated using Ficoll and SepMate tubes (Stemcell Technologies) and stained for flow cytometry or cryopreserved in liquid nitrogen in 10% DMSO and either 90% or 50% heat-inactivated fetal bovine serum supplemented with RPMI 1640 medium until use. For RNA extraction, cells were resuspended in RLT buffer (Qiagen) or Trizol (ThermoFisher Scientific) and stored at −80°C. Plasma was aliquoted and frozen at −80°C.

Flow cytometry

Fresh PBMC/CBMC was stained for 20 min on ice with fluorescently labelled antibodies (online supplemental table S3). Data were acquired using a Gallios flow cytometer (Beckman Coulter). Alternatively, PBMC/CBMC was thawed, stained with viability dye (ThermoFisher Scientific) and fluorescently coupled antibodies. For intracellular staining, cells were incubated with brefeldin A (10 µg/mL, Sigma-Aldrich) for 4 hours in 96-well plates with 5×10^5 cells per well. Stimulation with ionomycin (1 µM) and PMA (10 ng/mL) (Sigma-Aldrich) was used as positive control. Cells were then stained by viability dye and fluorescently labelled antibodies against cell surface markers, fixed and permeabilised (Fixation/Permeabilisation Kit, BD Biosciences), followed by intracellular staining for IFNγ, Granzyme-B and Perforin-1. Data were acquired by an LSR Fortessa flow cytometer (BD Biosciences) and analysed by FlowJo software V.10.6.2 (Tree Star). Gating strategies are shown in online supplemental figures S1-S4.

Microarrays

RNA from cells stored in RLT buffer was extracted using an RNeasy Mini Kit (Qiagen). RNA from cells stored in Trizol was

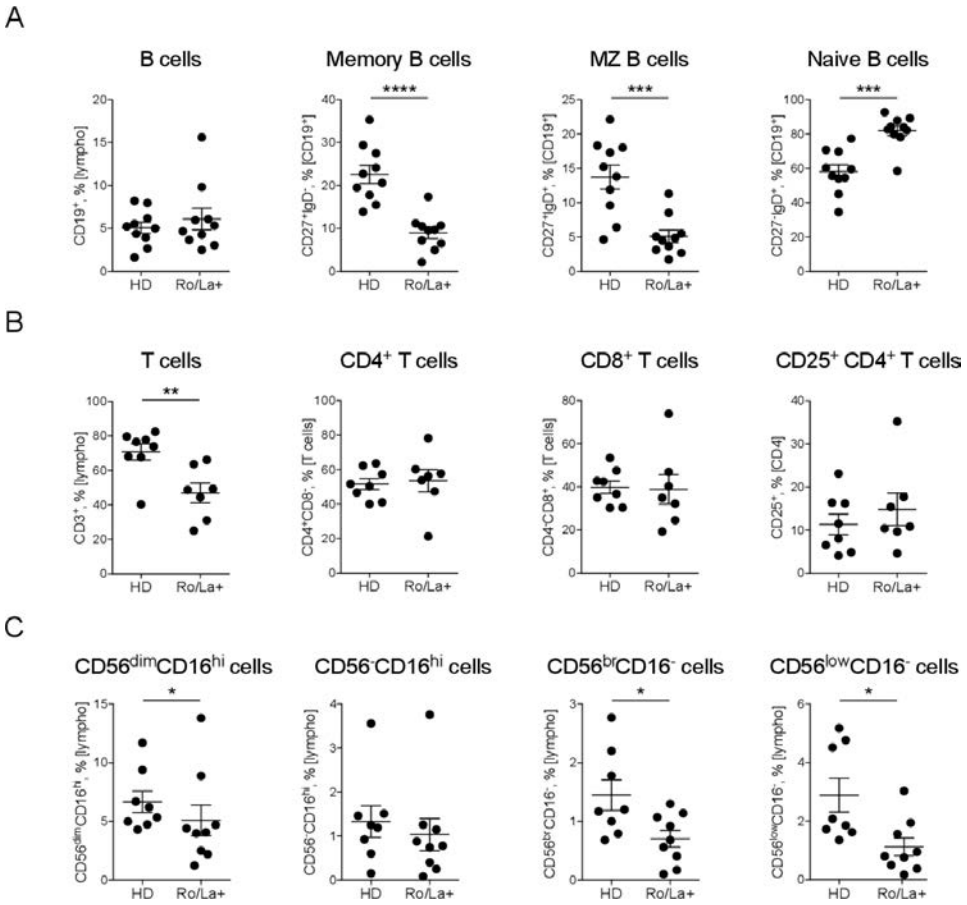

Figure 1 Frequencies of major cell populations in maternal PBMC at delivery. (A) Left-to-right: CD19$^+$ B cells, memory CD27$^+$IgD$^-$, MZ CD27$^+$IgD$^+$ and naive CD27$^-$IgD$^+$ B cells. (B) Left-to-right: CD3$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells and CD25$^+$ CD4$^+$ T cells. (C) Left-to-right: CD56^{dim}CD16^{high}, CD56$^-$CD16^{high}, CD56^{br}CD16$^-$ and CD56^{low}CD16$^-$ NK cells. Mann-Whitney U test; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars represent mean with SEM. Br, bright; HD, healthy donors; MZ, marginal zone; NK, natural killer; Ro/La+, anti-Ro/La-positive mothers.

extracted with chloroform. RNA expression was determined by the Human Transcriptome Array 2.0 chip (ThermoFisher Scientific) at the Bioinformatics and Expression Analysis core facility, Karolinska Institutet, Sweden.

Microarray data analysis

Differentially expressed genes (DEGs) were identified in log transformed data using the Comparative Marker Selection (CMS) module in GenePattern (Broad Institute; genepattern.org) and filtered for FDR <0.05 if not otherwise stated. For gene set enrichment analysis, DAVID (https://david.ncifcrf.gov/) and Enrichr (http://amp.pharm.mssm.edu/Enrichr/) tools were used. Type I and II IFN signatures, as well as natural killer (NK) cell modules were identified as suggested by Chiche *et al*.[22] Additional NK/Cytotoxicity gene lists were from WebSLE (websle.com).[23] The hypergeometric test (HGT) was run with R, *phyper* function. Euler diagrams were built with the R *eulerr* package. Morpheus (https://software.broadinstitute.org/morpheus/) was used for heatmap building and hierarchical clustering.

Elisa

IFNγ was measured using a high sensitivity IFNγ ELISA kit (ThermoFisher Scientific). Optimal ELISA conditions were determined using titrations, and samples were tested in duplicate at a dilution of 1:2.

Cardiomyocyte preparation, culture and stimulation

Fetal (9–12 weeks of gestation) cardiomyocytes were obtained from elective termination of pregnancies in healthy subjects and single cell suspensions prepared by stirring cut pieces of heart tissue with a magnetic bar at 37°C for 40 min in mincing solution (HBSS, 1 mM taurine, 1 mM BDM, 0.1 mg/mL BSA, 3800U collagenase-II, 0.0 4 uM EGTA). The single cell suspension was filtered through a cell strainer and washed with HBSS. Cardiomyocytes were plated in 12 or 24-well plates at 5×10^5 cells/mL in Claycomb medium with 10% FBS and 0.1 mM Norepinephrine for 24 hours at 37°C. Then, cells were supplemented or not with 1000 U/mL IFNα or 1000 U/mL IFNβ (PBL Assay Science). After 6 hours, cells were washed and harvested in Trizol (ThermoFisher Scientific) or RLT-ME buffer (Qiagen) and stored at −80.

Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad), except for microarray data. Differences in continuous variables between groups were analysed by the Mann-Whitney U test, Kruskal-Wallis or one-way analysis of variance with Dunn's correction for multiple comparison. A p<0.05 was considered significant.

RESULTS

Characterisation of peripheral cell populations in anti-Ro/La positive pregnancy

To define frequencies of immune cell populations in pregnancies at risk of CHB, we first performed flow cytometry of

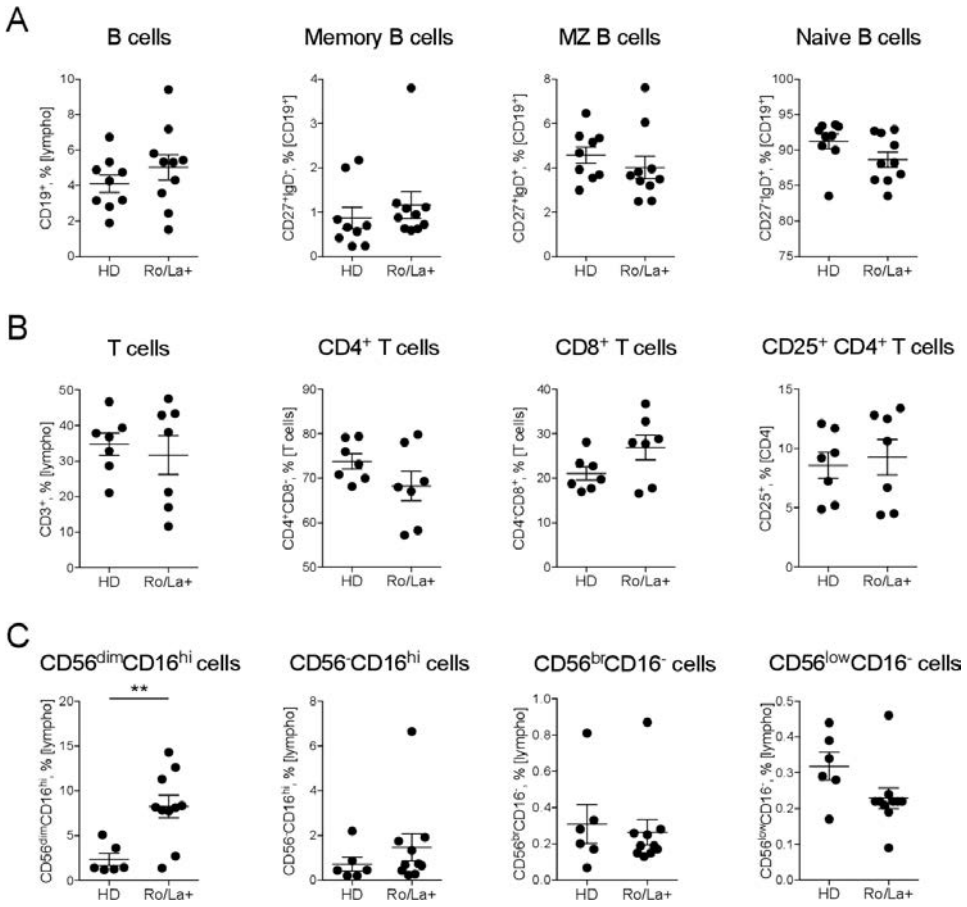

Figure 2 Frequencies of major cell populations in neonatal CBMC at birth. (A) Left-to-right: CD19$^+$ B cells, memory CD27$^+$IgD$^-$, MZ CD27$^+$IgD$^+$ and naive CD27$^-$IgD$^+$ B cells. (B) Left-to-right: CD3$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells and CD25$^+$ CD4$^+$ T cells. (C) Left-to-right: CD56^{dim}CD16^{high}, CD56$^-$CD16^{high}, CD56^{br}CD16$^-$ and CD56^{low}CD16$^-$ NK cells. Mann-Whitney U test; **p<0.01. Error bars represent mean with SEM. Br, bright; HD, healthy donors; MZ, marginal zone; NK, natural killer; Ro/La+, anti-Ro/La-positive mothers.

PBMC/CBMC from anti-Ro/La-exposed maternal-neonatal pairs in pregnancies during which the mother had not received immunomodulatory treatment. In anti-Ro/La+ mothers, we observed higher frequencies of naïve and lower frequencies of marginal zone and memory B cells than in healthy donors (figure 1A), as previously described in non-pregnant women with SS.[24 25] The anti-Ro/La+mothers also had a relative T-cell lymphopenia, without skewing of the CD4/CD8 T-cell ratio, as well as reduced NK-cell subset frequencies (figure 1B,C). Unlike their mothers, the B and T-cell frequencies were not affected in anti-Ro/La exposed newborns (figure 2A,B). Surprisingly, the Ro/La autoantibody-exposed neonates, however, presented with higher frequencies of CD56^{dim}CD16^{high} NK cells (figure 2C). This increased frequency was most pronounced in newborns of mothers with SS, although present also in newborns of mothers with other diagnoses (online supplemental figure S5). To confirm whether the NK-cell expansion is related to the Ro/La autoantibodies or maternal autoimmune disease in general, we phenotyped NK cells from neonates of Ro/La antibody positive and negative mothers with systemic autoimmunity. CD56$^-$CD16^{high}, and to a lesser extent CD56^{dim}CD16^{high}, NK-cell frequencies were increased in Ro/La antibody-exposed neonates compared with neonates born by mothers with rheumatic disease but no Ro/La autoantibodies (online supplemental figure S6). The frequency of NKT cells was around 0.2% of the lymphocytes and did not differ between Ro/La-antibody exposed and non-exposed newborns (online supplemental figure S6).

We next assessed the effect of treatment on immune cell frequencies. In CBMC from newborns with CHB and exposed to long-term high-dose steroids (online supplemental table S1), the B-, T- and NK-cell subset frequencies were decreased compared with controls (online supplemental figure S7). Maternal treatment in terms of hydroxychloroquine (HCQ), alone or in combination with azathioprine and/or prednisone during pregnancy, was also associated with normalisation of CD56^{dim}CD16^{high} and CD56$^-$CD16^{high} NK cell frequencies in the neonates (online supplemental figure S8), although the maternal B-, T- and NK-cell subset frequencies did not differ compared with those of non-treated mothers (online supplemental figure S9).

Gene expression supports NK-cell expansion in CBMC from anti-Ro/La exposed neonates

To substantiate the NK-cell expansion revealed by flow cytometry, we analysed RNA expression in CBMC from anti-Ro/La exposed neonates by microarrays. The common core markers of NK cells; CD56, CD16 and NKp46, were expressed at higher levels in anti-Ro/La exposed neonates (figure 3A). Next, DEG were identified in log transformed data using the CMS module in the GenePattern set with a two-sided t-test and without permutations. DEGs were filtered for FDR <0.05, and the resulting list was used for enrichment analysis with the Enrichr tool, Cell Types category. The list of DEG yielded a significant overlap with the NK Cells term from the ARCHS4 library and CD56$^+$

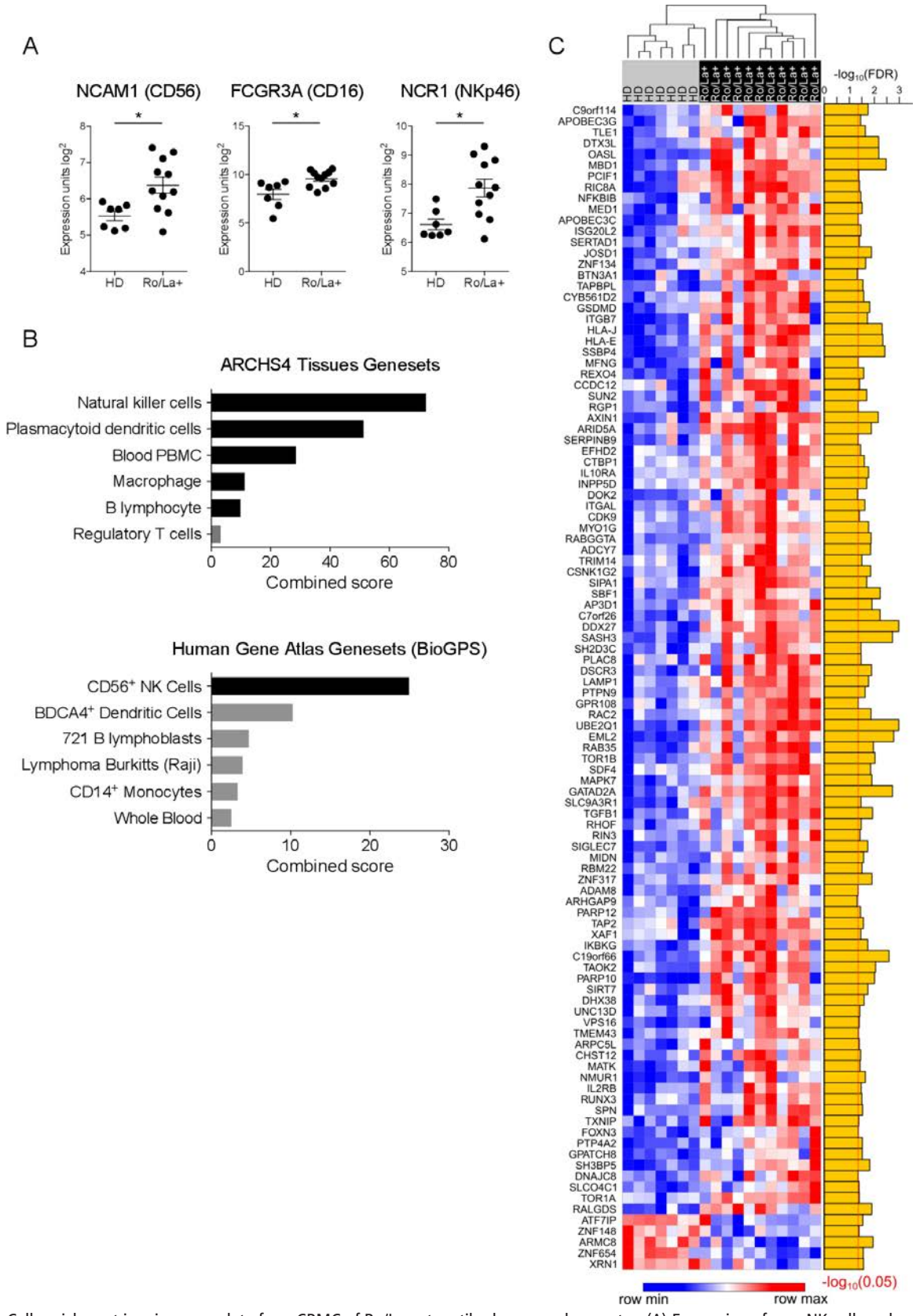

Figure 3 Cell enrichment in microarray data from CBMC of Ro/La autoantibody-exposed neonates. (A) Expression of core NK cell markers extracted from microarray data. Mann-Whitney U test. *p<0.05. Error bars represent mean with SEM. (B) Cell enrichment analysis performed using the Enrichr tool, cell types category. terms identified by running the list of DEG (n=1146 at FDR<0.05) From anti-Ro/La exposed neonates through the ARCHS4 library (upper panel) and human gene Atlas/BioGPS library (lower panel). The combined score is a resultant of three enrichment analysis methods suggested by Chen *et al*.[46] Bars of significantly overlapping terms are depicted in black, non-significant in grey. (C) Expression data for DEG (FDR <0.05) overlapping with entries from the BioGPS "CD56 +NK cell" term; unsupervised clustering by the Morpheus tool. CBMC, cord blood mononuclear cells; DEG, differentially expressed genes; HD, healthy donors; NK, natural killer; Ro/La+, anti-Ro/La exposed newborns.

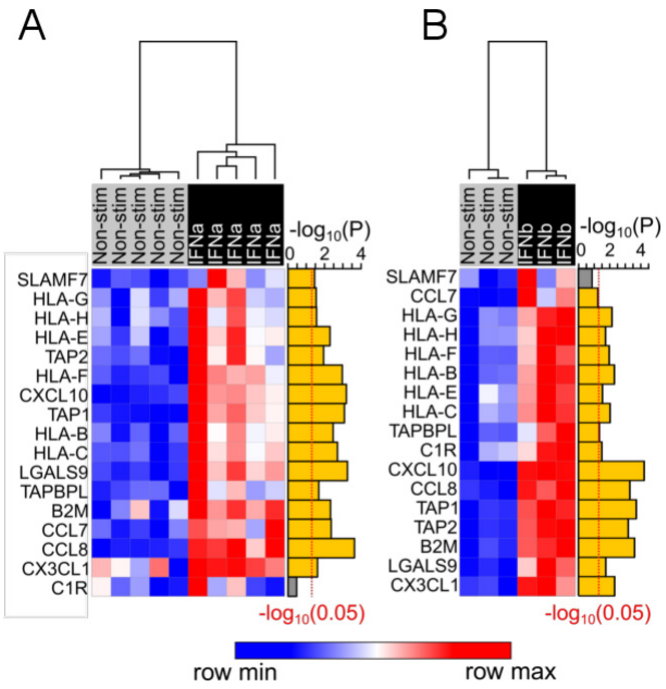

Figure 4 Gene expression in fetal cardiomyocytes exposed to type 1 IFN. Primary fetal cardiomyocytes were cultured with or without type I IFN for 6 hours before harvesting and gene expression analysed by microarrays. David and Enrichr tools were used to identify curated gene sets with significant overlap with the DEG between unstimulated and stimulated conditions. Expression of cytotoxic cell ligands and chemoattractants manually selected from the identified gene sets is illustrated. (A) Stimulation with 1000 U/mL IFNα. data represent five independent experiments. (B) Stimulation with 1000 U/mL IFNβ. Data represent three independent experiments. The DEG cut-offs for IFNα and IFNβ experiments were set to p<0.01 and p<0.05, respectively. unsupervised clustering by the Morpheus tool. DEG, differentially expressed genes; IFNα, interferon-α.

NK-cell term in the BioGPS database from the Human Gene Atlas library (figure 3B), well as the 3.6, but interestingly not the 4.15 cytotoxicity/NK-cell module in webSLE.com, a database generated with data derived from children with lupus[23] (online supplemental figure S10). Unsupervised clustering is visualised for the expression data of DEG overlapping with genes from the BioGPS CD56$^+$ NK-cell term (figure 3C). These results corroborate the observation that NK cells are enriched in CBMC of anti-Ro/La exposed neonates.

IFN-stimulation of fetal cardiomyocytes upregulates NK-cell ligands

Type I IFN and increased expression of type I IFN regulated genes has been demonstrated in anti-Ro/La exposed neonates.[14 17] Type I IFNs are known to upregulate NK-cell ligands such as MHC class I molecules, and interestingly, there is a genetic association of HLA-C with CHB.[26–28] Interactions between NK-cell receptors and their ligands may therefore contribute to the pathogenesis of the cardiac disease. We hypothesised that fetal cardiomyocytes upregulate cytotoxic cell attractants, ligands and activating molecules in the presence of type I IFN. To test this hypothesis, we stimulated primary human fetal cardiomyocytes with IFNα or IFNβ and subjected them to microarray assays. DEGs were run through the DAVID and Enrichr tools to identify curated gene sets with significant overlap. This analysis identified several cytotoxic activity-related sets. From these gene sets, expression data on 17 known cytotoxic cell ligands, chemokine and cytokine genes are illustrated as heatmaps (figure 4A,B). These genes were upregulated on type I IFN stimulation, suggesting that fetal cardiomyocytes can potentially attract NK cells and provide an activating environment as part of the pathogenesis in CHB.

Ifnγ and type II IFN signatures in anti-Ro/La exposed neonates

IFNγ is the prototype cytokine produced by NK cells,[29] and to further evaluate the functionality of NK cells in anti-Ro/La+exposed newborns, we performed intracellular IFNγ staining. Interestingly, in anti-Ro/La+exposed newborns, the frequency of IFNγ-positive cells was increased in all NK-cell subsets, with CD56^{low}CD16$^-$ NK cells being the main producers (figure 5A; gating strategy in online supplemental figure S11). The frequency of IFNγ-positive cells was also increased in CD8$^+$ and CD4$^+$ T cells from Ro/La exposed newborns (figure 5B). The frequency of cells expressing other effector cytotoxic molecules such as granzyme B and perforin-1 showed a tendency for being higher in the anti-Ro/La exposed newborns, but did not reach significance in the few samples available (online supplemental figures S11A-D). We also measured the IFNγ in plasma by ELISA, and detected elevated IFNγ in some of the anti-Ro/La exposed newborns (figure 5C).

We next analysed RNA expression of CBMC to understand if type II IFN activation is also a feature of anti-Ro/La exposed neonates. For that purpose, we used the modular analysis suggested by Chaussabel *et al*,[30] and analysed three of the defined modules: M1.2 (type I IFN stimulated gene signature), M3.4 (combined type I and type II IFN stimulated gene signature), and M5.12 (IFNγ gene signature). DEG in neonatal CBMC were filtered for FDR <0.05, and the gene lists were used for HGT. Unsupervised clustering of the expression data for the genes included in the modules is depicted in figure 5D–F. Genes from all three modules were significantly over-represented among our microarray hits (figure 5G).

We further substantiated the expression of IFNγ-regulated genes at the protein level by flow-cytometric analysis of HLA-DR on circulating monocytes. In this analysis we noted a significantly higher HLA-DR expression in the Ro/La autoantibody-exposed newborns (figure 5H). In all, these data suggest that in addition to the previously described type I IFNs, type II IFN is present in anti-Ro/La exposed neonates, which could lead to upregulation of IFNγ-stimulated genes such as HLA-DR.

DISCUSSION

The maternal and neonatal serology of neonatal lupus and CHB is well documented, but immune cell subsets in the mothers and newborns in anti-Ro/La risk pregnancies have not been delineated. Here, we profiled circulating immune cells in anti-Ro/La+mothers and their newborns as well as healthy donor mother-neonatal pairs. In Ro/La+mothers, we observed changes in B-cell subsets, relative T-cell lymphopenia and a lower NK-cell frequency in concordance with previous reports in patients with SS.[25 31 32] Notably, in the Ro/La autoantibody-exposed newborns, frequencies of CD56^{dim} CD16^{high} NK cells were increased. NK cells appear during the sixth week of fetal life, actively expand during the second trimester of pregnancy and are capable of cytotoxic activity, although limited.[33] Type I IFN expands and activates NK cells,[34] thus influencing cytotoxicity and NK-cell mediated immune responses. We recently demonstrated an increase of IFNα levels in Ro/La-antibody exposed neonates,[17] which may explain the expansion of NK cells in the newborns. The contrasting observation of low NK cell frequencies in the

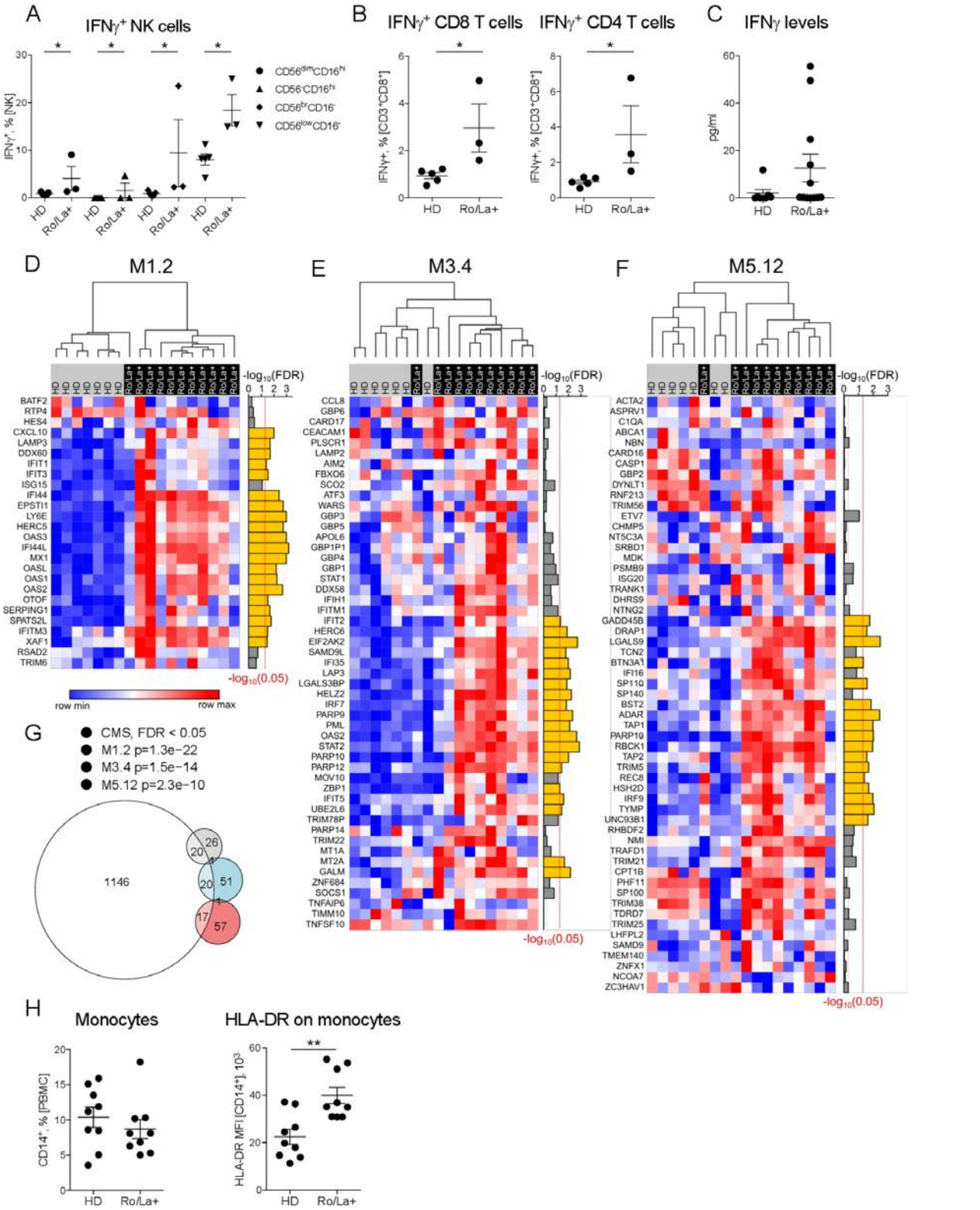

Figure 5 IFNγ and type I and type II IFN signatures in CBMC of anti-Ro/La exposed newborns. (A) Frequencies of IFNγ positive NK cells in cord blood defined by intracellular flow cytometry. (B) frequencies of IFNγ positive CD8$^+$ (left panel) and CD4$^+$ (right panel) T cells in cord blood measured by intracellular flow cytometry. (C) IFNγ in plasma measured by ELISA. (D) Expression data for genes from module M1.2,[22] mainly representing type I IFN signature genes. (E) Expression data for genes from module M3.4, mainly representing effects of both type I and type II interferons.[22] (F) Expression data for genes from module M5.12, mainly representing the influence of IFNγ.[22] Unsupervised clustering by the Morpheus tool. (G) Visualisation of numbers of overlapping genes between DEG in anti-Ro/La exposed neonates (n=1146 at FDR <0.05) and each of the modules. Hypergeometric test: DEG vs M1.2, p=1.3×10^{-22}; DEG vs M3.4, p=1.5×10^{-14}; DEG vs M5.12, p=2.2×10^{-10}. (H) Frequencies of monocytes in CBMC (left panel) and expression of HLA-DR on monocytes (right panel) by flow cytometry. Mann-Whitney U test; *p<0.05, **p<0.01. Error bars represent mean with SEM. CBMC, cord blood mononuclear cells; CMS, comparative marker selection; DEG, differentially expressed genes; HD, healthy donors; IFNγ, interferon-γ; Ro/La+, anti-Ro/La exposed subjects; MFI, median fluorescence intensity; NK, natural killer.

mothers is well documented and may relate to the differing effects of long-standing chronic IFN-exposure.[35]

CD56^{dim}CD16^{high} NK cells are characterised by their cytotoxic potential. Although the role of NK or other cytotoxic cells in the CHB pathogenesis is unclear, it is tempting to speculate that their antibody-dependent cellular cytotoxicity (ADCC) might contribute to targeted cardiomyocyte damage. During ADCC, CD16 (FcγRIII) on cytotoxic cells binds the Fc portion of IgG bound to specific epitopes on target cells to activate cell-mediated cytotoxicity.[36] CD16 primarily interacts with IgG1 and IgG3, which are the main Ro/La IgG subclasses found in the fetus.[37] In CHB, Ro/La autoantibodies bound to fetal cardiomyocytes[38] could be recognised by activated NK cells and induce the polarised release of granzymes and perforin-1 leading to cardiomyocyte death, fibrotic replacement and calcification. In favour of this scenario, an RNA-sequencing based study recent study reported NKT cells with low CD3 expression in a heart of a fetus with CHB.[39] Hence, disease specificity could be explained by the connection between Ro/La autoantibodies, type I IFN and cytotoxic cells.

Type I IFNs can regulate some of the key molecules involved in cytotoxicity. Primary fetal cardiomyocytes stimulated with type I IFN indeed upregulated several chemoattractants for NK cells, as well as NK-cell activating receptors, including MHC class I molecules. Interestingly, HLA-Cw has been consistently associated with CHB.[26–28] A robust protective association with the MHC class I allele Cw*06 has been demonstrated in the European population in family-based studies.[27] HLA-C is characterised by lower levels of cell surface expression and a more restricted peptide binding compared with the other MHC class I molecules.[40] The protective effect of this allele is consistent with NK cell and/or other cytotoxic cell activation being an important part of CHB pathogenesis. Interestingly, a pathological role for fetal NK cells has been demonstrated for other conditions, and for example, mediate the neonatal passively transferred autoimmune ovarian disease.[41] The condition was abrogated when fetal NK cells were replaced by maternal NK cells, demonstrating that major differences between maternal and fetal NK cell populations may occur even in the same pregnancy.

NK cells, and specifically, CD56^{bright}CD16^{-} cells, are known as high producers of IFNγ.[42] In our study, we observed increased IFNγ levels in several Ro/La autoantibody exposed neonates, further substantiated by an mRNA-based IFNγ signature and IFNγ production at the steady state in CD16^{-} NK cells. The frequencies of CD4+ and CD8+T cells with intracellular IFNγ were also increased in Ro/La autoantibody exposed neonates, suggesting that their inflammatory milieu is prone to induce IFNγ production. MHC class II was upregulated on monocytes of Ro/La autoantibody-exposed neonates further corroborating a role of IFNγ.

In Ro/La autoantibody-exposed neonates of whom the mother was treated with HCQ with/without azathioprine and prednisolone, the NK-cell frequency was significantly lower. Similarly, B-cell, T-cell and NK-cell subsets frequencies were decreased in CHB newborns from mothers with long-term high-dose steroids treatment. This decrease in blood circulation could result from redistribution of the immune cell population by migration to sites of inflammation, or, perhaps more likely, from the strong effect of steroids on immune cells.[43]

IFNs have been shown to drive a number of antenatal conditions. In congenital Zika infection, most of the antiviral functions in the fetus are carried by IFNλ, while effects of type I IFNs turned out to be detrimental.[44] The Acardi-Goutière syndrome, an umbrella term for several monogenic type I interferonopathies, is another disorder illustrating the effects of type I IFNs in the fetus. Mutations in several genes leading to type I IFN production have been identified as disease-causing, and manifesting as intracranial calcifications, vasculitis and skin lesions.[45] Further, *Trex1* (*DNase III*) knock-out mice develop cardiomyopathy, and present an IFN-dependent phenotype closely mimicking CHB.[45]

Limitations of our study are related to the rareness of the condition. Small sample size, heterogeneity of the study population in terms of the maternal diagnosis and immunomodulatory treatment are all evident limitations of the current study.

In summary, we demonstrate an increased frequency of CD56^{dim} CD16^{high} NK cells as well as type I and II IFN activation in anti-Ro/La exposed neonates. We further demonstrate the increased frequency of IFNγ-producing immune-cells in anti-Ro/La exposed neonates and an upregulation of ligands activating NK cells in fetal cardiac cells after type I IFN exposure. These novel observations indicate that, in the context of type I IFN stimulation, NK cell-related effector mechanisms may contribute to the pathogenesis of CHB.

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Contributors MI, MH, KC and MW-H conceived the study. MH, JT, SB, KB, ES-E, KG-D, S-ES recruited patients and provided biological material. MI, GET, MH, VO, AO, LM, KC and MW-H performed experiments and/or analysed data. MI, MH, GET generated figures and tables and MI wrote the first manuscript draft with input from MW-H, KC, GET and MH. All authors (MI, GET, MH, VO, LM, SB, AO, JT, KB, ES-E, KG-D, S-ES, KC, MW-H) participated in the editing of the manuscript until its final version.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Not required.

Ethics approval The study was approved by the Regional Ethics Committee in Stockholm (Dnr 2013/2201-31).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information.

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CLINICAL SCIENCE

Transcutaneous auricular vagus nerve stimulation reduces pain and fatigue in patients with systemic lupus erythematosus: a randomised, double-blind, sham-controlled pilot trial

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ABSTRACT

Objectives Musculoskeletal pain and fatigue are common features in systemic lupus erythematosus (SLE). The cholinergic anti-inflammatory pathway is a physiological mechanism diminishing inflammation, engaged by stimulating the vagus nerve. We evaluated the effects of non-invasive vagus nerve stimulation in patients with SLE and with musculoskeletal pain.

Methods 18 patients with SLE and with musculoskeletal pain ≥4 on a 10 cm Visual Analogue Scale were randomised (2:1) in this double-blind study to receive transcutaneous auricular vagus nerve stimulation (taVNS) or sham stimulation (SS) for 4 consecutive days. Evaluations at baseline, day 5 and day 12 included patient assessments of pain, disease activity (PtGA) and fatigue. Tender and swollen joint counts and the Physician Global Assessment (PGA) were completed by a physician blinded to the patient's therapy. Potential biomarkers were evaluated.

Results taVNS and SS were well tolerated. Subjects receiving taVNS had a significant decrease in pain and fatigue compared with SS and were more likely (OR=25, p=0.02) to experience a clinically significant reduction in pain. PtGA, joint counts and PGA also improved. Pain reduction and improvement of fatigue correlated with the cumulative current received. In general, responses were maintained through day 12. Plasma levels of substance P were significantly reduced at day 5 compared with baseline following taVNS but other neuropeptides, serum and whole blood-stimulated inflammatory mediators, and kynurenine metabolites showed no significant change at days 5 or 12 compared with baseline.

Conclusion taVNS resulted in significantly reduced pain, fatigue and joint scores in SLE. Additional studies evaluating this intervention and its mechanisms are warranted.

Key messages

What is already known about this subject?

- Pain and fatigue are common symptoms voiced by patients with systemiclupus erythematosus (SLE).
- The inflammatory reflex is a physiological mechanism diminishing inflammation. The inflammatory reflex may be engaged by stimulation of the vagus nerve.
- Vagus nerve stimulation with a surgically implanted device has shown clinical benefit in uncontrolled studies in rheumatoid arthritis and inflammatory bowel disease.

What does this study add?

- Non-invasive stimulation of the vagus nerve in patients with SLE in a double-blind sham-controlled study resulted in a significant reduction of both pain and fatigue.

How might this impact on clinical practice or future developments?

- Transcutaneous auricular vagus nerve stimulation, a non-pharmacological, non-invasive, safe approach to alleviate pain and fatigue in SLE would fulfil an unmet clinical need.

INTRODUCTION

Musculoskeletal pain and fatigue are common symptoms in systemic lupus erythematosus (SLE), affecting up to 95% of patients and contributing to a reduced quality of life. Safe and efficacious treatment remains an unmet need. The inflammatory reflex is a physiological mechanism that attenuates the innate inflammatory response. Stimulation of the vagus nerve results in the reduction of inflammatory mediators and beneficial effects have been demonstrated in multiple animal models of disease.[1–13] Vagus nerve stimulation (VNS) administered by a surgically implanted stimulator has been shown to be efficacious in uncontrolled studies and safe in human inflammatory diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease.[14 15] As the auricular branch of the vagus nerve innervates the cymba concha in the outer ear, the inflammatory reflex can be engaged non-invasively by stimulating this structure. Our objective was to obtain preliminary data evaluating the efficacy and safety of transcutaneous auricular vagus nerve stimulation (taVNS) in SLE and to explore the biological effects of this intervention.

PATIENTS AND METHODS

This pilot study was a randomised, double-blind, sham-controlled trial (ClinicalTrials.gov Identifier NCT02822989) of taVNS in subjects with SLE. Adult SLE subjects meeting 1997 Revised American College of Rheumatology (ACR) or Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for SLE with self-reported pain of at least 4 on a 10 cm anchored Visual Analogue Scale (VAS) (corresponding to a high level of pain previously described in patients with SLE[16]) and inflammatory musculoskeletal symptoms (British Isles Lupus Assessment Group (BILAG) C or greater on the BILAG-2004 musculoskeletal domain) were recruited. Stable doses of Disease modifying Anti-Rheumatic Drugs (DMARDs), biological therapy and/or prednisone ≤10 mg/day were permitted, defined as no change of dose within 28 days prior to baseline. Pertinent exclusion criteria included a diagnosis of fibromyalgia, tobacco use and use of anticholinergic medication.

Eighteen subjects were randomised (2:1) using the Biostatistics Randomization Management System, a web-based HIPAA compliant software package to receive 5 min of taVNS or sham stimulation (SS) for 4 consecutive days at the Feinstein Institutes for Medical Research. For taVNS, a spring-loaded clip consisting of opposing conductive silicone electrodes was placed around the left ear with one electrode on the concha and the other behind the ear. Stimulation pulses (30 Hz frequency, 300 μs pulse width) were generated by a commercial transcutaneous electrical nerve stimulation (TENS) unit (Roscoe TENS 7000), and the amplitude was increased to the maximum amount tolerated by the subject without pain. All subjects were told that they may or may not feel any sensation from the stimulation. For SS, the battery was removed from the TENS unit, the electrode clip placed on the ear lobe (a location without vagus nerve innervation) and the dial on the TENS unit advanced. After each advance of the dial, the subject was asked if they felt anything. After three advances, subjects receiving SS were informed that the 'target stimulation had been reached'. SS was then delivered for 5 min. To evaluate the effect(s) and durability of taVNS, subjects received comprehensive assessments at baseline, day 5 and day 12 by a physician blinded to the subject's treatment; all patient assessments were performed by an investigator who was not present during the stimulation. To provide additional assurance that the assessing physician would not inadvertently uncover a subject's treatment allocation, all participants were reminded not to mention any aspects of the stimulation procedures to the evaluating physician and Case Report Forms (CRFs) containing data relevant to the stimulation were maintained in a separate location.

The primary objective was the effect of taVNS on musculoskeletal pain. Safety and tolerability were also assessed throughout the study. Secondary objectives included determination of effects of taVNS on fatigue, tender and swollen joint counts and patient and physician assessments (PtGA and PGA) of disease activity at days 5 and 12. Patients were additionally asked if they felt better, worse or the same. Mechanistic objectives aimed to explore potential mechanisms known to be involved in pain and inflammation that might be affected by VNS.

In this pilot study, per protocol, subjects not receiving four consecutive stimulations were replaced. This study was approved by the Northwell Health Institutional Review Board (HS16-0171) and informed consent was obtained from all study participants prior to the initiation of any study procedures. Patients were not directly involved in the design, recruitment or conduct of the study.

Table 1 Baseline characteristics of 18 subjects

	taVNS (n=12)	SS (n=6)
Female, n (%)	12 (100)	6 (100)
Age (years), mean (SD)	45.7 (11.7)	54.2 (15.3)
Race, n (%)		
Black/African American	4 (33)	3 (50)
White	7 (58)	3 (50)
Other	1 (9)	–
Baseline pain on 10 cm VAS, mean (SD)	6.7 (1.0)	5.6 (1.5)
Baseline fatigue on FACIT-F,* mean (SD)	23.0 (9.1)	15.8 (5.4)
Tender joints, mean (SD)	7 (8.7)	13.3 (8.9)
Swollen joints, mean (SD)	2 (2.2)	4.8 (4.1)
Baseline musculoskeletal BILAG C	3 (25%)	1 (16.7 %)
Baseline musculoskeletal BILAG B	9 (75%)	5 (83.3%)
Baseline SLEDAI-2K, mean (SD)	4.8 (2.1)	5.8 (2.2)

*Higher FACIT-F scores correspond to lower fatigue.
BILAG, British Isles Lupus Assessment Group; FACIT F, Functional Assessment of Chronic Illness Therapy Fatigue Subscale; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index-2K; SS, sham stimulation; taVNS, transcutaneous auricular vagus nerve stimulation; VAS, Visual Analogue Scale.

Laboratory assessments

Laboratory assessments were performed on specimens collected at baseline before taVNS/SS, day 5 and day 12. Serum and plasma were batched and stored at −80°C until analysis. Commercial laboratory assessments including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), C3, C4 and anti-dsDNA were conducted at the Northwell Core Laboratories, Manhasset, New York, USA. HMGB1 ELISA (IBL International GmbH (Hamburg Germany), substance P ELISA (Cayman Chemical, catalogue 583751), neuropeptide Y ELISA (Millipore Sigma, catalogue EZHNPY-25K), calcitonin gene-related peptide (CGRP) EIA kit (Cayman Chemical, catalogue 589101), IL1RA, interleukin (IL)-18 multiarray assay (Meso Scale Discovery, catalogue K15067M-1) were performed according to the manufacturers' instructions. Serum levels of interferon (IFN)α, IL-1, IL-8, IL-10 and tumour necrosis factor (TNF) were determined at the Myriad RBM Central Laboratory using standardised Luminex multianalyte profiling. Assessments of components of the kynurenine pathway of tryptophan degradation (tryptophan, kynurenine and quinolinic acid) were performed by Charles River Laboratories (San Francisco, California, USA) using high-performance liquid chromatography with tandem mass spectrometry.

Cytokine release by unstimulated whole blood or whole blood stimulated by TLR 4, 7 and 9 agonists was determined using the TruCulture Myriad self-contained system. Whole blood was collected into null TruCulture tubes or TruCulture tubes containing 0.1 µg/mL LPS, 1 µg/mL gardiquimod or 30 µg/mL CpG/ODN2216. After incubation for 24 hours at 37°C, the supernatant was removed and stored at −80°C. Two panels of stimulated inflammatory mediators, HumanCytokine MAP A and MAP B, were measured in the supernatant by Myriad RBM Laboratories using a bead-based multiplex immunoassay.

Statistical analyses

The sample size of 18 subjects was based primarily on feasibility as there was no previous experience of taVNS in SLE.

The Wilcoxon Rank Sum test was used to compare the change in endpoints from baseline to day 5 and from baseline to day 12 in subjects receiving taVNS or SS and the Spearman Rank Order correlation was used to assess the strength of potential

Table 2 Change from baseline of trial endpoints

	Day 5–Day 1			Day 12–Day 1		
T	taVNS median (IQR)	SS median (IQR)	P value	taVNS median (IQR)	SS median (IQR)	P value
Δ VAS pain (0–10 mm)	−5.00 (−5.80 to −3.10)	0.10 (−10.0 to 1.0)	0.049	−5.35 (−5.80 to −1.45)	0.15 (−0.60 to 0.70)	0.079
Δ FACIT-F†	11.00 (4.50 to 16.00)	0.00 (−2.00 to 1.00)	0.003	12.00 (5.50 to 18.25)	−2.00 (−3.00 to 1.00)	0.003
% Tender joint reduction	100.0 (100.0 to 100.0)	5.27 (-11.1,80.0)	0.005	98.49 (50.0 to 100.0)	10.00 (0.00 to 34.61)	0.050
% Swollen joint reduction‡	100.0 (100.0 to 100.0)	9.09 (−8.33 to 57.15)	0.019	100.0 (80.0 to 100.0)	14.29 (−100.0 to 59.09)	0.056
Δ PtGA (0–100 mm)	−22.50 (−46.50 to −2.50)	4.00 (−2.00 to 9.00)	0.125	−18.50 (−64.00 to −2.00)	−0.52 (−16.00 to 9.00)	0.301
Δ PGA (0–3)	−0.51 (−0.99 to −0.30)	0.04 (−0.06 to 0.12)	0.053	−0.50 (−0.84 to −0.08)	0.03 (0.03 to 0.12)	0.107
Δ CRP (mg/dL)	0.00 (−0.23 to 0.00)	0.05 (-0.10 to 0.15)	0.165	0.00 (−0.20 to 0.00)	-0.05 (−0.23 to 0.08)	1.000
Δ Serum cytokine						
IFNα	0.00 (0.00 to 0.00)	0.12 (−0.12 to 1.10)	0.871	0.00 (−0.01 to 0.00)	0.25 (0.00 to 1.00)	0.080
IL-1β	−0.01 (−0.40 to 0.20)	−0.01 (−0.02 to 0.02)	0.820	−0.01 (−0.04 to 0.00)	−0.04 (−0.05 to 0.04)	0.874
IL-8	−0.21 (−2.25 to −0.01)	0.77 (−1.10 to 1.80)	0.144	−0. 17 (−2.54 to 0.45)	−0. 20 (−2.80 to 0.10)	0.963
IL-10	0.05 (−0.10 to 1.10)	−0.08 (−0.16 to 0.30)	0.600	0.12 (−0.17 to 0.23)	0.04 (−0.10 to 0.40)	0.569
TNF	−0.05 (−0.25 to 0.12)	0.00 (−0.80 to 0.20)	0.943	−0.05 (−0.25,0.12)	−0.05 (−0.20 to 0.20)	0.848
Il-6	−0.39 (−2.89 to −0.03)	0.17 (−3.35 to 2.6)	0.112	−0.17 (−3.13 to 0.67)	−0.20 (−3.35 to 0.25)	0.960
IL1-RA	−0.06 (−1.73 to 0.66)	2.92 (−2.63 to 10.30)	0.603	0.41 (−10.68 to 1.73)	−0.39 (−2.73 to 2.32)	0.741
Il-18	0.71 (−0.23 to 8.20)	−0.21 (−2.47 to 0.92)	0.208	−0.55 (−2.98 to 0.48)	0.10 (−9.37 to 1.21)	0.603
Δ Plasma neuropeptide						
Substance P	−2.76 (−4.79 to 0.94)	0.09 (−5.57 to 4.48)	0.008	4.90 (−2.41 to 8.23)	5.08 (0.24 to 9.98)	0.335
Neuropeptide Y	−0.80 (−7.43 to 2.28)	−2.25 (−4.38 to 5.48)	0.509	0.30 (−6.05 to 5.20)	2.0 (−1.38 to 14.98)	0.242
CGRP	0.0 (−2.0 to 4.7)	0.0 (−1.98 to 2.40)	0.960	−0.7 (−17.15 to 0.83)	0.0 (−1.68 to 0.99)	0.484
Δ Kynurenine pathway						
Kynurenine	−0.02 (−0.35 to 0.18)	0.155 (−0.06 to 0.51)	0.121	−0.17 (−0.56 to 0.18)	−0.27 (−0.01 to 0.65)	0.055
Quinolinic acid	−51.0 (−99.5 to −14.75)	−35 (−173.5 to 28.75)	0.674	−100.0 (−138 to −41.75)	−12.5 (−74.75 to 62.0)	0.121
Kynurenine/tryptophan	0.001 (−0.004 to 0.005)	−0.002 (−0.011 to 0.010)	0.603	−0.003 (−0.007 to 0.008)	0.001 (−0.004 to 0.008)	0.603

*Negative scores correspond to a reduction in the measured endpoint from baseline.
†Higher FACIT-F scores correspond to lower fatigue.
‡Data shown for seven taVNS and five SS subjects with swollen joints at baseline.
CGRP, calcitonin gene-related peptide ; CRP, C-reactive protein; IFNα, interferon alpha; IL, interleukin; IL1-RA, interleukin-1 receptor antagonist; PGA, Physician Global Assessment ; PtGA, Patient Global Assessment of disease activity ; SS, sham stimulation ; taVNS, transcutaneous auricular vagus nerve stimulation; TNF, tumour necrosis factor; VAS, Visual Analogue Scale.

associations between endpoints. A response was defined as a reduction in pain from baseline to day 5 of at least 1.58, as a 1.58 decrease on a 10 cm VAS is considered clinically meaningful.[17] An odd's ratio (OR) was determined to compare the odds of achieving a response between the two treatment groups. The odds of achieving a meaningful change in fatigue measured by the FACIT-F (a 4-point change)[18] was similarly determined. The Spearman rank-order correlation coefficient was used to examine the relationships between pain and fatigue and between cumulative current and changes in pain and fatigue.

RESULTS

Baseline characteristics of the 18 subjects completing the four daily stimulations (taVNS or SS) are shown in table 1, with no significant differences in any parameter between the two arms. All subjects noted musculoskeletal pain with tender and/or swollen joints and fatigue at baseline. One subject was replaced following two stimulations after developing an upper respiratory infection during the influenza season.

After four consecutive stimulations, subjects receiving taVNS achieved a significantly greater reduction in their pain compared with SS, (−5.00 vs 0.10, p=0.049) (table 2 and figure 1). As a clinical response was noted in 10 of 12 (83.3%) taVNS subjects, and 1 of 6 (16.7%) SS subjects, the odds of achieving a meaningful reduction in pain was 25 times greater in subjects receiving taVNS compared with subjects receiving SS (p=0.02). Subjects receiving taVNS also experienced a significant improvement of fatigue compared with subjects receiving to SS (table 2 and figure 2) and the odds of achieving a meaningful reduction in fatigue was 54.6 times greater in subjects receiving taVNS compared with those receiving SS (p=0.014), 10 of 12 taVNS subjects and 0 of 6 SS subjects achieved a meaningful reduction in fatigue. The change of reported pain at day 5 from baseline correlated significantly with the change in fatigue (r=0.69, p=0.013). Moreover, subjects receiving taVNS were more likely to report an overall improvement in their condition on day 5 compared with baseline on a Likert scale. Additionally, a greater numerical decrease from baseline to day 5 of both PtGA and

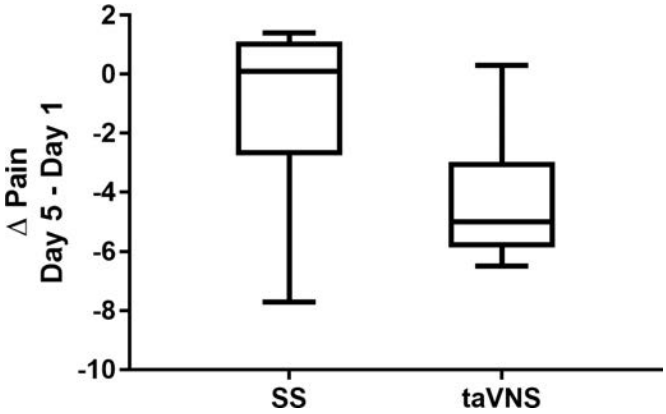

Figure 1 Change in patient-reported pain determined by a 10 cm VAS from baseline to day 5 (day 5–day 1) in subjects receiving SS or taVNS (p<0.05). SS, sham stimulation; taVNS, transcutaneous auricular vagus nerve stimulation; VAS, Visual Analogue Scale.

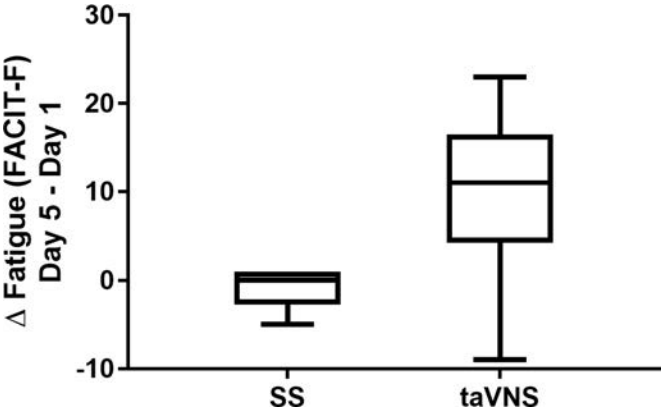

Figure 2 Change in patient-reported fatigue measure by the FACIT-F from baseline to day 5 (day 5–day 1) in subjects receiving SS or taVNS (p=0.003). An increase in the Functional Assessment of Chronic Illness Therapy Fatigue Subscale (FACIT-F) score correlates with less fatigue. SS, sham stimulation; taVNS, transcutaneous auricular vagus nerve stimulation; VAS, Visual Analogue Scale.

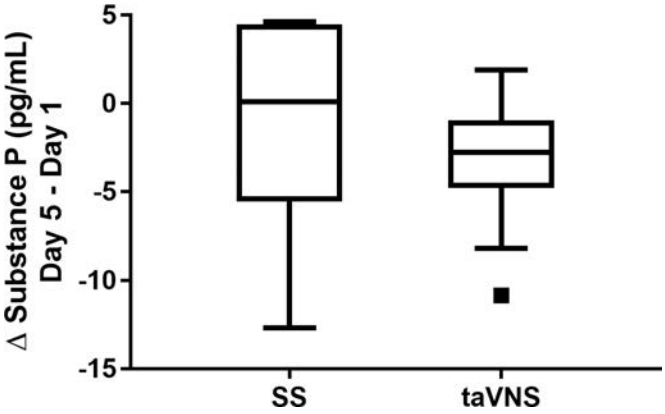

Figure 3 Change in plasma levels of substance P (pg/mL) from baseline to day 5 (day–5 day 1) in subjects receiving SS or taVNS (p=0.008). SS, sham stimulation; taVNS, transcutaneous auricular vagus nerve stimulation.

PGA was observed in subjects receiving taVNS compared with subjects receiving SS (table 2), however these differences were not statistically significant (p=0.125, p=0.053, PtGA, PGA respectively). Both the reduction in pain and the improvement of fatigue significantly correlated with the cumulative current received over 4 days of VNS (r=0.49, p=0.04, pain, r=0.83, p=0.003, fatigue). In general, these improvements continued through day 12.

Tender and swollen joints were present at baseline with no significant differences between the two groups. The median reduction of both tender and swollen joints for subjects receiving taVNS was 100%, compared with a median reduction of 5.3% tender and 9.1% reduction of swollen joints in subjects receiving SS (p=0.005, p=0.019, tender and swollen, respectively) (table 2).

Safety

taVNS was well tolerated with no adverse events attributed to the stimulation. There were no reports of headache, lightheadedness, tinnitus, ear irritation or changes to the external skin of the outer ear.

Mechanistic analyses

Baseline ESR and serum levels of High Mobility Group Box 1 (HGMB1) and CRP were low in this population with no significant changes from baseline to day 5 in either arm. Similarly, there were no significant changes in serum levels of IFNα, IL-1β, IL-6, IL-8, IL-10, IL1RA, IL-18 or TNF (table 2), and no significant changes in levels of C3, C4 or anti-DNA antibody titers. Levels of proinflammatory cytokines after stimulation with TLR 4, 7 or 9 agonists for 24 hours were variable with no differences observed from baseline to day 5 between subjects receiving taVNS or SS (data not shown). However, plasma levels of the neuropeptide substance P, were significantly lower in subjects receiving taVNS compared with those receiving SS at day 5 than at baseline p=0.008 (table 2, figure 3). Given the significant change observed between groups in levels of substance P, we evaluated two additional neuropeptides, neuropeptide Y and CGRP, but detected no significant difference between groups in plasma levels of either of these neuropeptides from baseline to day 5 (table 2). Lastly, the examination of changes from baseline of kynurenine and quinolinic acid levels and the kynurenine/tryptophan ratio, which have previously been shown to associate with severe fatigue in SLE,[19] did not correlate with a reduction in fatigue and showed no significant change at day 5 or 12 from baseline between subjects receiving taVNS compared with SS (table 2).

DISCUSSION

VNS is an approved treatment for refractory epilepsy, depression and migraine headaches. As stimulation of the vagus nerve engages the cholinergic anti-inflammatory pathway, this modality offers a promising, non-toxic intervention for the treatment of inflammatory disease. Clinical efficacy of VNS has been suggested in uncontrolled studies in other inflammatory diseases. In one small open-label pilot study, five of seven biologically naïve patients with active Crohn's disease received daily VNS administered by a surgically implanted device. Significant improvement was demonstrated on the Crohn's Disease Activity Index (CDAI). Moreover, CDAI remission was achieved in four of five patients at 6 months with only one patient requiring ongoing immunosuppressive medication. Inflammatory markers, that is, serum CRP and faecal calprotectin, were also significantly diminished.[15] A second pilot study included eight subjects with active Crohn's disease who were refractive to biological treatment and given 16 weeks of daily VNS delivered by a surgically implanted device.[20] At week 16, CDAI scores were significantly reduced meeting a predefined target reduction of 70 in six of eight patients; three patients achieved CDAI remission. Inflammatory markers (CRP and faecal calprotectin) were reduced in patients who exhibited clinical response.

An open-label study of VNS was completed in 18 patients with RA.[14] Subjects (eight non-responsive to methotrexate and 10 non-responsive to biologics) received daily stimulation delivered by an implanted device. At day 42, the significant improvement of Disease Activity Score (DAS) disease activity was observed in both cohorts and a EULAR response was achieved in 7 of 8 and 6 of 10 patients in each group. TNF secretion by ex vivo LPS-stimulated whole blood was attenuated by daily VNS and circulating levels of IL-6 were significantly reduced in those patients with a EULAR response. The treatment was well tolerated and the observed adverse events were those known to associate with an implanted device (transient hoarseness and events related to the actual surgery). Importantly, no infections were observed during this study. A study of VNS in treatment-resistant RA is ongoing in US centres (ClinicalTrials.gov Identifier: NCT03437473).

More recently, the effect of VNS on fatigue in patients with Sjogren's disease was evaluated using the gammaCore device.[21] This device stimulates the vagus nerve transcutaneously at the neck. In this uncontrolled 26-day open-label study, 15 patients received stimulation two times per day. Patients reported a significant reduction of fatigue. Moreover, LPS-stimulated production of IL-6, IL-1β, IP-10, MIP1α, IL-1β, TNF-α, IL-6 and IP-10, was also significantly reduced.

We now show that a short course of taVNS administered once daily for 4 consecutive days via non-invasive external electrodes to the auricular branch of the vagus nerve results in a significant reduction of pain and fatigue in patients with SLE. Our study population included individuals with significant pain and exemplifies the unmet need for adequate control of pain and fatigue in SLE. Importantly, this was a double-blind, sham-controlled study and neither the subject nor assessor was aware of a subject's intervention. Objective outcomes, that is, tender and swollen joint counts, were also significantly reduced in subjects receiving taVNS compared with those receiving SS. The stimulation was well tolerated with no adverse events attributed to the intervention, and, clinical benefits continued after taVNS was stopped.

Despite the impressive clinical benefits observed on pain and fatigue in our study in SLE after only 4 days of stimulation, we did not detect significant changes in circulating levels of most potential biomarkers. Reductions in serum proteins observed in studies in RA, inflammatory bowel disease or Sjogren's disease following VNS were reported following weeks or months of VNS[14 15 20] and four daily stimulations may not have been sufficient to effect changes in circulating levels of inflammatory markers, cytokines or components of the kynurenine pathway.

Previous studies investigating the effects of VNS have used ex vivo stimulation of whole blood with LPS before and after VNS to demonstrate the anti-inflammatory effects of engaging the inflammatory reflex and have shown that levels of LPS-stimulated proinflammatory cytokines including IL-1, IL-6 and TNF are reduced following VNS.[1 22–24] Decreased measurements of TNF, IL-1b, MCP-1 and IL-8 have also been observed in whole blood incubated, but not stimulated for 24 hours.[25] We, therefore, stimulated whole blood ex vivo, but did not observe reductions of inflammatory mediators or chemokines on day 5 or 12 in whole blood stimulated with TLR 4, 7 or 9 agonists, nor did we demonstrate a reduction of mediators in unstimulated whole blood after incubation for 24 hours. These assays were performed on day 5, 24 hours following the last stimulation and day 12. We do not know whether analysis of whole blood obtained shortly after stimulation of the vagus nerve would have resulted in different findings. Alternatively, the whole blood may have been overstimulated ex vivo with the stimulant concentrations used so that the anti-inflammatory biological effects of VNS could not be detected by these assays.

We did observe the change in plasma levels of substance P following 4 days of stimulation suggesting that the biological responsiveness of this neuropeptide to taVNS may be more rapid or sensitive than that of cytokines. Our finding of a reduction of plasma levels of substance P in subjects receiving taVNS but not in control subjects receiving SS is of interest, because substance P not only facilitates the transmission of nociceptive signals from the periphery to the brain but also has proinflammatory properties. In RA, a positive association between the levels of substance P and inflammation has been proposed.[26] The role of substance P in the inflammatory pain in SLE merits additional investigation.

The results of our short, sham-controlled pilot study engaging the cholinergic anti-inflammatory pathway by non-invasive stimulation of the vagus nerve for treatment of inflammatory musculoskeletal pain and fatigue in SLE are promising. Although we have not yet fully identified the molecular pathway(s) responsible for the observed clinical response, our findings suggest that SLE inflammatory symptoms are responsive to VNS and that substance P is affected by the cholinergic anti-inflammatory pathway. A non-toxic, non-pharmacological approach for control of these common SLE symptoms would be welcome. Additional studies of this intervention applied over a longer period of time are needed to assess the durability of the effects of VNS on pain, fatigue and other manifestations of SLE. A better understanding of the cellular and molecular pathways downstream of VNS are needed as well as biomarkers to identify those who will respond or that are early indicators of sustained response.

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Competing interests KJT reports a financial relationship with Set Point Medical and My String; Prof. CB and Assistant Professors TPZ and Datta-Chaudhuri have a provisional patent application: "Auricular stimulation device, system and methods of use".

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

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TRANSLATIONAL SCIENCE

Proteomic, biomechanical and functional analyses define neutrophil heterogeneity in systemic lupus erythematosus

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ABSTRACT

Objectives Low-density granulocytes (LDGs) are a distinct subset of proinflammatory and vasculopathic neutrophils expanded in systemic lupus erythematosus (SLE). Neutrophil trafficking and immune function are intimately linked to cellular biophysical properties. This study used proteomic, biomechanical and functional analyses to further define neutrophil heterogeneity in the context of SLE.

Methods Proteomic/phosphoproteomic analyses were performed in healthy control (HC) normal density neutrophils (NDNs), SLE NDNs and autologous SLE LDGs. The biophysical properties of these neutrophil subsets were analysed by real-time deformability cytometry and lattice light-sheet microscopy. A two-dimensional endothelial flow system and a three-dimensional microfluidic microvasculature mimetic (MMM) were used to decouple the contributions of cell surface mediators and biophysical properties to neutrophil trafficking, respectively.

Results Proteomic and phosphoproteomic differences were detected between HC and SLE neutrophils and between SLE NDNs and LDGs. Increased abundance of type 1 interferon-regulated proteins and differential phosphorylation of proteins associated with cytoskeletal organisation were identified in SLE LDGs relative to SLE NDNs. The cell surface of SLE LDGs was rougher than in SLE and HC NDNs, suggesting membrane perturbances. While SLE LDGs did not display increased binding to endothelial cells in the two-dimensional assay, they were increasingly retained/trapped in the narrow channels of the lung MMM.

Conclusions Modulation of the neutrophil proteome and distinct changes in biophysical properties are observed alongside differences in neutrophil trafficking. SLE LDGs may be increasingly retained in microvasculature networks, which has important pathogenic implications in the context of lupus organ damage and small vessel vasculopathy.

Key messages

What is already known about this subject?

- Low-density granulocytes (LDGs) are a subset of neutrophils expanded in systemic lupus erythematosus (SLE). These cells have been shown to have a pathogenic role through their enhanced ability to form neutrophil extracellular traps, promote type I interferon responses and damage the vasculature. Their levels and gene signature associate with enhanced vasculopathy and atherosclerosis in patients with lupus.

What does this study add?

- The findings from this study indicate that lupus LDGs display distinct proteomic and biomechanical properties that may impact their ability to travel through the vasculature, interact with the endothelium and enhance their trapping in the small vessels of various organs.

How might this impact on clinical practice or future developments?

- Increased retention of lupus LDGs in microvasculature could have pathogenic implications in lung or kidney damage, and in development of small vessel vasculopathy. These results suggest that development of therapeutics modulating neutrophil biomechanical properties could modulate deleterious responses in lupus and other autoimmune diseases.

INTRODUCTION

Neutrophil dysregulation may play critical roles in systemic lupus erythematosus (SLE) pathogenesis.[1] Enhanced release of neutrophil extracellular traps (NETs)—the externalisation of oxidised nucleic acids and granule proteins—promotes immune dysregulation, vasculopathy and organ damage associated with SLE.[2–5]

We previously identified a subset of SLE proinflammatory neutrophils (low-density granulocytes, LDGs), purified from the peripheral blood mononuclear cell (PBMC) layer.[6] In contrast to normal dense neutrophils (NDNs), LDGs spontaneously form proinflammatory NETs[7 8] induce endothelial damage,[6] and associate with in vivo vascular

inflammation, coronary atherosclerosis,[5 9–11] and T cell activation,[12] suggesting they play important roles in SLE pathogenesis.

Previous LDG studies focused on transcriptomic analysis, with little known about proteome modulation and protein function.[8 9 13–16] Proteomic analyses comparing SLE LDGs to SLE and healthy control (HC) NDNs identified differential phosphorylation of proteins associated with cytoskeletal organisation. Using real-time deformability cytometry (RT-DC) and a polydimethylsiloxane (PDMS) device mimicking neutrophil trafficking through the pulmonary microvasculature, we determined that SLE LDGs are biophysically distinct from other neutrophil subsets, which may affect their ability to traffic through small blood vessels.

METHODS

See online supplemental material.

RESULTS

Differential protein profiles of lupus and HC neutrophils

Proteomic/phosphoproteomic analyses were performed in SLE LDGs and NDNs, and HC NDNs (n=5/group; online supplemental tables 1 and 2). As controls, HC NDNs were also analysed following priming with N-formylmethionine leucylphenylalanine (fMLF), given that priming decreases HC NDN density.[17] Neutrophil preparations used identical protocols optimised to minimise biophysical or functional disruption of cells from their unstimulated state in whole blood (online supplemental figure 1).

Neutrophil mass spectrometry analysis identified 4109 proteins (figure 1A), of which 601 (14.6%) and 685 (16.6%) were identified only in HC or SLE neutrophils, respectively (online supplemental figure 2A,B). This is comparable to the most robust neutrophil proteomic analysis previously reported.[18] Results were aligned with SLE LDG, NDN, and HC NDN transcriptomics (GEO GSE139358)[16] to identify proteins not present at the mRNA level that may be of exogenous source (online supplemental figure 3). SLE LDGs and NDNs showed complete proteome overlap, although with considerable variation in protein abundance. Indeed, 9.4% of proteins expressed by SLE neutrophils were differentially abundant in SLE LDGs versus NDNs, with 270 more abundant and 60 less abundant (ratio cut-off >1.5 or<0.5 in at least 4/5 matched samples; figure 1C). Of the 2823 proteins common to both SLE and HC NDNs, 304 (10.7%) showed differential abundance. FMLF-primed and unstimulated HC NDNs showed complete proteome overlap with little variation in protein abundance, except for decreased abundance of L-selectin in primed HC NDNs, suggesting protein shedding from in vitro activation[19] (online supplemental figure 2D–H). Overall, many proteins were uniquely present in either HC or SLE neutrophils and protein abundances varied between subsets, indicating neutrophil proteome heterogeneity.

We identified 875 proteins phosphorylated on serine, threonine, and/or tyrosine residues in neutrophils (figure 1B). Some proteins were phosphorylated at multiple sites (online supplemental table 3). Of these phosphoproteins, 48 (5.4%) and 366 (41.8%) were only identified in HC NDNs and SLE neutrophils, respectively. The same phosphoproteins were identified in HC unstimulated and fMLF-primed NDNs, and one phosphoprotein was uniquely identified in SLE LDGs (round spermatid basic protein 1-like protein, pRSBN1L; online supplemental figure 2C). When comparing SLE LDGs and NDNs, 95 phosphoproteins (11.5%) were differentially abundant, with 11 less and 84 more abundant in SLE LDGs (figure 1D). Of the 509 phosphoproteins coexpressed in fMLF-primed and unstimulated HC NDNs, 167 (32.8%) were differentially abundant (online supplemental figure 2E). Of the 460 phosphoproteins common to all neutrophils, 100 (21.7%) were differentially abundant between HC and SLE NDNs (online supplemental figure 2G). These data support neutrophil phosphoproteome heterogeneity.

The LDG proteome displays a distinct profile

Using ShinyGO[20] and MetaScape,[21] we mapped proteins differentially abundant in at least 4/5 samples to known gene-ontology biological processes. Proteins more abundant in SLE NDNs relative to HC NDNs mapped to neutrophil activation networks, including proteins facilitating migration to inflammatory sites and release from bone marrow.[22–24] Some proteins associated with neutrophil activation were most abundant in SLE LDGs (figure 1E,F).

SLE subjects express elevated type 1 IFN-stimulated genes (ISGs) in various organs and cells, including NDNs and LDGs.[16 25] While ISG-encoded proteins were not uniformly upregulated in SLE NDNs versus HC NDNs, many were upregulated in SLE LDGs relative to SLE or HC NDNs (figure 1G). ISG transcription is mediated by phosphorylation of signal transducer and activator of transcription (STAT) molecules[26] but we did not detect phospho-STATs, possibly because pTyr residues are less abundant than pSer.[27] Limited LDG numbers prevented immunoprecipitation of pTyr residues alongside phosphopeptide enrichment. Collectively, the SLE neutrophil proteome suggests an activated status, while the IFN-associated protein signature is distinct to SLE LDGs.

Neutrophil priming/activation facilitates interactions with the endothelium.[28] There were no differences in adhesion molecule or integrin expression among neutrophil subsets. However, phosphoproteins regulating neutrophil–endothelial interactions were more abundant in fMLF-primed HC NDNs than other neutrophil subsets (figure 1H,I). This suggests differences between SLE LDGs/NDNs and fMLF-primed HC NDNs.

Proteins with differential phosphorylation in SLE NDNs versus HC NDNs were associated with organelle organisation and actin cytoskeletal organisation, including phospho-coronin 1A (pCORO1A) and phospho-heat shock protein 90AA1 (pHSP90AA1; online supplemental figure 2H). Some proteins less abundant in SLE LDGs versus SLE NDNs associated with neutrophil degranulation but key granule proteins, including myeloperoxidase and cathepsin-G, were not decreased (figure 2A,B). Rather, lower abundance of membrane proteins, particularly ficolin-1-rich granule membrane proteins, accounted for downregulated degranulation-associated networks in SLE LDGs. Differences in degranulation capabilities did not explain changes in the neutrophil proteome between neutrophil subsets.

Abundant proteins in SLE LDGs versus SLE NDNs clustered in neutrophil activation, coagulation, platelet and intracellular trafficking networks (figure 2C). The SLE biological network (false discovery rate=$10^{-11.119}$) was upregulated in SLE LDGs versus autologous NDNs, primarily driven by complement proteins (figure 2D). Immunoglobin chains and apolipoproteins were more abundant in SLE LDGs versus other neutrophils. Differential phosphorylation in SLE LDGs versus NDNs also associated with neutrophil activation and intracellular trafficking. In addition, SLE LDGs expressed higher abundances of ribosomal proteins (figure 2E–G).

SLE LDGs are a heterogeneous group comprising $CD10^-$ (immature, less abundant) and $CD10^+$ (intermediate-mature, most abundant) subsets. $CD10^-$ LDGs have decreased *CEBPD* and *SPI1*

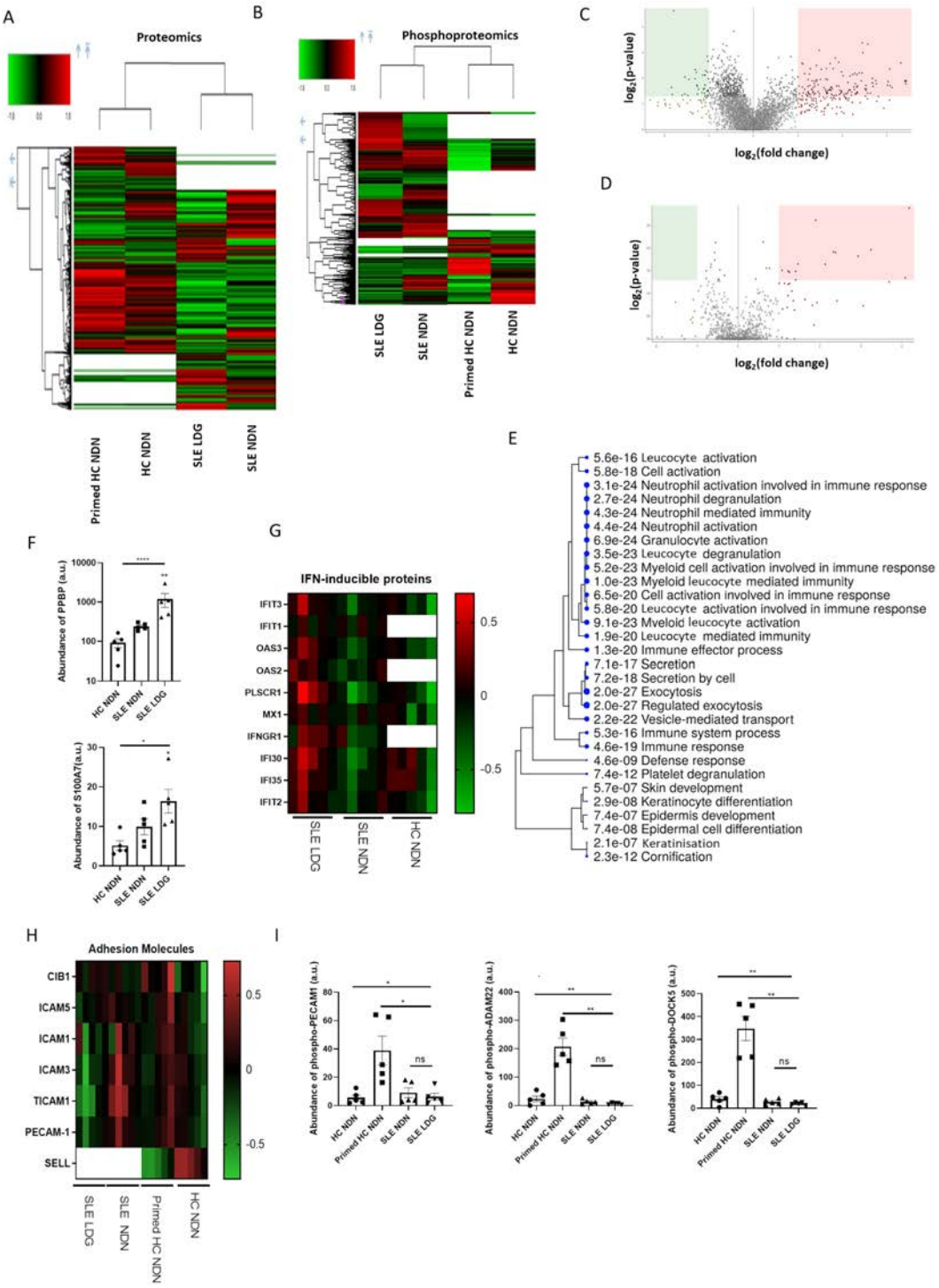

Figure 1 Systemic lupus erythematosus (SLE) normal dense neutrophils (NDNs) differ in their proteome and phosphoproteome compared with healthy control (HC) NDNs. (A) A total of 4109 proteins and (B) 875 phosphoproteins were identified by mass spectrometry in low-density granulocytes (LDGs) and NDNs from subjects with SLE (n=5) and unstimulated and primed NDNs from HC volunteers (n=5). Volcano plots depict differences between SLE NDN and LDG proteomes (C) and phosphoproteomes (D). The upregulated (red) and downregulated (green) proteomes are in LDGs, while NDNs are the reference proteome. (E) Gene ontology biological process analysis highlighting biological networks associated with proteins more abundant in at least 4/5 SLE NDN samples relative to HC NDNs. Proteins with abundance ratios greater than 1.5 were included and significance was established by false discovery rate. (F) Proteins responsible for upregulation of networks associated with neutrophil activation in SLE NDNs relative to HC NDNs in arbitrary units. Significance was established by Kruskal-Wallis test with post hoc Dunn's tests for multiple comparisons (three comparisons) or by Mann-Whitney U test (two comparisons). (G) Relative abundance of interferon-inducible proteins in SLE NDNs and HC NDNs relative to SLE LDGs. SLE NDNs were compared with autologous SLE LDGs. HC NDNs were compared with the mean protein abundance in SLE LDGs. Open boxes in heatmaps indicate the given protein was not identified in the sample. (H) Abundance of cell integrins and adhesion-related proteins in SLE NDNs and HC NDNs relative to autologous SLE LDGs and autologous primed HC NDNs, respectively. (I) Abundance of phosphoproteins associated with regulation of neutrophil–endothelial interactions in all neutrophil subsets, in arbitrary units. Significance was established by Kruskal-Wallis test with post hoc Dunn's tests for multiple comparisons. All results are mean±SEM and significance was set at *p≤0.05, **p≤0.01, ns=not significant.

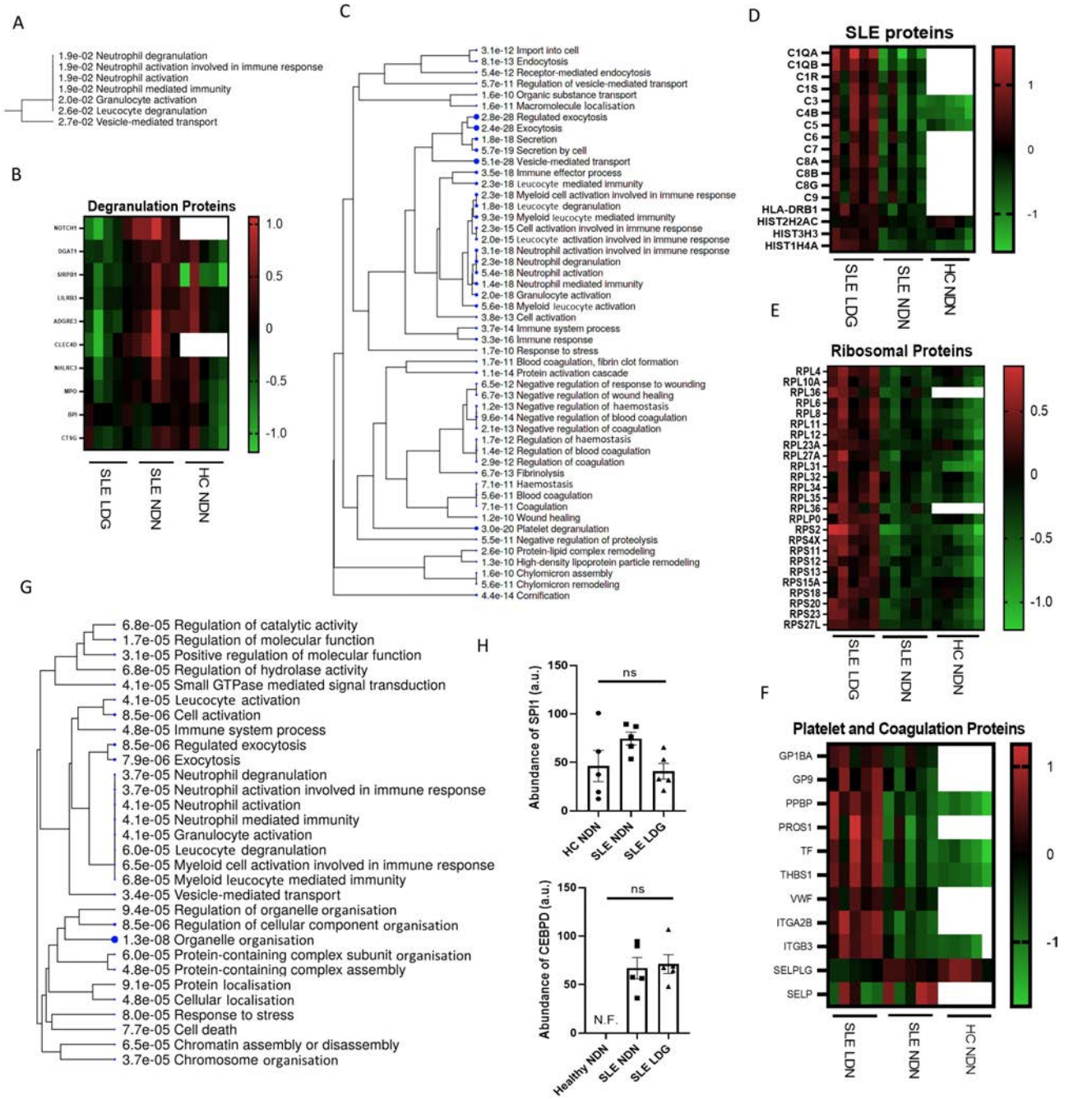

Figure 2 Systemic lupus erythematosus (SLE) low-density granulocytes (LDGs) have a distinct proteomic profile characterised by enhanced pathways associated to translational activity, intracellular trafficking and type I interferon-induced protein pathways. (A) Gene ontology biological process analysis highlighting biological networks associated with proteins less abundant in SLE LDGs (n=5) relative to SLE normal dense neutrophils (NDNs; n=5). Proteins with abundance ratios less than 0.5 in at least 4/5 matched samples were included and significance was established by false discovery rate (FDR). (B) Relative abundance of degranulation network-associated proteins in SLE NDNs and healthy control (HC) NDNs relative to SLE LDGs. SLE NDNs were compared with autologous SLE LDGs. HC NDNs were compared with the mean protein abundance in SLE LDGs. (C) Gene ontology biological process analysis highlighting biological networks associated with proteins more abundant in at least 4/5 SLE LDG samples relative to SLE NDNs. Proteins with abundance ratios greater than 1.5 were included and significance was established by FDR. (D) Relative abundance of SLE network-associated proteins in SLE NDNs and HC NDNs relative to SLE LDGs. SLE NDNs were compared with autologous SLE LDGs. HC NDNs were compared with the mean protein abundance in SLE LDGs. (E) Relative abundance of eukaryotic translation network-associated proteins in SLE NDNs and HC NDNs relative to SLE LDGs. SLE NDNs were compared with autologous SLE LDGs. HC NDNs were compared with the mean protein abundance in SLE LDGs. Open boxes in heatmaps indicate the given protein was not identified in the sample. (F) Relative abundance of coagulation and platelet network-associated proteins in SLE NDNs and HC NDNs relative to SLE LDGs. SLE NDNs were compared with autologous SLE LDGs. HC NDNs were compared with the mean protein abundance in SLE LDGs. (G) Gene ontology biological process analysis highlighting biological networks associated with phosphoproteins differentially abundant in SLE LDGs and NDNs. Phosphoproteins with abundance ratios less than 0.5 or greater than 1.5 in at least 4/5 matched samples were included and significance was established by FDR. (H) Abundance of transcription factors CEBPD and SPI1 in arbitrary units. CEBPD not identified in HC NDNs. Results are mean±SEM, with comparisons between autologous SLE LDG and NDNs. Significance established by Kruskal-Wallis test with post hoc Dunn's tests for multiple comparisons and set at *p≤0.05, ns=not significant. N.F.=not found.

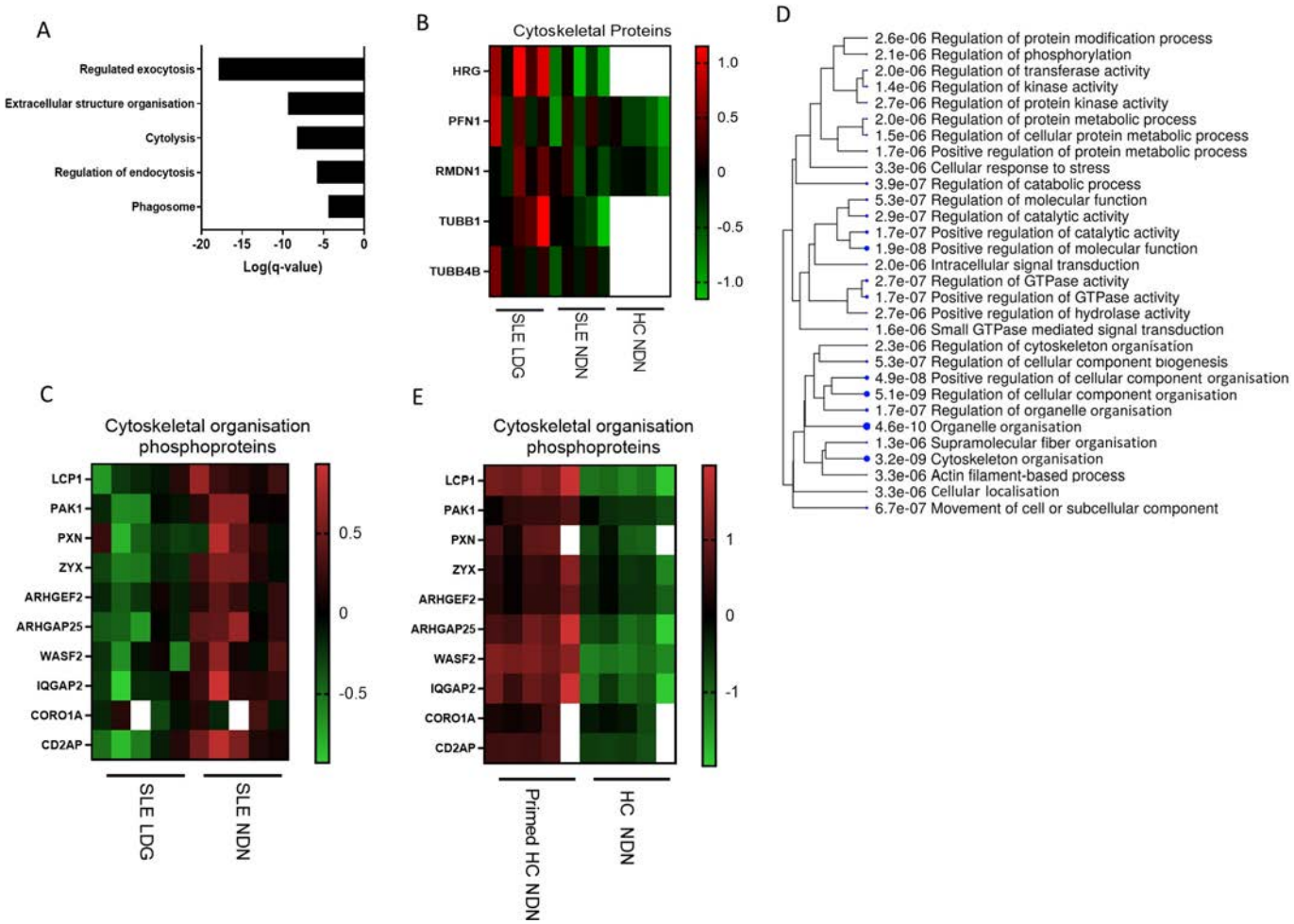

Figure 3 Proteomic and phosphoproteomic analyses indicate differential expression of proteins associated to cytoskeleton function between systemic lupus erythematosus (SLE) normal dense neutrophils (NDNs) and low-density granulocytes (LDGs). (A) Gene ontology biological process analysis highlighting biological networks related to the cytoskeleton and associated with proteins more abundant in at least 4/5 SLE LDG samples relative to their matched SLE NDNs. Proteins with abundance ratios greater than 1.5 were included and significance was established by false discovery rate (FDR). (B) Abundance of cytoskeleton-associated proteins in SLE NDNs and HC NDNs relative to SLE LDGs. SLE NDNs were compared with autologous SLE LDGs. Healthy control (HC) NDNs were compared with the mean protein abundance in SLE LDGs. Open boxes in heatmaps indicate that the given protein was not identified in one of the two autologous samples being compared. (C) Abundance of cytoskeleton network-associated phosphoproteins in SLE LDGs relative to abundance in autologous SLE NDNs. (D) Gene ontology biological process analysis highlighting biological networks associated with phosphoproteins differentially abundant in primed HC NDNs (n=5) and unstimulated HC NDNs (n=5). Proteins with abundance ratios greater than 1.5 or less than 0.5 in at least 4/5 matched samples were included and significance was established by FDR. (E) Abundance of phosphoproteins regulating the cytoskeleton in primed HC NDNs relative to autologous HC NDNs.

transcripts relative to SLE NDNs and CD10$^+$ LDGs.[16] Proteomic analysis was completed on unfractionated SLE LDGs and displayed similar SPI1 protein abundance across neutrophil subsets. CEBPD was not identified in HC NDNs but was similar in SLE LDGs and NDNs (figure 2H). Most SLE LDGs had multilobulated nuclei (online supplemental figure 1C), confirming intermediate-mature cells represent the most abundant LDG subset.[16]

SLE LDGs and NDNs differ in expression of cytoskeleton-associated proteins

Consistent with evidence that cytoskeleton-associated transcriptional networks are enhanced in SLE LDGs,[8] we found upregulation at the protein level when compared with autologous NDNs (figure 3A). Many proteins differentially expressed and/or phosphorylated in LDGs regulate intracellular trafficking (figure 3B,C).[29–33] In addition, we assessed modulation of the HC NDN phosphoproteome by fMLF.[17 34 35] Many proteins differentially phosphorylated in fMLF-primed HC NDNs were associated with cytoskeletal organisation (figure 3D,E). Upregulation of cytoskeleton-associated networks in the SLE LDG proteome, alongside phosphoproteomic findings suggestive of differential cytoskeletal reorganisation among neutrophil subsets, prompted investigation of neutrophil biomechanical properties.

Neutrophil biomechanical properties are altered in clinically active SLE

RT-DC is a high-throughput technique that analyses biomechanical properties of thousands of cells in suspension.[36] An inverted microscope with a high-speed camera captures images of individual cells moving through a narrow constriction channel within a PDMS microfluidic chip, where cells are deformed by hydrodynamic shear stress. Cell tracing algorithms generate biomechanical profiles per cell, including cell size (cross-sectional area), roughness (cell surface perturbations quantified by dividing the convex hull area by the cross-sectional area), and deformability (one minus the value of circularity within a

constriction channel). Cell populations are identified in blood by size and brightness (figure 4A).[37] These measurements were obtained in peripheral blood from HC (n=11), clinically quiescent (n=11) or clinically active SLE (n=4). In some experiments, fMLF was added directly to HC peripheral blood to prime neutrophils prior to analysis. Neutrophils from HC and clinically quiescent SLE were biomechanically identical, while active SLE neutrophils had larger areas, enhanced deformability and roughness. FMLF-primed HC neutrophils were also larger and more deformable than unstimulated neutrophils and significantly rougher than any unstimulated neutrophil subsets (figure 4B). Overall, neutrophils from active SLE subjects displayed altered biomechanical properties and cell membrane perturbations.

SLE LDGs and NDNs are biomechanically distinct

Biomechanical properties of purified SLE LDGs/NDNs from clinically quiescent subjects and HC NDNs were quantified, using optimised purification strategies to avoid disruption of biomechanical properties (online supplemental figure 1). Gating strategies allowed for identification of neutrophils, monocytes, lymphocytes and eosinophils in mixed cell fractions (figure 4A).[37] Biomechanical properties did not differ in lymphocytes and monocytes between SLE and HC subjects (online supplemental figure 4). In contrast, SLE LDGs displayed distinct biomechanical features relative to other neutrophil subsets (figure 4E). While HC and SLE NDNs were round and smooth, SLE LDGs had significantly rougher cell surfaces that correlated with age but not with other clinical/demographic characteristics (online supplemental figure 5). HC NDNs incubated for various timepoints with Sm/RNP immune complexes[7] and/or recombinant IFN-α displayed no changes in neutrophil roughness (online supplemental figure 6). Overall, SLE LDGs display distinct biomechanical properties seemingly unrelated to exposure to immune complexes or type I IFNs.

Neutrophil percentages were higher in SLE than HC PBMC fractions (figure 4C,D), consistent with higher LDG numbers.[9] It is unclear whether LDGs are present in small numbers in healthy individuals but expanded in SLE.[38] We compared biomechanical properties of SLE LDGs to the small population of HC LDGs. Like autologous HC NDNs, and not consistent with the SLE LDG biomechanical phenotype, HC LDGs displayed smooth, non-polarised surfaces. HC LDGs were also more deformable than HC NDNs (figure 5C). These differences in biomechanical properties support SLE LDGs do not represent expansion of a minor LDG population found in HCs.

While fMLF-primed HC NDNs are morphologically rougher[17] and localise to the PBMC interphase on density gradients (figure 4D),[17] they were consistently larger than autologous unprimed NDNs. This contrasts with SLE LDGs, which were similar in size to autologous SLE NDNs (figure 4E), supporting primed HC NDNs and SLE LDGs are biomechanically distinct. These biomechanical differences were confirmed by brightfield (figure 4F,G) and lattice light-sheet fluorescence microscopy (online supplemental file 1). The cell surface of fMLF-primed HC NDNs appeared to ruffle, with small membrane perturbations moving inwards and outwards. In contrast, SLE LDGs' cell surface was smooth, except for sections of dramatic protrusions, which were irregularly shaped (figure 4F,G). This suggests SLE LDGs have distinct biophysical properties not consistent with acutely primed phenotypes.

SLE LDGs are retained in a microfluidic microvasculature mimetic (MMM)

Neutrophil biomechanical properties can modulate transit through the pulmonary microvasculature. Primed neutrophils are retained in pulmonary capillary beds,[39] possibly due to enhanced cell stiffness and/or irregular cell shape.[40] To mimic trafficking through the pulmonary microvasculature, we developed an MMM formed of a branched pyramidal network within a PDMS chip (figure 5A). Neutrophils flowed through this network at physiologically relevant pressures (10 and 50 mbar, or 10.2 and 51.0 cmH_2O, respectively)[40] without impact on viability (online supplemental figure 8). As previously reported,[39 40] fMLF-primed HC NDNs were increasingly retained in the MMM, with >80% unable to fully navigate it. In contrast,>80% unprimed HC NDNs navigated the MMM within 3 s. Trafficking patterns of SLE LDGs resembled those of primed HC NDNs, with >75% SLE LDGs retained in the MMM versus approximately 50% SLE NDNs (figure 5B). Of neutrophils transiting the entire MMM, SLE and unprimed HC NDNs averaged a transit time of <0.9 s, while SLE LDGs and primed HC NDNs averaged transit times of 1.87 and 2.89 s, respectively (figure 5C).

HC NDNs treated with cytochalasin D, which disassembles filament actin and decreases neutrophil deformability,[36] were increasingly retained in the MMM (>75% retention vs <20% in vehicle-treated HC NDNs (figure 5D)), supporting biomechanical modulation alters neutrophil trafficking. Like primed HC NDNs,[39] SLE LDGs may be preferentially retained in microvasculature due to biomechanical property differences.

The MMM evaluated effects of cellular biomechanical properties on trafficking but not the putative role of neutrophil–endothelial interactions. By decoupling effects of biophysical properties and cell-surface markers on neutrophil trafficking, we evaluated their independent contributions. A two-dimensional assay evaluated neutrophil interactions—rolling alongside or adherence to microvascular endothelium—in a circulatory flow system mimicking physiological conditions (flow rate 0.4 mL/min). Over 3 min, 15% and 60% primed HC NDNs interacted with unstimulated or stimulated endothelium, respectively. In contrast,<10% and<20% HC NDNs, SLE LDGs and NDNs interacted with unstimulated and stimulated endothelium, respectively ($p<0.01$ compared with primed HC NDNs and $p>0.05$ comparing other neutrophil subsets; figure 5E,F; online supplemental file 1). These observations suggest that, while enhanced neutrophil–endothelium interactions may contribute to microvasculature retention of primed HC NDNs, they do not explain differences in microvasculature trafficking observed between SLE LDGs and NDNs. Overall, SLE LDGs may be retained in microvasculature networks,[39] by intrinsic changes in cellular biomechanical properties rather than by specific neutrophil–endothelium interactions.

DISCUSSION

We identified significant differences between the SLE and HC neutrophil proteomes as well as heterogeneity in the proteome of SLE neutrophils including proteins involved in formation/rearrangement of the cytoskeleton. In addition, we identified biomechanical differences in SLE LDGs with implications for neutrophil trafficking in the microvasculature.

Consistent with the proteomics data and previous transcriptomic analyses reporting differential gene expression associated with the actin cytoskeleton in SLE LDGs,[8] we found SLE LDGs are biomechanically distinct and showed cell membrane perturbations differing from fMLF-primed neutrophils.[17 41] While

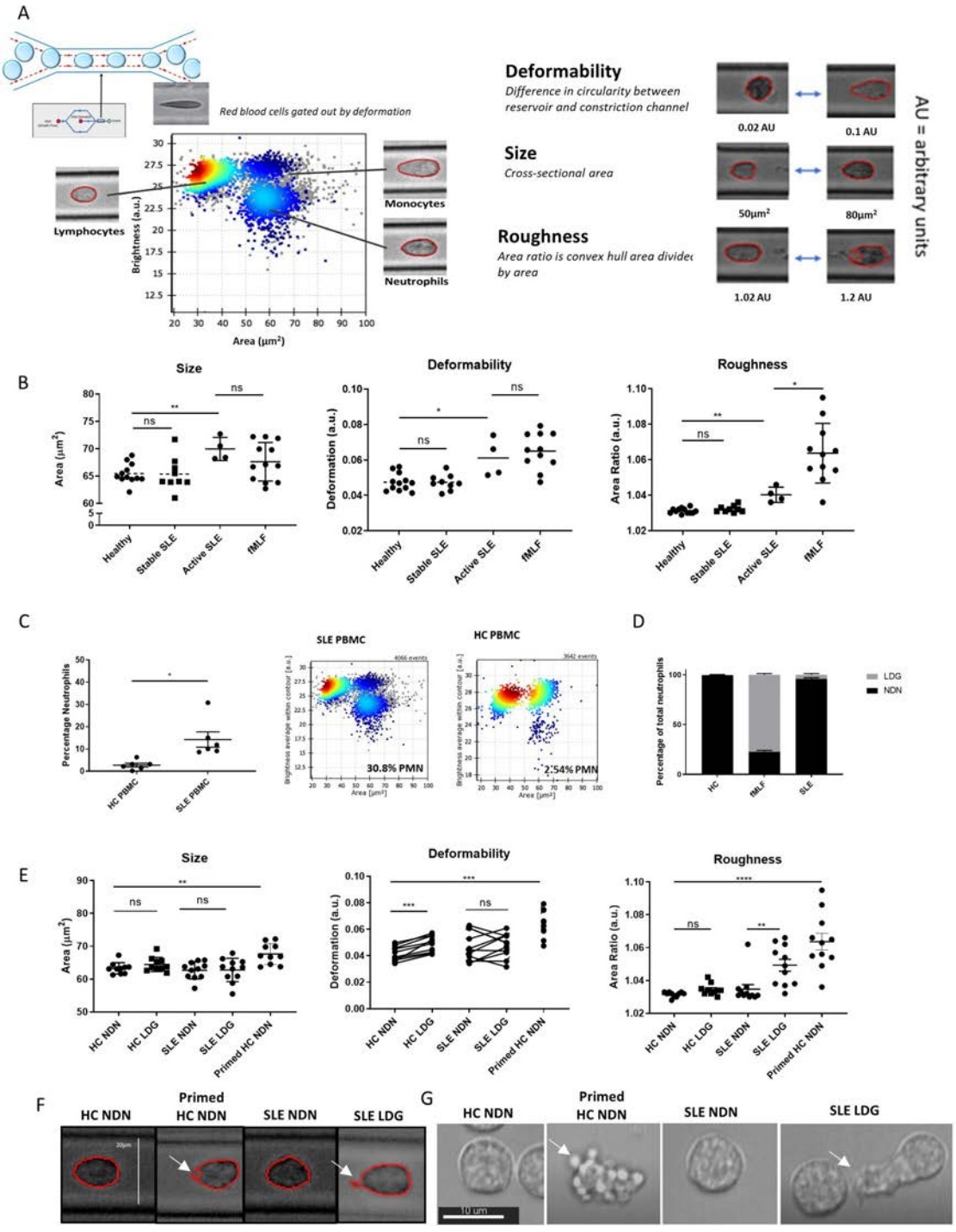

Figure 4 Systemic lupus erythematosus (SLE) low-density granulocytes (LDGs) are biomechanically rougher than other neutrophil subsets by real-time deformability cytometry (RT-DC). (A) RT-DC was used to biomechanically characterise the shape, size and deformability of neutrophil subsets. (B) Biomechanical profiling of neutrophils in blood samples obtained from healthy volunteers (n=12), clinically quiescent patients with SLE (n=9), and active patients with SLE (n=4) by RT-DC. In some instances, 100 nM N-formylmethionine leucyl-phenylalanine (fMLF) was used to prime neutrophils within the healthy blood. (C) Percentage of LDGs identified in SLE (n=6) and healthy control (HC) peripheral blood mononuclear cells (PBMCs; n=6) by RT-DC. (D) LDGs as a percentage of total neutrophils in patients with SLE (n=6), HC volunteers (n=6), and fMLF-primed HC blood (n=6). (E) Biomechanical profiling of isolated NDNs and LDGs from HC volunteers (n=11) and clinically quiescent patients with SLE (n=11) by RT-DC. Some HC NDNs were primed with fMLF prior to isolation. For each sample analysed by RT-DC, the median measurement of over 500 neutrophils is graphed and the mean±SEM for each neutrophil subset is depicted. Autologous unstimulated/primed HC NDNs or autologous SLE NDN/LDGs were compared and significance was established by Wilcoxon matched-pairs signed rank tests. In other comparisons, significance was established by Mann-Whitney U tests. Significance was set at *p≤0.05, **p≤0.01, ns=not significant. (F) Images of NDNs and LDGs captured during RT-DC, representative of >500 images of each neutrophil subset from n=11 patients with SLE and n=11 HC volunteers, 10× objective. White arrows identify a concave cell surface feature common in primed HC NDNs and an irregular protrusion in the cell surface common in SLE LDGs. (G) Brightfield microscopy of NDNs and LDGs (n=3). White arrows identify rounded, protruded, membrane features observed in nearly 100% of primed HC NDNs and an irregular protrusion observed in ~30% of SLE LDGs. See online supplemental videos 1–4.

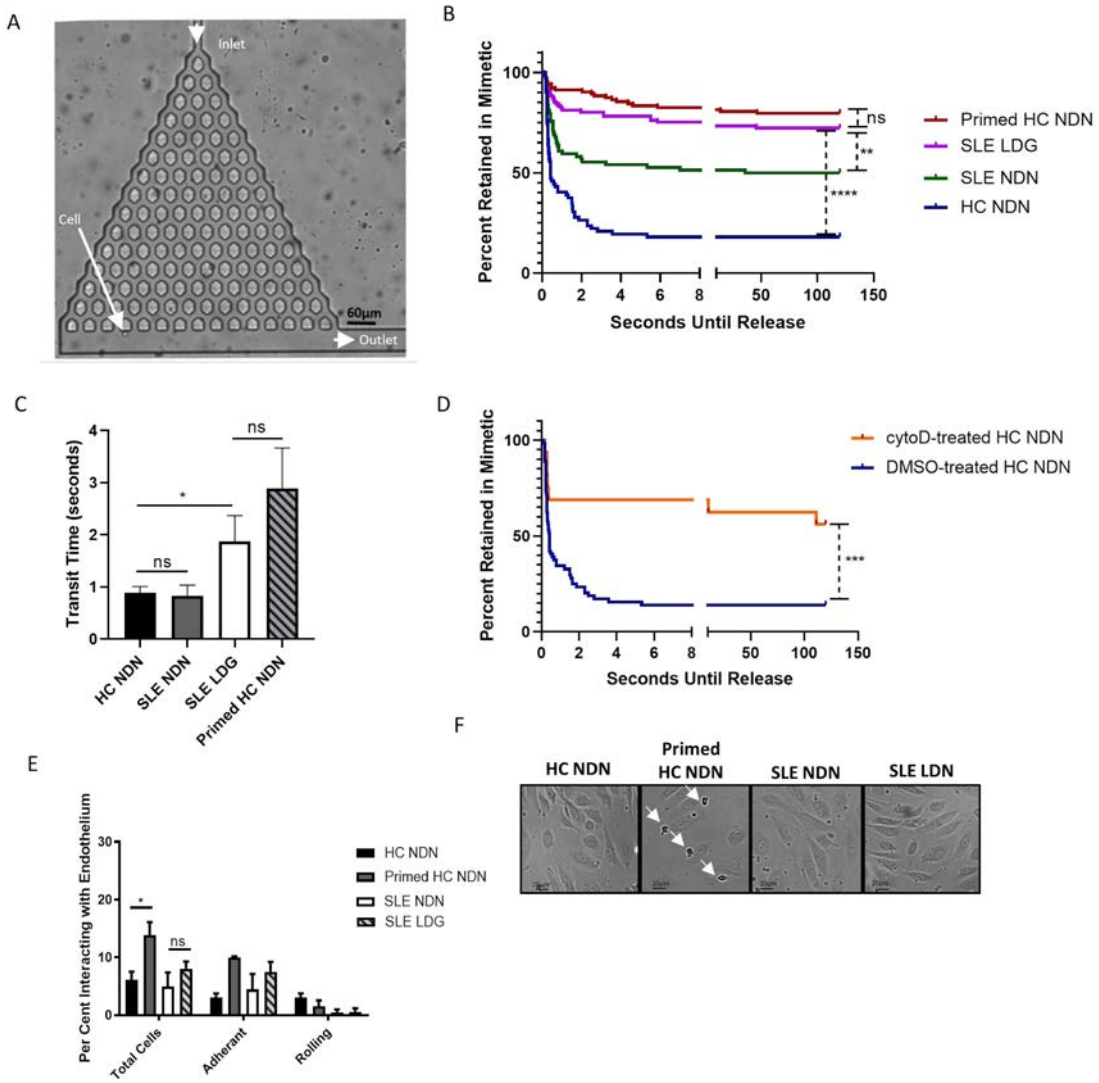

Figure 5 Systemic lupus erythematosus (SLE) low-density granulocytes (LDGs) are increasingly retained within a three-dimensional pulmonary microvasculature mimetic, but do not display enhanced adherence to endothelial cells in a two-dimensional system of flow. (A) A branching microfluidic mimetic of the pulmonary microvasculature was designed. Arrows indicate the inlet, outlet and a cell navigating the network, 10× objective. (B) Retention of normal dense neutrophils (NDNs) and LDGs in the microvasculature mimetic (n>150 neutrophils per subset from n=6 healthy control (HC) volunteers and n=6 patients with SLE). Seconds until release refers to the amount of time each neutrophil was retained within the mimetic, measured from entry at the inlet to exit at an outlet. Seconds until release was recorded as >120 s if cells did not exit the mimetic within the 2 min video. Times were determined manually with a timer superimposed on the video during data collection. Significance was determined by log-rank test. (C) Transit times through the microvasculature mimetic for all NDNs and LDGs navigating the entire device. Significance assessed by Mann-Whitney U test. (D) Retention of HC NDNs treated with dimethyl sulfoxide or cytochalasin D in the microvasculature mimetic (n>50 neutrophils from n=3 HC volunteers). Significance was determined by log-rank test. (E) Percentage of neutrophils interacting with endothelium under 0.4 mL/min flow. Significance was determined by Kruskal-Wallis test with post hoc Dunn's tests for multiple comparisons. (F) Light microscopy of neutrophil binding to endothelium post-flow assay. Arrows show enhanced binding of primed NDNs. Images representative of n=4 images obtained for each neutrophil subset isolated from n=6 patients with SLE or n=6 HC volunteers. All results are mean±SEM with significance was set at *p≤0.05, **p≤0.01, ****p≤0.0001, ns=not significant.

mechanisms promoting enhanced SLE LDG cytoskeletal changes remain unclear, differential abundance of proteins associated with extracellular structure organisation and cytolysis may be implicated. For example, profilin 1 (PFN1) modulates actin/microtubule dynamics[30 42] and actin polymerisation,[43] while histidine rich glycoprotein (HRG) induces neutrophil morphological changes[29] implicated in neutrophil retention in microvasculature.[29 44] Furthermore, the enhanced ability of LDGs to form NETs may contribute to cytoskeleton perturbations and disruptions in cell membrane integrity.[15 45–47] Although SLE LDGs did not morphologically resemble HC neutrophils treated with Sm/RNP immune complexes or phorbol myristate acetate (PMA) to induce NETosis (online supplemental figures 6 and 7), differences in spontaneous LDG NET formation and PMA-induced NET formation have been reported.[7 8 48] The potential link between NET formation, neutrophil proteome and biomechanical properties of LDGs should be studied further.

Previous studies indicate that rougher, primed neutrophils are retained in the lungs.[29 40] Our MMM data suggest that LDG roughness may similarly hinder LDGs' ability to traffic through narrow capillaries and biophysical properties, not enhanced binding to endothelium.[40] Increased retention in microvasculature networks could have pathogenic implications in lung or kidney damage, and in development of small vessel vasculopathy.

SLE lung manifestations are associated with blood vessel damage triggered by neutrophils.[49–51] Circulating immune complexes can activate neutrophils, promote endothelial cell barrier dysfunction and perturbed vascular permeability.[52 53] While distinct biomechanical properties of SLE LDGs did not align with preferential binding to microvascular endothelial cells, LDGs have potent deleterious effects on endothelium through NET formation.[7 54 55] Accordingly, we propose a model where slow LDG microvasculature transit, coupled to enhanced NETosis, promotes vasculopathy. Future studies should assess mechanisms of enhanced SLE LDG roughness and in vivo significance of its effect on LDG trafficking.

In contrast to SLE LDGs, fMLF-primed NDNs showed enhanced adherence to endothelium and higher abundance of phosphoproteins linked to cell adhesion.[56–59] Actin-regulatory proteins are dephosphorylated in LDGs but phosphorylated in primed neutrophils, suggesting both actin depolymerisation and polymerisation may induce biophysical changes affecting trafficking. Indeed, imaging showed primed NDNs with contracted cortical actin rings while LDGs appeared irregularly shaped with incomplete actin rings (online supplemental figure 7, online supplemental videos 1–4). Overall, SLE LDGs differ from acutely primed neutrophils and interact with the vasculature differently.

The type I IFN pathway is linked to SLE pathogenesis and neutrophils responding to these cytokines exhibit proinflammatory responses.[4] ISG-encoded proteins were higher in SLE LDGs, consistent with transcriptome reports.[16] Why SLE LDGs express higher ISG-encoded proteins than autologous SLE NDNs, exposed to similar levels of cytokines in vivo, could be related to differences in JAK-STAT activity or to differences in activation status.[16] SLE LDGs have enhanced ISG hypomethylation relative to HC neutrophils, perhaps modulating the protein response.[60] Future studies should address how enhanced IFN responses modulate pathogenic differences linked to LDGs' ability to NET and damage vasculature.

The SLE LDG proteome contained increased acute phase response proteins associated with complement and coagulation.[61] Corroborating our findings, LDGs display significantly enhanced transcription of several complement components (online supplemental figure 3). Some complement proteins identified by proteomics were not identified by transcriptomics. These proteins, including C6–C9, may bind to circulating neutrophils. This aligns with findings of C6–C9 contributing to formation of MAC-induced lytic pores in rheumatoid arthritis neutrophils.[62] Additionally, activated HC NDNs upregulate *C3* transcription (online supplemental figure 10), suggesting activated LDGs may behave in a similar manner. This LDG–complement relationship should be investigated further.

Some proteins associated to platelet biology were more abundant in SLE LDGs, similar to descriptions in psoriasis LDGs.[63] This was confirmed by fluorescence microscopy (online supplemental figure 1) and suggests commonalities in the proteome of LDGs across inflammatory diseases associated with enhanced vascular damage. Platelet–neutrophil interactions can drive inflammation and thrombosis[64]; thus, increased platelet presence in LDG samples may contribute to their upregulation of coagulation and some neutrophil activation-associated proteins relative to NDNs. Ultimately, LDG–platelet interactions may play distinct pathophysiological roles in vasculopathy development.

Alongside the proteomics, the biomechanical profile and trafficking pattern of SLE LDGs support reports that LDGs represent a distinct neutrophil subset rather than expansion of immature/primed neutrophils present in healthy subjects.[15 65 66] SLE LDGs have a distinct proteomic signature and specific biomechanical features impacting transit through the microvasculature. This study adds to the understanding of neutrophil heterogeneity in the context of blood vessel trafficking, with important implications for development of small vessel vasculopathy and organ damage and development of therapeutics modulating neutrophil biomechanical properties.[67]

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CLINICAL SCIENCE

Progressive interstitial lung disease in patients with systemic sclerosis-associated interstitial lung disease in the EUSTAR database

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ABSTRACT

Objectives To identify overall disease course, progression patterns and risk factors predictive for progressive interstitial lung disease (ILD) in patients with systemic sclerosis-associated ILD (SSc-ILD), using data from the European Scleroderma Trials And Research (EUSTAR) database over long-term follow-up.

Methods Eligible patients with SSc-ILD were registered in the EUSTAR database and had measurements of forced vital capacity (FVC) at baseline and after 12±3 months. Long-term progressive ILD and progression patterns were assessed in patients with multiple FVC measurements. Potential predictors of ILD progression were analysed using multivariable mixed-effect models.

Results 826 patients with SSc-ILD were included. Over 12±3 months, 219 (27%) showed progressive ILD: either moderate (FVC decline 5% to 10%) or significant (FVC decline >10%). A total of 535 (65%) patients had multiple FVC measurements available over mean 5-year follow-up. In each 12-month period, 23% to 27% of SSc-ILD patients showed progressive ILD, but only a minority of patients showed progression in consecutive periods. Most patients with progressive ILD (58%) had a pattern of slow lung function decline, with more periods of stability/improvement than decline, whereas only 8% showed rapid, continuously declining FVC; 178 (33%) experienced no episode of FVC decline. The strongest predictive factors for FVC decline over 5 years were male sex, higher modified Rodnan skin score and reflux/dysphagia symptoms.

Conclusion SSc-ILD shows a heterogeneous and variable disease course, and thus monitoring all patients closely is important. Novel treatment concepts, with treatment initiation before FVC decline occurs, should aim for prevention of progression to avoid irreversible organ damage.

Key messages

What is already known about this subject?

- A subset of patients with systemic sclerosis-associated interstitial lung disease (SSc-ILD) develop progressive ILD, which is associated with higher mortality, but the prevalence of progressive ILD and the overall disease course and patterns of SSc-ILD are unknown. Current clinical practice emphasises treatment initiation of SSc-ILD patients with progressive ILD.

What does this study add?

- Around 30% of SSc-ILD patients experienced ILD progression during any 12-month period, and 67% of all SSc-ILD patients experienced progression at any time over the mean 5-year follow-up.
- ILD patterns in patients with SSc-ILD are very heterogeneous, with most patients showing both progressive and stable periods.
- Of all progressive SSc-ILD patients, only a minority showed a pattern of rapid, continuously declining forced vital capacity (FVC) with several consecutive episodes of FVC decline and no periods of FVC stability or improvement.

How might this impact on clinical practice or future developments?

- These results highlight a pitfall in current clinical practice, where treatment is often initiated after FVC decline has happened, and thus when lung damage has already occurred. Novel treatment concepts are needed and should aim for prevention of progression to avoid irreversible organ damage. This study defines factors that can identify patients at risk for progression. The results also stress the heterogeneity and variability of the course of ILD in SSc, and highlight the need for close monitoring of all patients with SSc-ILD.

INTRODUCTION

Systemic sclerosis (SSc) is a rare autoimmune disease, frequently complicated by interstitial lung disease (ILD), which is associated with worse outcomes.[1–5] Some patients with SSc-associated ILD (SSc-ILD) develop progressive ILD, showing decline in lung function and/or increasing extent of fibrosis by high-resolution CT (HRCT).[4–10] The proportion of patients with SSc-ILD who develop progressive ILD and the pattern of disease course in these patients are incompletely understood. Prior

Table 1 Overall baseline demographic and clinical characteristics of all patients with SSc-ILD and characteristics stratified by ILD progression over the 12±3-month observation period

Progression criteria: ΔFVC% predicted	Total (N=826)	Significant progression (n=100) <–10	Moderate progression (n=123) –10 to –5	Stable (n=396) >–5 to <5	Improvement (n=207) ≥5
Age, years (SD)*	56 (13.1)	59 (13.1)	56 (12.4)	55 (13.5)	58 (12.4)
Male, n (%)*	150 (18)	17 (17)	16 (13)	81 (20)	36 (17)
Disease characteristics at baseline					
Disease duration, years* (SD)	9.7 (8.3)	8.8 (7.7)	10.2 (8.2)	10.2 (8.5)	8.9 (8.3)
Disease duration <3 years*, n (%)	175 (21)	26 (26)	27 (22)	68 (17)	54 (26)
Diffuse cutaneous SSc, n (%)	365/732 (50)	44/96 (46)	55/106 (52)	182/357 (51)	84/173 (49)
Limited cutaneous SSc, n (%)	367/732 (50)	52/96 (54)	51/106 (48)	175/357 (49)	89/173 (51)
Anti-topoisomerase I Ab, n (%)	421/789 (53)	41/97 (42)	64/117 (55)	218/378 (58)	98/197 (50)
Anti-centromere Ab, n (%)	141/783 (18)	19/97 (20)	18/113 (16)	59/376 (16)	45/197 (23)
Anti-RNA polymerase III Ab, n (%)	23/451 (5)	3/54 (6)	3/60 (5)	10/217 (5)	7/117 (3)
Follow-up period, years*, mean (SD)	5.4 (2.0)	5.8 (1.4)	5.6 (2.0)	4.8 (3.2)	5.0 (3.2)
Lung characteristics					
FVC% predicted,* mean (SD)	87 (21.1)	95 (23.3)	90 (21.8)	85 (20.4)	85 (19.7)
DL_{CO}% predicted,* mean (SD)	59 (18.3)	61 (17.8)	60 (17.9)	58 (19.3)	60 (16.8)
ΔFVC% predicted,† mean (SD)	–0.1 (10.2)	–18 (7.9)	–7 (1.3)	0.3 (2.2)	12 (7.0)
ΔDL_{CO}% predicted,† mean (SD)	–0.7 (12.2)	–4 (15.4)	2 (12.8)	–0.3 (10.9)	0.9 (11.9)
NYHA class, n (%)	N=797	n=99	n=119	n=377	n=202
1	363 (44)	44 (44)	57 (46)	167 (42)	95 (46)
2	317 (38)	42 (42)	44 (36)	152 (38)	79 (38)
3	103 (13)	10 (10)	17 (14)	50 (13)	26 (13)
4	14 (2)	3 (3)	1 (1)	8 (2)	2 (1)
Other characteristics					
mRSS, mean (SD)	N=747 10 (8.1)	n=96 11 (7.6)	n=112 10 (8.5)	n=352 10 (7.6)	n=187 10 (8.8)
ΔmRSS,† mean (SD)	N=698 –0.4 (4.6)	n=88 0.5 (4.3)	n=103 –0.4 (3.1)	n=337 –0.3 (4.4)	n=170 –1.2 (5.6)
Reflux/dysphagia symptoms, n (%)	547/822 (67)	76/100 (76)	83/122 (68)	261/393 (66)	127/207 (61)
Digital ulcers, n (%)	266/808 (32)	35/100 (35)	38/118 (31)	141/386 (36)	5/2042 (25)
Tendon friction rubs, n (%)	73/804 (9)	7/99 (7)	10/119 (8)	35/383 (9)	21/203 (10)
Synovitis, n (%)	117/810 (14)	18/100 (18)	15/120 (13)	60/386 (16)	24/204 (12)
Muscle weakness, n (%)	182/814 (22)	25/100 (25)	31/120 (25)	78/388 (20)	48/206 (23)
Scleroderma renal crisis, n (%)	11/818 (1)	4/100 (4)	3/120 (2)	6/391 (2)	1/206 (0.5)
ESR, mean (SD)	766 (93) 26 (20.6)	98 (98) 29 (23.9)	115 (93) 25 (21.7)	361 (91) 26 (19.5)	192 (93) 25 (20.2)
Elevated CRP, n (%)	217/797 (27)	40/99 (30)	25/120 (33)	98/377 (26)	49/201 (24)
Immunosuppressant use, n (%)	89/244 (37)	8/20 (40)	8/31 (26)	51/121 (42)	22/72 (31)

Significant progression (FVC decline of >10%); moderate progression (FVC decline of 5% to 10%); stable ILD (FVC decline or improvement of <5%); moderate improvement (FVC improvement of 5% to 10%). Definitions of organ manifestations were described previously.[3 28] All characteristics were assessed before or on the index date. The following treatment options were received by the included patients at baseline, and for this study were defined as immunosuppressive: prednisone >10 mg/day, azathioprine, cyclophosphamide, mycophenolate, methotrexate or rituximab.

*Available for all 826 patients.

†Change from baseline to 12 months.

Ab, antibody; CRP, C-reactive protein; DL_{CO}, diffusion capacity of the lungs for carbon monoxide; ESR, erythrocyte sedimentation rate; FVC, forced vital capacity; mRSS, modified Rodnan skin score; NYHA, New York Heart Association; SSc, systemic sclerosis; SSc-ILD, systemic sclerosis-associated interstitial lung disease.

analyses of disease course in SSc-ILD have found different disease patterns in different patient cohorts.[8–11] However, these studies are limited by their small sample size, selected patient populations, significant referral biases and statistical instabilities of the trajectories. Randomised clinical trials provide valuable data, but the 12-month or 24-month duration often used[12–14] is insufficient for assessment of long-term disease course. There also remains a high unmet need to specifically identify patients with SSc at risk of progressive ILD. Risk factors for SSc-ILD progression have been proposed by several studies;[15–21] however, their clinical applicability and specific power to predict progression are limited. The optimal combination of risk factors to accurately predict progression has not been identified.

Treatments are available for SSc-ILD, but to date, nintedanib is the only approved treatment shown to reduce lung function decline in patients with SSc-ILD.[14 22 23] Current clinical practice emphasises treatment of patients with progressive ILD,[24] and a recent study showed that nintedanib reduces decline of forced vital capacity (FVC) in progressive ILD associated with various underlying conditions, including connective tissue disease-associated ILD.[25] However, waiting for FVC decline and/or extensive ILD involvement neglects the opportunity of early

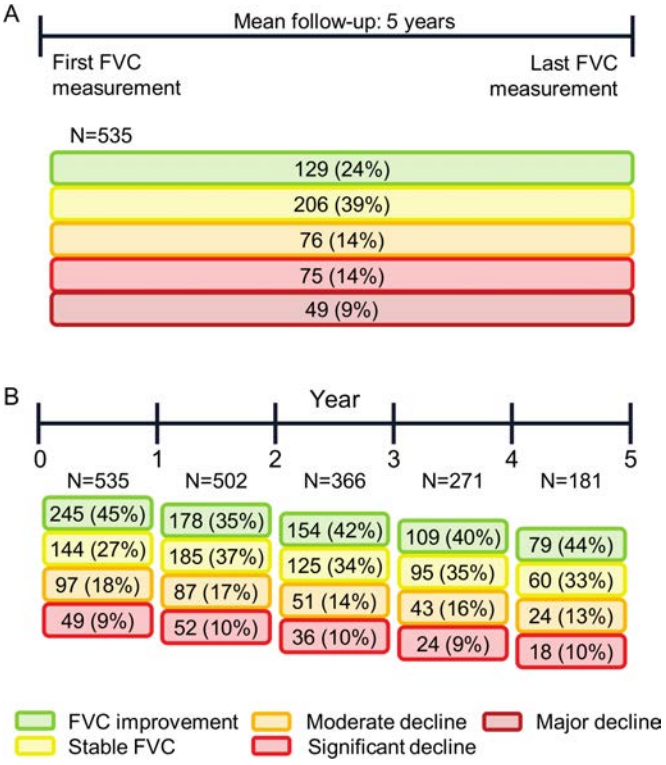

Figure 1 FVC changes among patients with SSc-ILD in the EUSTAR database (number of patients per category): (A) overall change during the 5-year follow-up period; (B) changes during each 12-month follow-up period. (A) Patients for whom ≥3 serial FVC measurements were available were divided into five disease course subgroups based on the overall difference between the first and last FVC measurement (% predicted): major decline (FVC decline of >20%); significant decline (FVC decline of >10% to 20%); moderate decline (FVC decline of 5% to 10%); stable (FVC decline or improvement of <5%); and improvement (FVC improvement of ≥5%). (B) Disease course each year was evaluated by determining the magnitude of FVC changes (% predicted) in each 12-month period during the mean 5-year follow-up defined as follows: significant decline (FVC decline of >10%); moderate decline (FVC decline of 5% to 10%); stable (FVC decline or improvement of <5%); and improvement (FVC improvement of ≥5%). EUSTAR, European Scleroderma Trials And Research; FVC, forced vital capacity; SSc-ILD, systemic sclerosis-associated interstitial lung disease.

treatment intervention until after clinically meaningful lung damage has occurred. Novel treatment concepts are therefore aiming to prevent progression and avoid irreversible damage to organs. This requires an understanding of the course and patterns of ILD progression, and reliable prediction algorithms that allow the specific detection of patients at risk of progression at a very early stage. Unfortunately, in SSc-ILD, this knowledge is currently lacking, and treatment initiation is often delayed in clinical practice by waiting for lung function decline over the preceding year before initiation.

The European Scleroderma Trials And Research (EUSTAR) group database is a large, real-world database representative of the general SSc population. It includes a wide range of patients with SSc-ILD, from those with mild and stable to advanced progressive disease.[26 27]

Thus, the aims of this study were: to assess the prevalence of progressive ILD over 12-month periods; to examine disease course and identify patterns of ILD progression in SSc over a 5-year period; and to identify risk factors predictive for progressive ILD in patients with SSc-ILD, using the EUSTAR database.

MATERIALS AND METHODS

Study design

Post hoc analyses of prospectively collected patient data from the EUSTAR database were conducted. The structure of the online database, the collected data set and definitions of clinical variables have been described in detail previously.[3 28]

Patient population and characteristics

Patients registered since 2010 in the EUSTAR database (start of the online version), aged ≥18 years, who fulfilled the 2013 American College of Rheumatology/European League Against Rheumatism SSc classification criteria;[29 30] with presence of ILD by HRCT or X-ray; recorded disease duration; and with available measurements of FVC and diffusion capacity of the lungs for carbon monoxide (DL_{CO}) at baseline and after 12±3 months were included.

Progressive ILD measured by FVC changes in a 12-month period

To reflect clinical practice with respect to patient follow-up, and the usual study duration in clinical SSc-ILD trials, absolute changes in FVC% predicted were first evaluated over a 1-year period (baseline to 12±3 months).[8 14 31 32] FVC decline ≥10% predicted is frequently used to define significant ILD progression and was therefore selected in this study as the main outcome measure for progressive ILD. Furthermore, an FVC decline >5% predicted is greater than the estimated minimum clinically important difference at a group level and has previously been associated with increased mortality in SSc.[33 34] Patients were therefore divided into four progressive ILD subgroups based on absolute change in FVC% predicted from baseline to 12±3 months: significant progression (decline of >10%); moderate progression (decline of 5% to 10%); stable ILD (decline or improvement of <5%); and improvement (improvement of ≥5%) (table 1). The prevalence of annual FVC changes was assessed prospectively in patients with available data over a mean follow-up of 5 years, using the definitions of progressive ILD described above.

Progressive ILD measured by changes in FVC and DL_{co} over 12 months

A decline in FVC of ≥10%, or a decline in FVC of 5% to 10% along with a decline in DL_{CO} of 15%, is a proposed definition of progressive fibrosis.[8 31 35 36] Therefore, we also assessed the prevalence of this combined endpoint.

Disease course and patterns in patients with SSc-ILD, measured by change in FVC from baseline to last available measurement

Because annual FVC patterns can change over time, we evaluated the overall lung function course in patients who had at least two 12-month periods with FVC measurements. These periods could be, but did not need to be, consecutive. For the overall FVC course, patients were divided into five subgroups based on the difference between the first and last available FVC measurement: major decline (FVC decline of >20%); significant decline (FVC decline of >10 and ≤20%); moderate decline (FVC decline of 5% to 10%); stable (FVC decline or improvement of <5%); and improvement (FVC improvement of ≥5%).

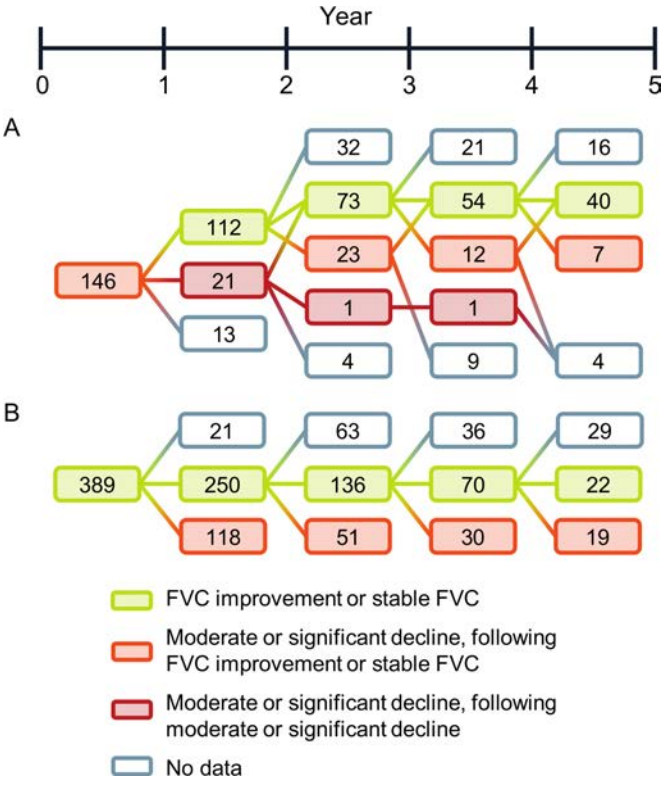

Figure 2 FVC changes in consecutive 12-month periods among patients with SSc-ILD in the EUSTAR database (number of patients per category): (A) subsequent course among patients with stable or improved FVC during the first year of follow-up; (B) subsequent course among patients with minor or moderate decline during the first year of follow-up and those who had further declines. Disease course each year was evaluated by determining the magnitude of FVC changes (% predicted) in individual patients in each 12-month period during the mean 5-year follow-up, defined as follows: significant decline (FVC decline of >10%); moderate decline (FVC decline of 5% to 10%); stable (FVC decline or improvement of <5%); and improvement (FVC improvement of ≥5%). EUSTAR, European Scleroderma Trials And Research; FVC, forced vital capacity; SSc-ILD, systemic sclerosis-associated interstitial lung disease.

The numbers of patients who experienced no 12-month period of decline, one period of decline (moderate or significant) or multiple periods of decline (moderate, significant or both) across all periods with data available over the 5-year follow-up were assessed. Patients with ILD progression were split into different progression patterns according to the number of FVC decline periods: rapid progression (no periods of FVC stability or improvement); progression (more periods of decline than stability/improvement); and slow progression (more periods of stability/improvement than decline).

Mortality

All-cause mortality was assessed in all patients with SSc-ILD, and in patients with progressive ILD, until last available follow-up.

Risk factors predictive for progressive ILD

Candidate baseline variables to predict progressive ILD were selected based on reports from the published literature and expert opinion: sex,[15] age,[16] reflux/dysphagia symptoms,[17 18] SSc subtype,[16] antibody status (anti-topoisomerase antibody (ATA) anti-centromere antibody (ACA), anti-RNA polymerase III antibody (ARA)),[16 19] baseline FVC,[16 20] baseline DL_{CO},[16 21] disease duration,[11 15 37 38] skin involvement measured by modified Rodnan skin score (mRSS),[16 19 21 39] erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) level, dyspnoea class, treatment, synovitis and muscle weakness.[16] Extent of lung fibrosis on HRCT was not included due to extensive missing data.

Statistical methods

All analyses were performed using IBM SPSS Statistics V.25 and Stata V.15. Pearson χ^2 test, Fisher's exact test or independent sample t-test was used, as appropriate. For correlation analyses, Pearson or Kendall's tau-b coefficients were applied as appropriate. All multivariable analyses were preceded by estimation of correlation between risk factors. Univariable and multivariable logistic regression analyses with OR and 95% CI were applied to analyse the predictive ability of baseline variables for progressive ILD. In the multivariable analyses, 10 events per variable were needed, and the variables were selected by expert opinion.

Univariable and multivariable linear mixed-effect models were performed to identify risk factors of longitudinal changes in FVC (% predicted) over the maximum 5-year follow-up period (baseline, 1, 2, 3, 4 and 5 years). Only patients with at least three serial FVC measurements were included in the analyses. Time and risk factors were fixed effects. Interaction effects between time and fixed factors were checked by including product terms in the models. Only significant interaction terms in the univariable analysis are presented, and they were further included in the multivariable model. Risk factors selected for multivariable analyses were based on expert opinion. All models included random intercept and slope, and an unstructured correlation matrix was used.

Patient and public involvement

EUSTAR is part of the World Scleroderma Foundation, which has patient representatives from the Federation of European Scleroderma Associations (FESCA) in its governing board.

RESULTS

Patient population

Within the EUSTAR database, 6004 patients included since 2010 aged ≥18 years fulfilled the SSc classification criteria and had lung imaging data available. Of these, 2259 (38%) had evidence of SSc-ILD on imaging, of which 826 had valid lung function data available after 12±3 months follow-up and were eligible for inclusion.

Demographic and clinical characteristics of all eligible patients are shown in table 1. No significant difference was observed in the baseline characteristics of the 826 eligible patients and the 1433 ineligible patients (online supplementary table S1).

Prevalence and risk factors of progressive ILD at 12 months

When analysing the prevalence of progressive ILD within the initial 12-month period, we found that 100 patients (12%) had significant ILD progression, 123 (15%) had moderate progression, 396 (48%) were stable and 207 (25%) had improvement.

In multivariable logistic regression analyses, FVC (OR 1.02; 95% CI 1.01 to 1.03; p<0.001), presence of reflux/dysphagia symptoms (OR 1.97; 95% CI 1.14 to 3.40; p=0.016) and mRSS (OR 1.06; 95% CI 1.00 to 1.12; p=0.036) at baseline were predictive for significant ILD progression at 12±3 months. No association was seen with age, sex, disease duration, antibody status, SSc subtype or immunosuppressant treatment.

Table 2 Number of patients (n (%)) with SSc-ILD in the EUSTAR database with 12-month periods of FVC decline, stratified by overall FVC decline from first to last available FVC measurement

		One 12-month period with FVC decline		Two or more 12-month periods with FVC decline		
Overall FVC change from baseline to last FVC	No decline (n=178)	Moderate decline (n=113)	Significant decline (n=107)	Only moderate declines (n=65)	One significant and ≥1 moderate decline (n=25)	Only significant declines (n=47)
Improved (n=129)	79 (44)	22 (20)	21 (20)	1 (2)	3 (12)	3 (6)
Stable (n=206)	99 (56)	59 (53)	29 (27)	13 (20)	1 (4)	5 (11)
Moderate decline (n=76)		28 (25)	17 (16)	25 (39)	1 (4)	5 (11)
Significant decline (n=75)		2 (2)	29 (27)	23 (35)	10 (40)	11 (23)
Major decline (n=49)		2 (2)	11 (10)	3 (5)	10 (40)	23 (49)

Overall FVC change from baseline to last FVC: major decline (FVC decline of >20%); significant decline (FVC decline of >10 and ≤20%); moderate decline (FVC decline of 5% to 10%); stable (FVC decline or improvement of <5%); and improvement (FVC improvement of ≥5%).
EUSTAR, European Scleroderma Trials And Research; FVC, forced vital capacity; SSc-ILD, systemic sclerosis-associated interstitial lung disease.

Prevalence and prediction of progressive ILD measured by the combined FVC and DL_{CO} definition over the initial 12-month period were comparable to these data (online supplementary table S1 and figure S1).

Disease course and ILD patterns in patients with SSc-ILD

A total of 535 (65%) patients with SSc-ILD had ≥3 FVC measurements available during the mean 5-year (±2.2) follow-up period, allowing for assessment of long-term ILD course. Baseline characteristics did not differ between patients with ≥3 and patients with <3 FVC measurements (n=291 (35%)).

To assess the overall disease course, we assessed FVC changes between baseline and last available FVC. We found that 49 (9%) showed major FVC decline (FVC decline of >20%); 75 (14%) had significant decline (FVC decline 10% to 20%); 76 (14%) had moderate decline (FVC decline 5% to 10%); 206 (39%) were stable (FVC changes <5%); and 129 (24%) experienced improvement in FVC (FVC improvement >5%) over the overall disease course (mean 5-year follow-up) (figure 1A). The prevalence of significant ILD progression was between 13% and 18% and the prevalence of moderate progression was between 9% and 10% in each 12-month period over this 5-year follow-up (figure 1B). These progressive periods rarely appeared in consecutive 12-month periods, and progressive periods were mostly followed by stable periods (figure 2A). Stable periods were followed by a progressive period in about 30% of cases (figure 2B). Irrespective of the severity of overall FVC decline (major, significant or moderate), most patients still experienced at least one 12-month period of stable or improving FVC (table 2). On the other hand, patients with stable or improved overall FVC could still experience 12-month periods of FVC decline; these declines were more frequently moderate (FVC decline 5% to 10%) than significant (FVC decline 10% to 20%). Only 178 (33%) patients experienced no period of FVC decline of ≥5% during any 12-month period (table 2).

Most patients with SSc-ILD with an overall FVC decline over 5 years had a slow pattern of lung function decline, with more periods of stability/improvement than decline (58%); 34% showed a progressive pattern, with more periods of decline than stability/improvement and slow progression. Only 16 (8%)

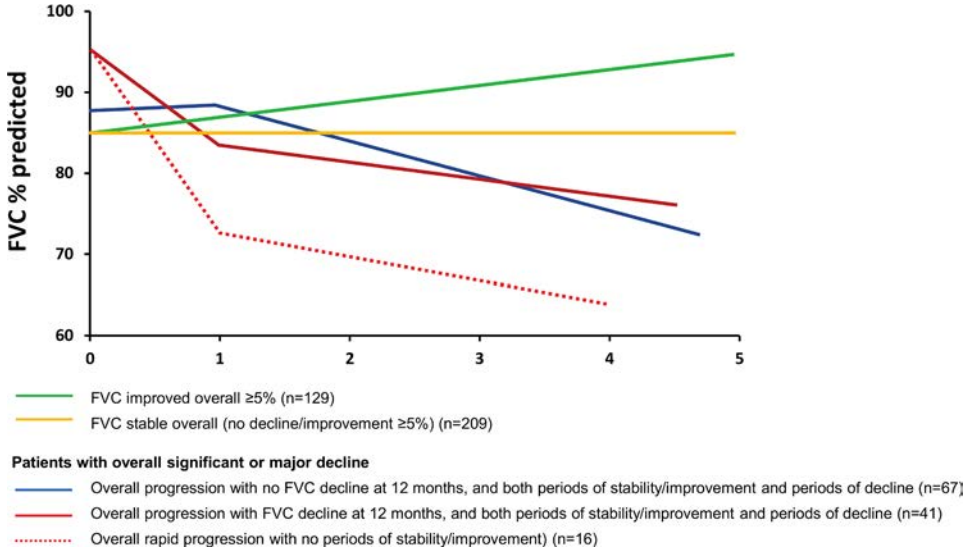

Figure 3 Patterns of disease course in SSc-ILD. Overall disease course was evaluated by determining the magnitude of FVC changes (% predicted) in individual patients from baseline to the end of follow-up defined as follows: major decline (FVC decline of >20%); significant decline (FVC decline of 10% to 20%); moderate decline (FVC decline of 5% to 10%); stable (FVC decline or improvement of <5%); and improvement (FVC improvement of ≥5%). Patterns of disease progression are shown in patients with improved FVC, stable FVC and those with significant or major decline. FVC, forced vital capacity; SSc-ILD, systemic sclerosis-associated interstitial lung disease.

Table 3 Risk factors for change in FVC over the 5-year follow-up in patients with ≥3 serial FVC measurements in univariable and multivariable linear mixed-effect regression analysis

	Univariable			Multivariable		
Predictor variable	Coefficient	95% CI	P value	Coefficient	95% CI	P value
Time	−0.45	−0.72 to −1.7	0.002	0.8	0.22 to 1.39	0.007
Reflux/dysphagia symptoms	−2.06	−5.06 to 0.94	0.180	0.58	−2.18 to 3.34	0.681
Time×reflux/dysphagia symptoms	−0.76	−1.34 to −0.17	0.011	−0.72	−1.34 to −0.10	0.024
mRSS	−0.51	−0.69 to −0.33	<0.001	−0.31	−0.47 to −0.15	<0.001
Time×mRSS	−0.05	−0.07 to −0.01	0.011	−0.06	−0.10 to −0.02	0.002
Sex	−5.25	−8.91 to −1.59	0.005	−3.90	−7.29 to −0.53	0.024
Time×sex	−0.97	−1.72 to −0.21	0.012	−1.30	−2.10 to −0.49	0.002
Age	0.42	0.31 to 1.53	<0.001	0.47	0.37 to 0.57	<0.001
DL_{CO}	0.55	0.47 to 0.62	<0.001	0.45	0.37 to 0.52	<0.001
ESR	−0.14	−0.21 to −0.01	0.001	−0.09	−0.15 to −0.03	0.005
NYHA class	−14.59	−18.7 to −10.49	<0.001	−4.76	−6.59 to −2.92	<0.001
ACA	11.42	7.65 to 15.19	<0.001			
ARA	10.95	1.62 to 20.27	0.021			
ATA	−5.01	−7.98 to −2.05	0.001			
CRP	−7.72	−11.01 to −4.43	<0.001			
dcSSc	−6.37	−7.43 to −3.32	<0.001			

ACA, anti-centromere antibody; ARA, anti-RNA polymerase III antibody; ATA, anti-topoisomerase I antibody; CRP, C-reactive protein; dcSSc, diffuse cutaneous systemic sclerosis; DL_{CO}, diffusion capacity of the lungs for carbon monoxide; ESR, erythrocyte sedimentation rate; FVC, forced vital capacity; mRSS, modified Rodnan skin score; NYHA, New York Heart Association.

patients showed a rapidly declining FVC pattern, with several consecutive episodes of FVC decline and no periods of FVC stability or improvement. Patterns of progression in patients with moderate, significant and major overall decline in FVC%, stratified by the presence or absence of a decline in the first 12 months, are shown in figure 3.

Risk factors predictive of 5-year FVC decline

To identify SSc-ILD patients at risk of ILD progression, we assessed the predictive value of baseline clinical variables on FVC measurements over the 5-year follow-up period. In multivariable linear mixed-effect models, we identified male sex, presence of reflux/dysphagia symptoms and high baseline mRSS as the strongest predictors, with significant interaction effects between time and these variables. This indicates that FVC changed differently over time as a function of one of these predictors (ie, different slopes). Older age, higher DL_{CO}, dyspnoea (New York Heart Association class 3 or 4) and increased ESR were also significantly predictive for FVC decline but without a time interaction effect, indicating that the FVC changed significantly over time but not differently between patients with or without these clinical features (table 3). Immunosuppressive treatment was not predictive for FVC decline over time.

Mortality

Of 826 patients with SSc-ILD, 85 (10%) died during follow-up. There were no significant differences in mortality rate between patients with significant ILD progression (11 (12%)), moderate progression (18 (15%)) or stable ILD (36 (9%)) over the initial 12±3-month period. In patients with overall FVC changes measured between baseline and last available FVC, death occurred in 9 of 49 (19%) patients with major decline; 7 of 75 (9%) patients with significant decline; 12 of 76 patients (16%) with moderate decline; 18 of 206 (9%) patients who were stable; and 9 of 129 (7%) patients with improvement, with differences not statistically significant. As there were only a small number of events, no regression analyses were performed.

DISCUSSION

This is the largest study to prospectively analyse the prevalence of progressive ILD in patients with SSc-ILD, and the first to describe comprehensively the disease course and patterns of ILD progression in patients with SSc over the long term.

The proportion of patients with SSc-ILD who experienced progressive ILD during the initial 12±3-month period was 27%, and in each 12-month period over the mean 5-year follow-up, 23% to 27% of patients experienced progression. These findings are in agreement with estimates of progressive ILD prevalence of 31% to 32% derived from serial lung function data in patients with SSc[6] and an international physician survey.[40]

Here, we show that patterns of FVC are frequently inconsistent between consecutive 12-month periods. Most patients who experienced an overall decline in FVC had periods of FVC improvement and, conversely, some patients whose FVC improved overall had periods of FVC decline. Patients with overall major FVC decline (FVC decline >20% over the entire study period) usually had several 12-month periods with FVC decline >10% rather than FVC decline of 5% to 10%. Others experienced a slower, but cumulative course of declining FVC. Such patients with slower progression can easily be overlooked in clinical practice and in current treatment strategies that target patients who progress rapidly and with significant FVC changes. Smaller changes in FVC (5% to 10%) may in themselves be clinically significant, as seen in patients with idiopathic pulmonary fibrosis.[41 42] In clinical practice, this means that FVC decline >5% should alert physicians, especially when multiple declines occur, even when not in consecutive periods. These results highlight a pitfall in current clinical practice, where treatment is often initiated after FVC decline has happened, and thus when lung damage has already occurred.[24] Novel treatment concepts are needed and should aim for prevention of progression to avoid irreversible organ damage. These results also stress and highlight the need for close monitoring of all patients with SSc-ILD, as also recently suggested by the European expert consensus.[43] Respiratory symptoms, changes in HRCT findings and desaturation on

exercise tests should all be implemented in clinical practice to assess ILD progression and aid treatment decisions.

Robust predictive risk factors are very important for the early identification of progressive patients. Our large, multicentre study demonstrates that skin fibrosis (higher mRSS), male sex and the presence of reflux/dysphagia symptoms are the strongest predictors for FVC decline over time. Other predictive parameters included the presence of inflammation (higher ESR) and shorter disease duration, which are already frequently used as enrichment strategies for clinical studies. These parameters may also be applied in daily clinical practice, helping to identify patients who should receive treatment early, even before the first FVC decline has occurred. However, if earlier treatment of patients at risk for FVC decline truly leads to better outcomes, it needs to be analysed in appropriate randomised controlled clinical trials in the future. Risk factors identified in this study are potential inclusion criteria for such a trial. Interestingly, contrary to our finding that higher FVC at baseline was predictive for ILD progression, previous studies suggested that lower FVC at baseline is a risk factor for progressive ILD.[16 20] These studies included some SSc patients without ILD, and one study assessed patients within 3 years of SSc diagnosis. Furthermore, definitions of progression (FVC decline of ≥15%,[20] FVC or DL_{CO} decline of ≥15%, or FVC or DL_{CO} falling below 55%[16]) differed from those in our study. The strongest association with further FVC decline was seen in patients with baseline FVC <65% predicted,[16] lower than the mean value in our study (86%). Our contrasting findings may reflect these differences and should be assessed in other unselected cohorts.

Strengths of our study include the use of a large set of prospective, representative real-world data, which increases the applicability of our results to clinical practice and different definitions of ILD progression. Nonetheless, this study has several limitations. While the data were gathered prospectively, this was a post hoc analysis. No central lung function reading was conducted, increasing the variability of FVC and DL_{CO}. Most patients with SSc-ILD in the database (1433/2259) did not have serial lung function data. Data on immunosuppressant use were only available for 244/826 eligible patients, and the exact date of initiation, treatment indication and cumulative doses are unknown. Several studies[7 8 15] have suggested that the extent of lung fibrosis by HRCT is prognostic for disease progression and mortality in SSc-ILD. Although data regarding the extent of lung fibrosis were not available in the database for the present analysis, they may be a valuable addition in future studies. Recent analyses also suggest that mRSS progression is an important risk factor for later FVC progression,[39] which was not analysed in this study. A lead time bias cannot be excluded, as this was not an incident cohort. Finally, ILD-specific mortality was not available in the EUSTAR database. Here, all-cause mortality was not influenced by ILD progression; as recently highlighted,[5] it is likely that ILD-specific mortality differs between progressive and stable ILD patients.

CONCLUSION

This study provides novel insights into the occurrence of progressive ILD in SSc-ILD. The results stress the heterogeneity and variability of the course of ILD in SSc. Close monitoring of patients with SSc-ILD and awareness of the variable course of progression is of high importance in considering when to initiate treatment.

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TRANSLATIONAL SCIENCE

Machine learning integration of scleroderma histology and gene expression identifies fibroblast polarisation as a hallmark of clinical severity and improvement

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ABSTRACT

Objective We sought to determine histologic and gene expression features of clinical improvement in early diffuse cutaneous systemic sclerosis (dcSSc; scleroderma).

Methods Fifty-eight forearm biopsies were evaluated from 26 individuals with dcSSc in two clinical trials. Histologic/immunophenotypic assessments of global severity, alpha-smooth muscle actin (aSMA), CD34, collagen, inflammatory infiltrate, follicles and thickness were compared with gene expression and clinical data. Support vector machine learning was performed using scleroderma gene expression subset (normal-like, fibroproliferative, inflammatory) as classifiers and histology scores as inputs. Comparison of w-vector mean absolute weights was used to identify histologic features most predictive of gene expression subset. We then tested for differential gene expression according to histologic severity and compared those with clinical improvement (according to the Combined Response Index in Systemic Sclerosis).

Results aSMA was highest and CD34 lowest in samples with highest local Modified Rodnan Skin Score. CD34 and aSMA changed significantly from baseline to 52 weeks in clinical improvers. CD34 and aSMA were the strongest predictors of gene expression subset, with highest CD34 staining in the normal-like subset ($p<0.001$) and highest aSMA staining in the inflammatory subset ($p=0.016$). Analysis of gene expression according to CD34 and aSMA binarised scores identified a 47-gene fibroblast polarisation signature that decreases over time only in improvers (vs non-improvers). Pathway analysis of these genes identified gene expression signatures of inflammatory fibroblasts.

Conclusion CD34 and aSMA stains describe distinct fibroblast polarisation states, are associated with gene expression subsets and clinical assessments, and may be useful biomarkers of clinical severity and improvement in dcSSc.

Key messages

What is already known about this subject?

- Systemic sclerosis (SSc) histologic features (collagen score, alpha-smooth muscle actin (aSMA) and biopsy weight) have been shown to correlate with the Modified Rodnan Skin Score.

What does this study add?

- CD34 staining decreases with worsening clinical severity and subsequently increases with clinical improvement. Conversely, aSMA staining increases with worsening clinical severity and subsequently decreases with clinical improvement.
- Fibroblast polarisation, according to aSMA and CD34 staining intensity, can be used to distinguish between scleroderma gene expression subsets.
- We identify a robust fibroblast polarisation gene expression signature that decreases over time in those with clinical improvement, but not in those who do not improve.

How might this impact on clinical practice or future developments?

- Dermal fibroblast polarisation between aSMA and CD34 may be used to describe clinical improvement among individuals with diffuse cutaneous SSc.

INTRODUCTION

Systemic sclerosis (SSc; scleroderma) is an autoimmune disorder characterised by vasculopathy, inflammation and fibrosis of the skin and internal organs.[1] Among rheumatic diseases, SSc carries the highest mortality rate, in part due to limited treatment options that do not address both the fibrotic and the inflammatory disease features.[2] Progress in the field is limited by patient heterogeneity and imperfect outcome measurements,[3] and there is a growing need to discover novel treatment targets.

Most SSc treatment trials have used the Modified Rodnan Skin Score (MRSS) as the primary outcome measurement tool. This validated measure of skin thickness has limitations including interobserver variability.[4] The Combined Response Index in Systemic Sclerosis (CRISS) is a composite outcome measure that incorporates new scleroderma renal crisis, decline in forced vital capacity (FVC)

>15%-predicted, new heart failure and pulmonary hypertension requiring treatment, as well as change in MRSS, patient and physician global assessments, Health Assessment Questionnaire Disability Index (HAQ-DI) and FVC. The CRISS output is a probability of clinical improvement (0—1), and a threshold of ≥0.6 has been proposed.[5]

Many SSc trials use skin histology and/or gene expression as exploratory outcomes. Skin biopsies have good face, content, criterion and construct validity[6]; however, no standardised approach exists for interpreting histology in clinical trials. Previous studies have described correlations of skin biopsy weight,[7] collagen and alpha-smooth muscle actin (aSMA) with MRSS.[8] CD34, a dermal fibroblast marker, is decreased in SSc versus normal skin.[9] Skin gene expression may also describe SSc clinical severity and heterogeneity. Previous studies identified three gene expression subsets in diffuse cutaneous SSc (dcSSc) skin: normal-like, fibroproliferative and inflammatory.[10 11] These subsets have been incorporated into stratified clinical trial analyses to better understand patient heterogeneity and treatment response.[12–18]

The purpose of this study was to determine which histologic features of SSc lesional skin are most informative of gene expression subset and to use those histologic features to focus subsequent gene expression analyses to gain insights relevant to clinical improvement. We aimed (1) to define SSc skin histologic correlates of clinical improvement, (2) to assess the power of histologic features to predict gene expression subsets using an unbiased machine learning approach and (3) to integrate histology-based gene expression analyses with 52-week clinical improvement.

METHODS

Patient data

Fifty-eight forearm skin biopsies from 26 individuals with early dcSSc were assessed by physical examination, DNA microarray and histology in the context of two clinical trials at Hospital for Special Surgery (New York): the nilotinib in SSc trial (N=8)[15] and the belimumab in SSc trial (N=18).[12] The nilotinib trial was an open-label, single-arm pilot trial where background immunosuppressive treatment was not permitted and all participants received nilotinib, a tyrosine kinase inhibitor. The belimumab trial was a randomised, controlled pilot trial where all participants received background mycophenolate mofetil and were randomised to receive either intravenous belimumab or placebo. Clinical data were collected including disease duration, autoantibodies, clinical assessments of lung and renal SSc involvement, C reactive protein, erythrocyte sedimentation rate (ESR), FVC, total MRSS, local (biopsy site) MRSS (scored by a single assessor using 'averaging' approach[19]), physician and patient global assessments and HAQ-DI. Fifty-two-week CRISS was calculated.

Sample processing

Two 3 mm punch biopsies of extensor surface, forearm lesional skin were obtained (nilotinib: baseline, 26, 52 weeks; belimumab: baseline, 52 weeks).[12 15] Subsequent biopsies were performed 1 cm from baseline procedure. One biopsy was formalin-fixed, paraffin-embedded and stained for H&E, aSMA (Leica PA0943, RTU) and CD34 (Leica PA0212, RTU). The other biopsy was analysed by DNA microarray as described previously and in online supplemental methods.[12 15] Microarray data were log2-lowess normalised and filtered for probes with intensity ≥1.5-fold over local background. Probes with >20% missing data were excluded. Missing expression values were imputed using GenePattern module (ImputeMissingValues.KNN) with default parameters, and probe expression set was collapsed to gene expression set using respective GenePattern modules.[20] Nilotinib samples were processed in a single batch. Belimumab samples were processed in two batches. These three batches did not exhibit a significant batch bias (as determined by gPCA, p=0.434, online supplemental figure 1); therefore, no batch adjustment was performed. Expression data are accessible at NCBI GEO (accession nos GSE65405 and GSE97248, respectively).

Histologic evaluation

Each sample was assessed using a histology scoring system that includes seven histologic/immunophenotypic features: thickness (epidermis to subcutis, measured by micrometre), follicle count and a semiquantitative (0—3) assessment of global histologic severity, infiltrate intensity and collagen density detectable by H&E stain, and two fibroblast markers: CD34 and aSMA (online supplemental figure 2). Similar to a patient or physician global assessment, the pathologist global assessment of histologic severity is a summary assessment of the histologic features assessed by H&E stain. Blinded to prior scores, a dermatopathologist (CM) and second pathologist (YZ) analysed a subsample of biopsies (N=12) for reliability.

Statistical analysis

Intraclass correlation coefficients were calculated for inter-rater and intrarater reliability for each histology domain. Median (IQR) score of each histology feature was calculated by local MRSS. In a paired analysis (baseline and 52 weeks), Wilcoxon signed-rank test was used to evaluate histologic change, stratified by CRISS 0.6 probability threshold to differentiate clinical improvers and non-improvers.[5] Spearman correlation was used to correlate histologic change with 52-week CRISS and change in each clinical outcome included in CRISS. Kruskal-Wallis and Mann-Whitney U tests, Bonferroni-adjusted for multiple comparisons, were used to determine differences in clinical characteristics and histologic features by gene expression subset assignment.

Predicting gene expression subset assignment using histologic features

Online supplemental figure 3 outlines the data processing pipeline. Samples were assigned to gene expression subsets using multinomial elastic net supervised classifier (GLMnet), as previously developed.[21] For each sample, the classifier assigns a probability for belonging to each gene expression subset (sum of probabilities equals 100%), and samples are assigned to the subset with the highest probability. Then, using histologic features as inputs and gene expression subsets as classifiers, support vector machine learning was performed to determine histologic features most predictive of gene expression subset. To binarise continuous variables (ie, thickness), quantiles were generated. The area under the curve (AUC) of the receiver operating characteristic (ROC) curves generated for each gene expression subset was calculated to evaluate algorithm performance. Mean absolute weight for each histology score was determined using 'w-vector' to identify histologic features and associated scores most predictive of subset assignment.

Differential gene expression by histologic features

Using binarised scores for the histologic/immunophenotypic features with the highest weight for classifying samples (CD34

Table 1 Baseline characteristics of study cohort

Patient characteristics	Total cohort (N=26)
Age, mean (SD)	50.7 (13.6)
Disease duration* (years), median (IQR)	0.8 (0.6–1.2)
Sex, female, n (%)	20 (77)
Race, n (%)	
White	19 (73)
Black	5 (19)
Asian	2 (8)
SSc-specific autoantibodies, positive, n (%)	
Anti-topoisomerase I (Scl-70)	6 (23)
Anti-centromere	2 (8)
Anti-RNA polymerase III	15 (58)
ILD present, n (%)	8 (31)
FVC %-predicted, mean (SD)	86 (16)
DLCO %-predicted, mean (SD)	79 (17)
History of renal crisis, n (%)	2 (8)
MRSS, total, median (IQR)	25 (22–31)
MRSS, forearm (local), mean (SD)	2 (0.75)
Physician Global Assessment (0–10), median (IQR)	5.8 (5.0–6.8)
Patient Global Assessment (0–10), median (IQR)	2.9 (1.1–4.7)
HAQ-DI, mean (SD)	0.85 (0.59)

*Time since first non-Raynaud's disease symptom.
DLCO, diffusing capacity for carbon monoxide in the lungs; FVC, forced vital capacity; HAQ-DI, Health Assessment Questionnaire Disability Index; ILD, interstitial lung disease; MRSS, Modified Rodnan Skin Score; SSc, systemic sclerosis.

and aSMA), differentially expressed genes (DEGs) were identified using an unequal sample size and unequal variance t-test between groups. Significant genes were defined as those with Bonferroni-adjusted p value <0.05. Hierarchical clustering was performed for DEG identified according to binarised histology scores. DEGs identified by histology scores were similarly analysed in paired samples, stratified by improvement status. Average DEG expression was plotted by clinical improvement status. Hierarchical clustering, supervised by baseline or 52 weeks, was performed for significant genes in improvers and non-improvers.

Pathway analysis

Pathway analysis was performed for 47 identified genes differentially expressed according to aSMA and CD34 scores (goseq R package).[22] The background gene set was the 16 645 genes expressed in the skin samples (genes with intensity >1.5-fold over background Cy3 and Cy5 in the microarray). All gene ontology (GO) pathways were considered. Benjamini-Hochberg adjusted false discovery rate <5% was considered significant.

RESULTS

Histologic features underlying biopsy site assessments of SSc severity

Fifty-six biopsies were analysed from 26 individuals with dcSSc (median disease duration 0.8 years). Patient characteristics are presented in table 1. Reliability of histology scores are presented in online supplemental table 1. To describe histologic features underlying biopsy site clinical assessments of SSc severity, median histology scores were calculated for each local MRSS score (table 2). Higher global severity, aSMA, and collagen density and lower CD34 scores were observed for samples with highest (worse) local MRSS. The reverse was true for lowest local MRSS samples where global severity, aSMA and collagen density was low and CD34 was high.

Histologic correlates of clinical improvement

Paired baseline and 52-week skin biopsies were available for 24 individuals. Samples were stratified according to clinical improvers (CRISS ≥0.6) versus non-improvers (online supplemental table 2). As expected, clinical improvers had significant improvements in total and local MRSS, physician global assessment, patient global assessment and HAQ-DI (figure 1A). Among improvers, there were significant changes in baseline versus 52-week global histologic severity (2.5 to 1.5, p<0.01), aSMA (2 to 0.5, p=0.04), CD34 (1 to 2, p<0.001) and collagen density (2 to 1, p<0.01) (figure 1B). Among six non-improvers, there were no significant changes in any baseline versus 52-week histology scores. Because the CRISS threshold for improvement is still provisional, we also compared histology changes to CRISS as a continuous measure. Consistent with the previous analysis using a CRISS threshold of ≥0.6, increasing (more favourable) CRISS correlated with decreasing global histologic severity (r=−0.52, p=0.01), aSMA (r=−0.44, p=0.03) and collagen density (r=−0.44, p=0.03), and increasing CD34 (r=0.53, p=0.01) (table 3).

Histologic features to predict gene expression subset

Twenty-four, 16 and 18 samples were assigned to normal-like, fibroproliferative or inflammatory gene expression subsets, respectively. Skin biopsy sites from normal-like, fibroproliferative or inflammatory samples were associated with increasing clinical severity with median local MRSS of 1, 2 and 3, respectively (p<0.01) (figure 2A). There was also a significant trend of increasing total MRSS and ESR and decreasing disease duration across the three gene expression subsets (figure 2A). Histologic/immunophenotypic features also associated with gene expression subset. CD34 staining was highest in normal-like compared

Table 2 Histologic features underlying local (biopsy site) MRSS for 26 individuals with diffuse cutaneous systemic sclerosis contributing 58 skin biopsy samples

Histologic feature	Local MRSS=0 (N=3)	Local MRSS=1 (N=23)	Local MRSS=2 (N=17)	Local MRSS=3 (N=15)
Global	0.5 (0–2.5)	1.5 (0.5–2.5)	2 (2–2.5)	2 (2–3)
aSMA	0 (0–1)	0.5 (0–1.5)	1 (0–2)	2 (1–2)
CD34	3 (1.5–3)	2 (1–3)	1 (1–2)	1 (0–1)
Collagen	0 (0–2.5)	1 (0.5–2)	2 (1.5–2.5)	2.5 (2–3)
Infiltrate	0.5 (0.5–1)	0.5 (0–1)	0.5 (0.5–1)	0.5 (0.5–1)
Follicles	2 (2–3)	1 (0–2)	0 (0–1)	0 (0–1)
Thickness	1.9 (1.3–2)	2.6 (2.1–3.0)	2.4 (2.1–2.7)	2.8 (2.2–3.0)

Thickness is measured in micrometres from epidermis to subcutis. Median (IQR) is reported.
aSMA, alpha-smooth muscle actin; MRSS, Modified Rodnan Skin Score.

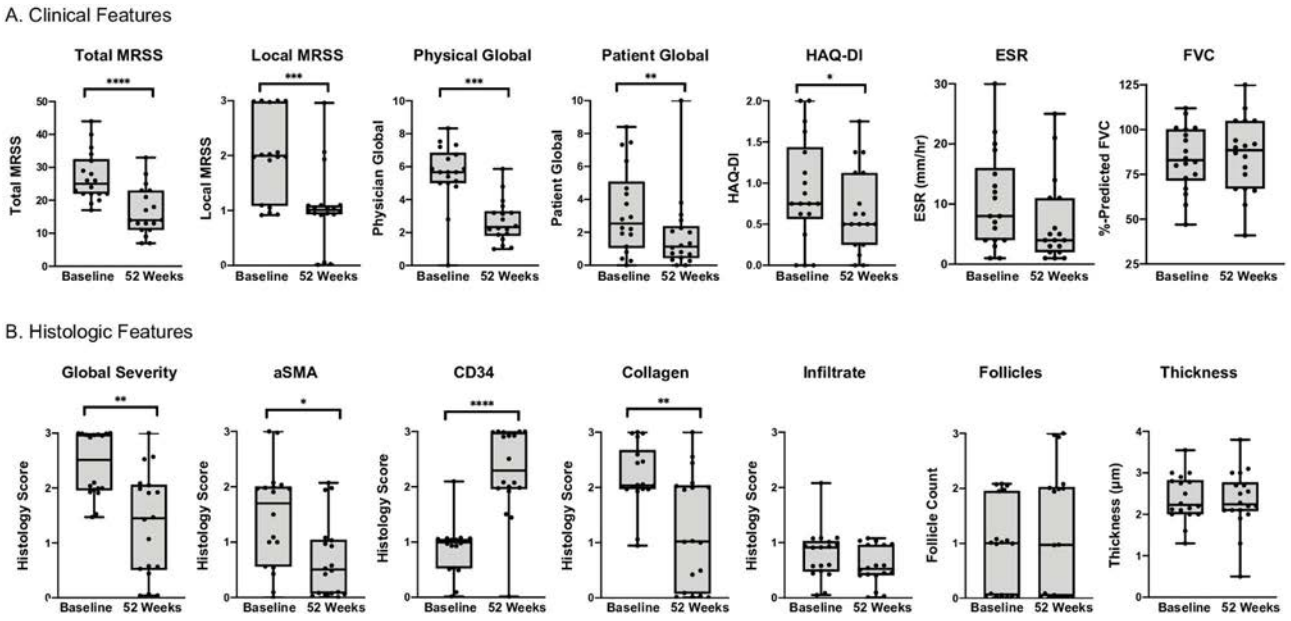

Figure 1 Clinical and histologic correlates of CRISS ≥0.6 (N=18). Among 18 individuals with diffuse cutaneous systemic sclerosis with 52-week CRISS ≥0.6, (A) clinical and (B) histologic changes in paired baseline and 52-week samples are demonstrated. Boxplots have whiskers from minimum to maximum values, a horizontal line at median value, and box edges at lower (Q1) and upper quartiles (Q3). P values represent results of Wilcoxon signed-rank test. ****p≤0.0001, ***p≤0.001, **p≤0.01, *p≤0.05. Adjusting for multiple comparisons, local MRSS, total MRSS, physician global assessment, patient global assessment, global histologic severity and CD34 remain statistically significant. aSMA, alpha-smooth muscle actin; CRISS, Combined Response Index in Systemic Sclerosis; ESR, erythrocyte sedimentation rate; FVC, %-predicted forced vital capacity; HAQ-DI, Health Assessment Questionnaire Disability Index; MRSS, Modified Rodnan Skin Score.

with fibroproliferative and inflammatory samples (median score 2, 1, 0.5, respectively; p<0.001) (figure 2B). Conversely, aSMA staining was lowest in normal-like versus fibroproliferative and inflammatory samples (0.5, 0.75, 2, respectively; p=0.02). There were also significant differences in global severity (1.5, 2.25, 2, respectively; p=0.01) and collagen density (1, 2, 2.5, respectively; p=0.01).

We next tested the performance of a machine learning algorithm using histologic features to predict gene expression subset. The AUC of the ROC curve of models predicting inflammatory, normal-like and fibroproliferative gene expression subsets were 0.72, 0.66 and 0.52, respectively (figure 2C). The histology features with strongest predictive values (highest mean weight) were CD34 and aSMA (figure 2D). In subsequent machine learning models, using only either CD34 or aSMA as inputs, CD34 predicted fibroproliferative subsets (AUC 0.77) and normal-like subsets (AUC 0.76), while aSMA predicted inflammatory subsets (AUC 0.73).

Gene expression signature of high aSMA versus high CD34 scleroderma skin

aSMA was highest and CD34 was lowest in samples with high MRSS and high inflammatory gene expression and reversed with clinical improvement (table 2, figures 1B and 2B). Taken together, these data support a strong and clinically relevant inverse relationship between the two dermal fibroblast markers. To visualise this, we plotted aSMA and CD34 scores for all samples (figure 3A). Samples with highest aSMA and lowest CD34 scores were often assigned to the inflammatory gene expression subset, while the inverse was true of the normal-like subset. We next sought to uncover gene expression signatures that characterise fibroblast polarisation. We identified one DEG when constraining the analysis to aSMA high versus low and 32 DEGs when constraining the analysis to CD34 high versus low. When we further focused the analysis to samples with either extreme of the immunophenotype ($aSMA^{low}/CD34^{high}$ vs

Table 3 Correlation of 52-week histology change† with CRISS and change in clinical findings among 24 individuals with diffuse cutaneous systemic sclerosis

Histology	CRISS	Total MRSS	Physician global	Patient global	HAQ-DI	FVC
Global	−0.52*	0.43*	0.11	0.07	0.27	−0.31
aSMA	−0.44*	0.46*	0.20	0.30	0.14	−0.02
CD34	0.53*	−0.30	−0.42*	−0.46*	−0.51*	0.35
Collagen	−0.44*	0.32	0.09	0.22	0.12	−0.25
Infiltrate	−0.09	0.11	0.04	0.14	0.08	−0.16
Follicles	−0.09	0.23	0.26	0.10	−0.21	0.39
Thickness	−0.20	0.25	0.33	0.41*	0.13	−0.10

*Significant p value <0.05. Spearman correlation coefficients reported.
†Histology change categorised as decreased, unchanged or increased score from baseline to 52 weeks.
aSMA, alpha-smooth muscle actin; CRISS, Combined Response Index in Systemic Sclerosis; FVC, %-predicted forced vital capacity; HAQ-DI, Health Assessment Questionnaire Disability Index; MRSS, Modified Rodnan Skin Score.

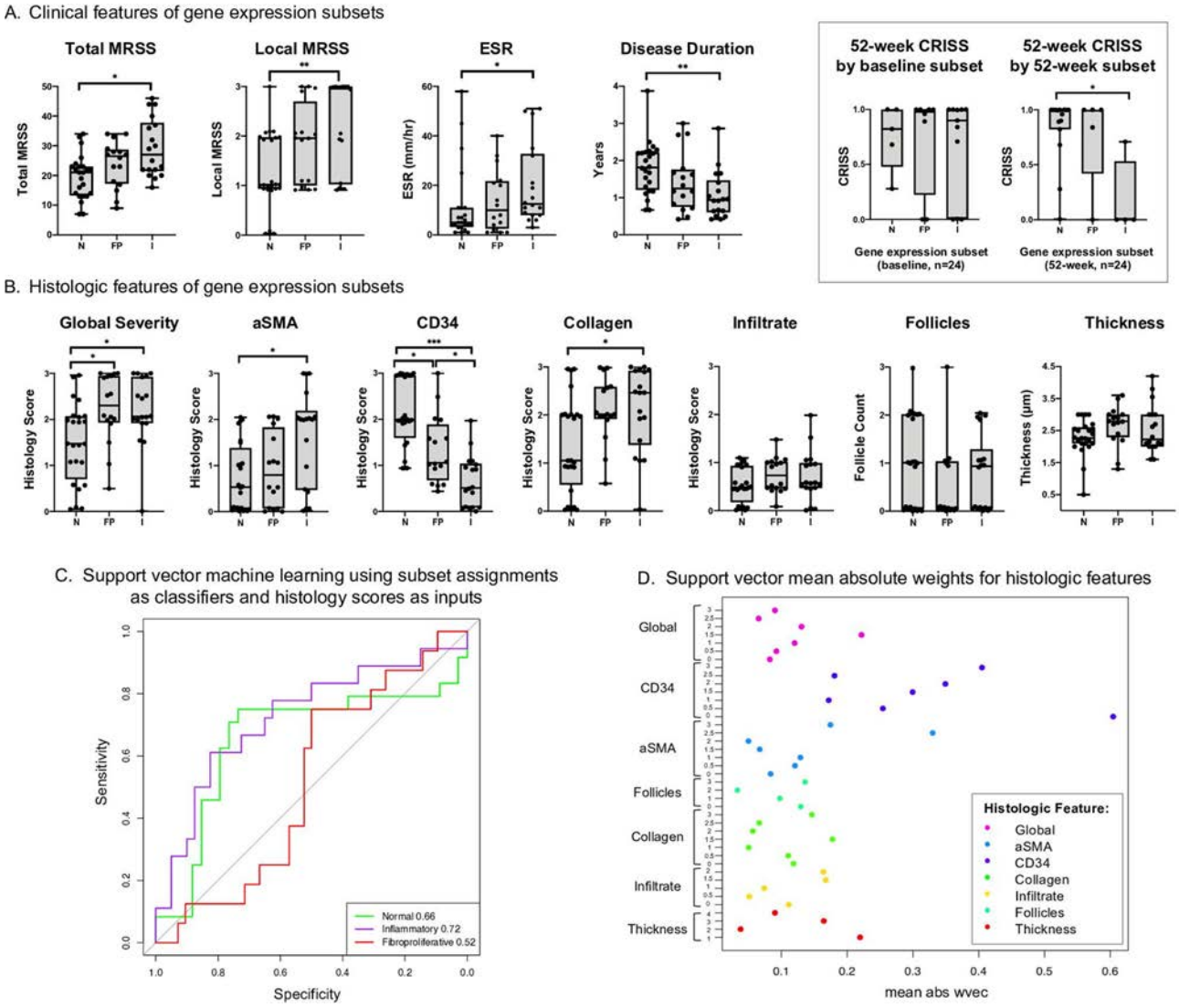

Figure 2 Clinical and histologic correlates of three gene expression subsets among 26 individuals with diffuse cutaneous systemic sclerosis. (A) Clinical features of samples assigned to each gene expression subset (N=normal like, FP=fibroproliferative, I=inflammatory). Box displays 52-week CRISS, stratified by baseline and 52-week gene expression subset for 24 individuals with paired biopsy samples. Boxplots have whiskers from minimum to maximum values, a horizontal line at median value and box edges at lower (Q1) and upper quartiles (Q3). (B) Histologic features of samples assigned to each gene expression subset. (C) Support vector machine learning was performed using gene expression subset as classifiers and the seven histology feature scores (global severity, aSMA, CD34, collagen density, infiltrate, follicle count and thickness) as inputs. For continuous variables (ie, thickness), quantiles were generated. Area under the curve (AUC) of the receiver operating characteristic curves assessed algorithm performance and are shown in the lower right legend. The p values for the AUC for normal-like, inflammatory and fibroproliferative subsets are 0.02, 0.003 and 0.58, respectively. (D) Support vector mean absolute weights for each binarised histology score from 'w-vector' model identified histologic features most predictive of subset assignment. P values represent results of Mann-Whitney U test, adjusted for multiple comparisons using Bonferroni correction. ***p≤0.001, **p≤0.01, *p≤0.05. aSMA, alpha-smoothmuscle actin; CRISS, Combined Response Index in Systemic Sclerosis; ESR, erythrocyte sedimentation rate; MRSS, Modified Rodnan Skin Score.

aSMA^{high}/CD34^{low}), we identified 36 DEGs. The union of these results yielded a total of 47 genes, which we refer to as aSMA/CD34 polarisation genes (figure 3B).

We compared unsupervised hierarchical clustering of the aSMA/CD34 polarisation genes (figure 3C) to local MRSS (figure 3C top colour bar), gene expression subset and immunophenotypic assessments of CD34 and aSMA (figure 3C bottom colour bar). Pathway analysis confirmed these genes relate to fibroblast activation state, including FGF13, COL4A4, MMP3, TNFSF4 (OX40L), THY1 (CD90) and JAK3. The top 10 most highly DEGs include COL8A1, COL10A1, SERPINE2, SYNDIG1, TNFSF4, MATN3 and HAPLN1. Online supplemental table 3 summarises the functional enrichment analysis for 47 aSMA/CD34 polarisation genes. Significant GO biologic pathways include 'extracellular matrix organisation' (adjusted p value <0.0001), 'cell adhesion' (adjusted p value <0.001), 'regulation of leucocyte activation' (adjusted p value=0.017), 'interleukin-12 production' (adjusted p value=0.011) and 'skeletal system development' (adjusted p value <0.001).

Expression of aSMA/CD34 polarisation genes and clinical improvement

We compared gene expression of 47 aSMA/CD34 polarisation genes at baseline versus 52 weeks in 18 improvers and 6 non-improvers (figure 4A). Among improvers, 30 of the 47

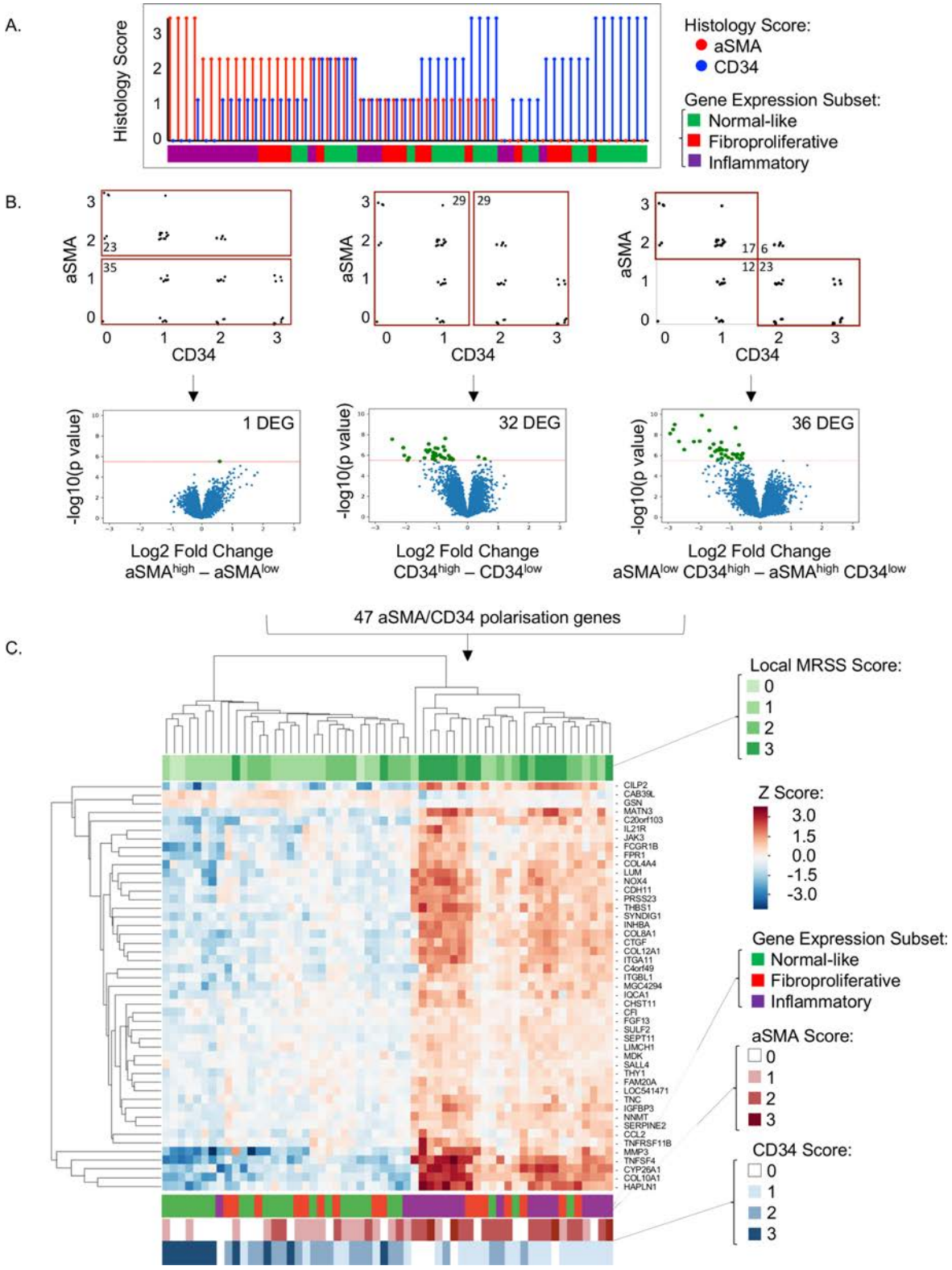

Figure 3 Gene expression according to fibroblast polarisation. (A) aSMA and CD34 scores with corresponding gene expression subset assignments for N=58 samples. aSMA scores (red vertical lines) were sorted in descending order and associated CD34 scores (blue vertical lines) were visualised for each patient sample. The horizontal bar below depicts each sample's gene expression subset assignment. (B) Upper panel: Three sample gating strategies according to aSMA and CD34 staining. $aSMA^{high}$ versus $aSMA^{low}$ (N=58 samples), $CD34^{high}$ versus $CD34^{low}$ (N=58 samples) and $aSMA^{low}$/$CD34^{high}$ versus $aSMA^{high}$/$CD34^{low}$ (N=40 samples). Lower panel: volcano plots of significantly DEGs according to aSMA and CD34 scores. Significant DEGs are highlighted in green and quantified in top right corner. The threshold for significance (horizontal red line) was 0.000003, determined using Bonferroni correction for multiple comparisons of 16 645 genes evaluated. (C) Unsupervised hierarchical clustering using single linkage method and Euclidean distance metric of 47 aSMA/CD34 polarisation genes identified in analysis outlined in B. Top horizontal bar indicates local MRSS for each skin biopsy sample. Bottom horizontal bars indicate gene expression subset, aSMA score (red) and CD34 score (blue) for each sample. aSMA, alpha-smooth muscle actin; DEG, differentially expressed gene; MRSS, Modified Rodnan Skin Score.

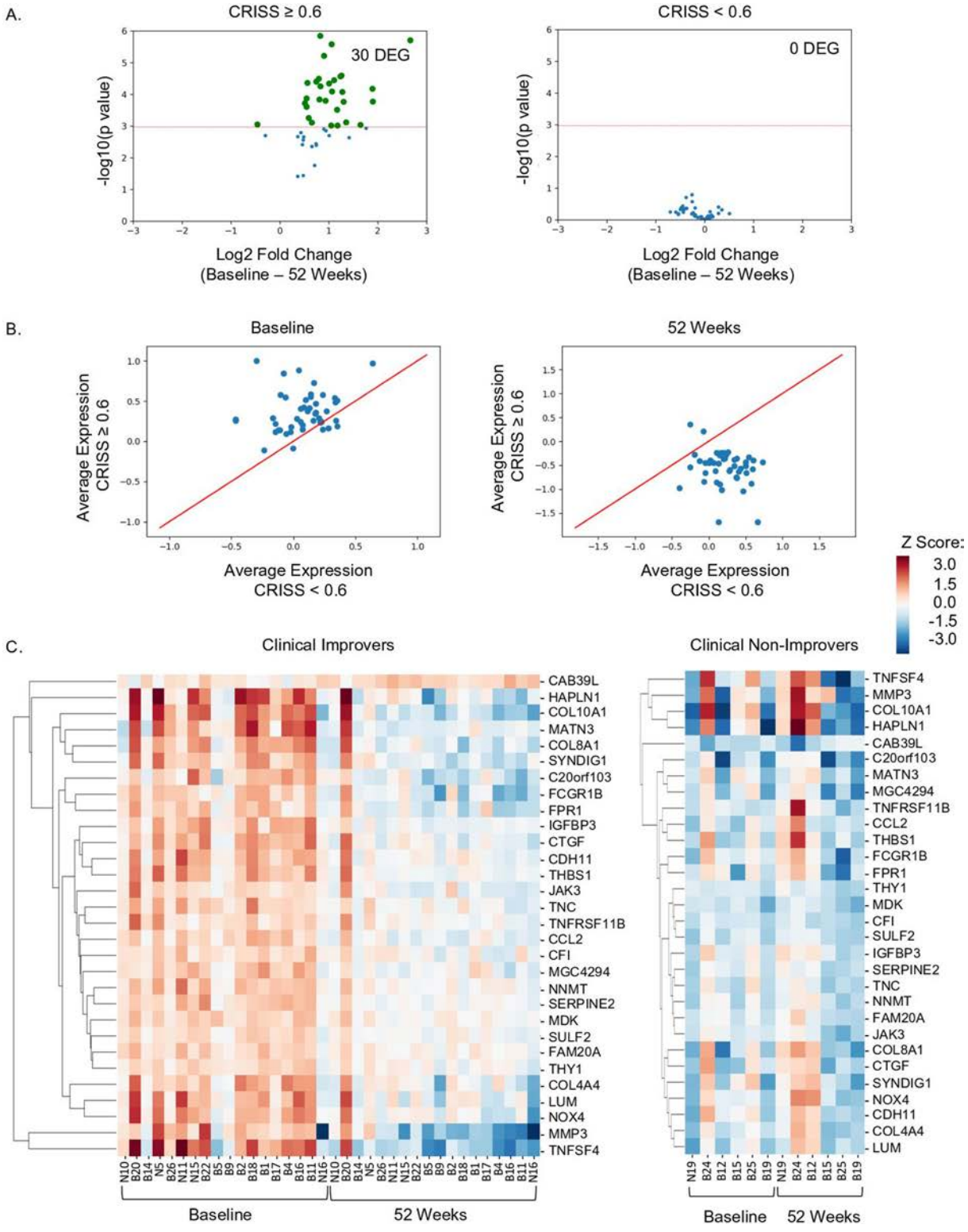

Figure 4 Change in 47 aSMA/CD34 polarisation genes from baseline to 52 weeks among individuals with and without 52-week clinical improvement. (A) Volcano plot of 47 aSMA/CD34 polarisation genes (log2 fold change of baseline vs 52 weeks) in 18 individuals with diffuse cutaneous systemic sclerosis who were classified as clinical improvers (CRISS ≥0.6; N=36 samples), left panel, or six individuals classified as non-improvers (CRISS <0.6; N=12 samples), right panel. The threshold for significance (horizontal red line) was 0.001, determined using Bonferroni correction for multiple comparisons of 47 aSMA/CD34 polarisation genes evaluated. Significant DEGs are highlighted in green and quantified in top corner. (B) Average expression of 47 aSMA/CD34 polarisation genes at baseline and 52 weeks by clinical improvement status. (C) Hierarchical clustering using single linkage method and Euclidean distance metric, supervised by either baseline or 52 weeks, of 30 significant DEGs identified in A in improvers and non-improvers. aSMA, alpha-smooth muscle actin; CRISS, Combined Response Index in Systemic Sclerosis; DEG, differentially expressed gene.

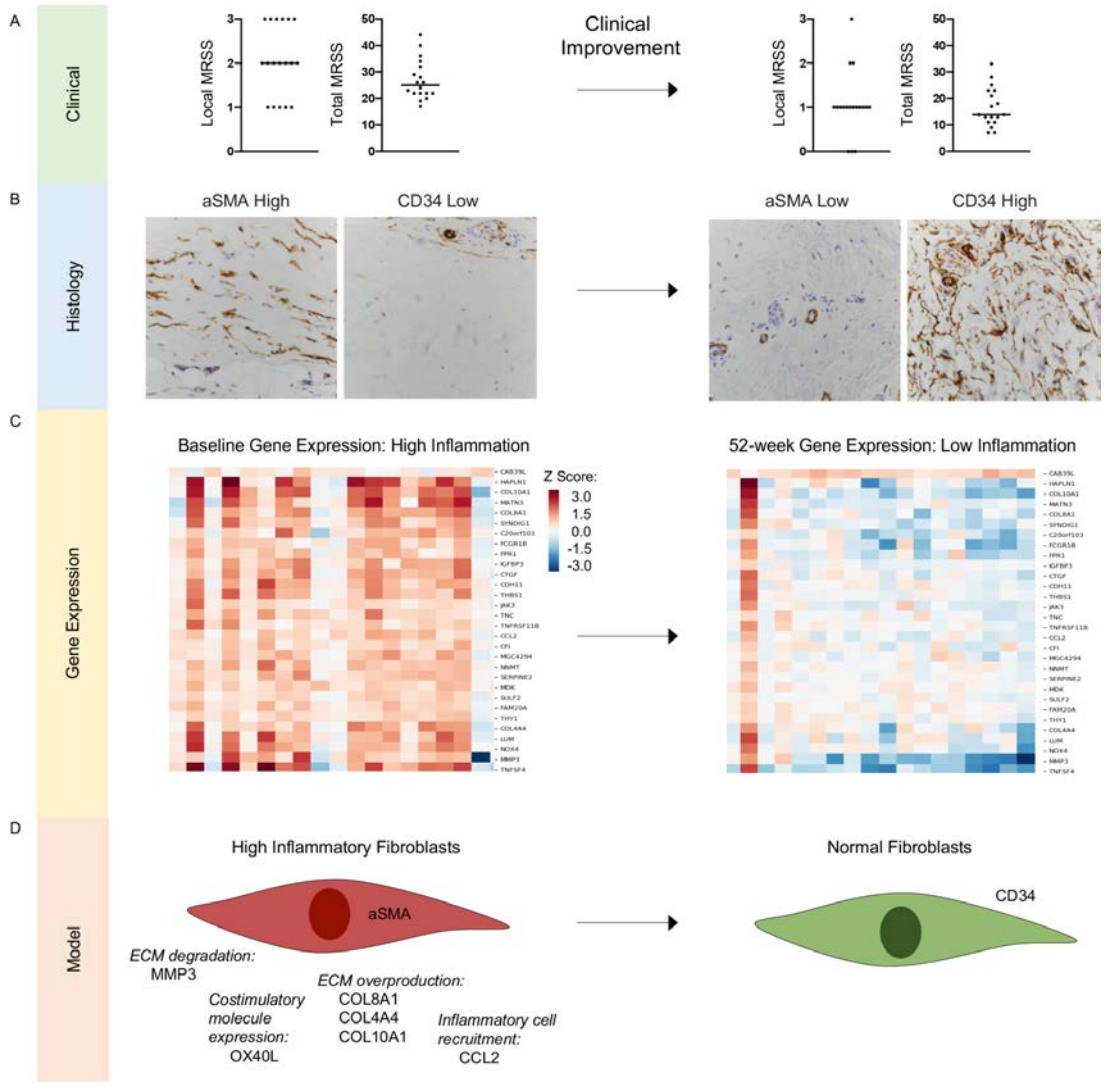

Figure 5 Model integrating analysis of clinical, histologic and gene expression features of clinical improvement. (A) Baseline (left) and 52-week (right) total and local Modified Rodnan Skin Score (MRSS) among 18 individuals with diffuse cutaneous systemic sclerosis (SSc) who experienced 52-week clinical improvement, defined by the Combined Response Index in SSc ≥0.6. Baseline median total MRSS is 25 and local MRSS is 2; 52-week median total MRSS is 14 and local MRSS is 1. (B) Representative fibroblast stains (magnification 40×) at baseline (aSMA high and CD34 low) and 52 weeks (aSMA low and CD34 high) in an individual who experienced clinical improvement. (C) Gene expression heatmap of 30 significant aSMA/CD34 polarisation genes differentially expressed from baseline to 52 weeks (N=18 skin samples) from individuals who experienced clinical improvement from baseline to 52 weeks. (D) Model proposing that fibroblast polarisation is a hallmark of clinical severity in SSc. aSMA, alpha-smooth muscle actin; ECM, extracellular matrix.

aSMA/CD34 polarisation genes were significantly differentially expressed between baseline and 52 weeks. There were no significant differences in baseline versus 52-week expression of any gene in non-improvers. Average expression of the 47 aSMA/CD34 polarisation genes was plotted for improvers versus non-improvers for baseline and 52-week samples. Average gene expression in improvers (vs non-improvers) was higher at baseline and lower at 52 weeks (figure 4B). A heat map of the 30 significant genes differentially expressed from baseline to 52 weeks further demonstrates the increased expression at baseline relative to 52 weeks in improvers but not non-improvers (figure 4C). Of these 30 genes, MMP3, TNFRSF11B and THBS1 were most strongly correlated with CRISS, that is, the more these decreased from baseline to 52 weeks, the more likely a patient was to have clinically improved (online supplemental figure 4). Taken together, this work indicates clinically severe scleroderma skin harbours aSMA high and CD34 low fibroblasts with associated high inflammatory gene expression, and these histologic and gene expression signatures of fibroblast polarisation can reverse with clinical improvement (figure 5).

DISCUSSION

There has been an unmet need to standardise the approach to skin histology assessment in SSc research. We report that of seven tested histologic/immunophenotypic features, global severity, aSMA and collagen negatively correlated with clinical improvement, measured by CRISS, while CD34 positively correlated. In a parallel, unbiased machine learning analysis, two fibroblast markers (aSMA and CD34) also emerged as most strongly predictive of gene expression subset. These findings are consistent with prior investigations that describe positive correlations between aSMA and collagen with local MRSS[8]

and decreased CD34 in SSc and morphoea.[23 24] Further, in a cross-sectional study of individuals with morphoea (N=50) and healthy controls (N=50), Lee *et al* found that individuals with morphoea (vs healthy controls) had higher aSMA and lower CD34 scores, and these were associated with assessments of fibrosis severity (mild vs severe).[25 26] We add to the literature by demonstrating that baseline aSMA/CD34 immunophenotype among improvers changes to resemble at 52 weeks the $aSMA^{low}$/$CD34^{high}$ immunophenotype of normal skin.

The aSMA/CD34 polarisation transcripts included genes under investigation as possible SSc treatment targets: TNFSF4,[27] JAK3,[28] CDH11[29] and TGF-β-regulated genes.[30] TNFSF4/OX40L, a costimulatory molecule expressed on antigen presenting cells[31] and SSc fibroblasts, is a genetic risk factor for dcSSc and SSc-associated autoantibodies,[32] and its blockade leads to fibrosis regression in mice.[27] Additionally, several TGF-β-regulated genes (eg, THBS1, SERPINE2 and CTGF) were increased in samples with $aSMA^{high}$/$CD34^{low}$ immunophenotype. Expression of THBS1 (thrombospondin-1) has been shown to correlate with MRSS.[33] SERPINE2/PN-1 is induced by TGF-β in models of cardiac fibrosis[34] and induces collagen promoter activity in 3T3 fibroblasts.[35] In an open-label study, 15 individuals with dcSSc received fresolimumab, a neutralising antibody against TGF-β, and post-treatment (vs baseline) dermal SERPINE2 and CTGF expression decreased and MRSS improved.[36] We also identified DEGs involved in MEK/ERK signalling: Integrin Subunit Alpha 1 (ITGA1) and Hyaluronan And Proteoglycan Link Protein 1 (HAPLN1). Inhibiting MEK/ERK pathway in vitro reduces fibroblast contractility, suggesting that the MEK/ERK pathway is dysregulated in SSc fibroblasts.[37] Nazari *et al*[9] described an inverse staining pattern between CD34 and two other fibroblast markers: podoplanin and CD90/Thy1. We similarly observed significantly decreased expression of CD90/Thy1 in CD34 high versus low samples. Together these studies suggest that fibroblasts can be polarised towards an inflammatory fibroblast/myofibroblast state in the context of scleroderma-related inflammation. Indeed, this transition can be induced in vitro in response to tumour necrosis factor, IL-1β or acute skin injury.[9] Our data add to the growing literature implicating inflammatory fibroblasts in SSc by showing that inflammatory fibroblast polarisation can be reversed as scleroderma improves clinically.

Our data also suggest that gene expression profiles might be useful for identifying patients more likely to improve, as demonstrated by higher baseline expression of aSMA/CD34 polarisation genes in improvers versus non-improvers. Lofgren *et al*[38] developed an SSc-specific 415 gene expression signature and defined an SSc skin severity score (4S) based on these genes. The study results showed that the 4S correlated with MRSS, and the 4S at 12 months predicted 24-month MRSS. The aSMA/CD34 polarisation genes identified herein includes eight overlapping genes with the 4S gene expression signature: CHST11, FPR1, GSN, HAPLN1, LUM, PRSS23, THY1 and TNFSF4. Our results may help to refine the 415 gene signature and improve the ability for gene expression to function as an outcome measure and predictive tool. By synchronising histology with gene expression and clinical data, we also suggest that fibroblast polarisation is the likely foundation of this gene expression signature.

Study strengths include dermatopathologist collaboration and use of a histology-centred approach to gene expression analysis to better understand disease heterogeneity and clinical improvement. We also acknowledge study limitations. Data was retrospectively analysed from single-centre trials for early, dcSSc with a high proportion of RNA polymerase III autoantibody positivity. This limits generalisability. Also, while there was no statistically significant batch bias, minor batch effects could still exist and potentially influence results of downstream analyses. The majority (18 of 26) of individuals were classified as 52-week improvers. As a result, our analysis of non-improvers was likely underpowered and findings regarding these individuals can only be considered descriptive. We used the provisional classification of CRISS ≥0.6 to distinguish clinical improvers versus non-improvers; however, CRISS does not allow us to describe histologic and gene expression features of clinical stability versus worsening, and this cut-off may evolve over time as clinical trials aggregate data. Additionally, it is not possible to know the precise cell types that express each inflammatory gene identified. Future approaches using single-cell RNA sequencing are needed to better understand the cellular sources of these genes.

In conclusion, histologic features reflect disease severity, while dually enhancing our understanding of fibroblasts as contributors to SSc disease heterogeneity and behaviour over time.

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CLINICAL SCIENCE

Impact of the COVID-19 pandemic on the disease course of patients with inflammatory rheumatic diseases: results from the Swiss Clinical Quality Management cohort

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ABSTRACT

Objectives To investigate whether the transient reduction in rheumatology services imposed by virus containment measures during the COVID-19 pandemic was associated with disease worsening in axial spondyloarthritis (axSpA), rheumatoid arthritis (RA) or psoriatic arthritis (PsA).

Methods Patient-reported disease activity assessed during face-to-face visits and/or via a smartphone application were compared between three periods of each 2 months duration (before, during and after the COVID-19-wave) from January to June 2020 in 666 patients with axSpA, RA and PsA in the Swiss Clinical Quality Management cohort.

Results The number of consultations dropped by 52%, whereas the number of remote assessments increased by 129%. The proportion of patients with drug non-compliance slightly increased during the pandemic, the difference reaching statistical significance in axSpA (19.9% vs 13.2% before the pandemic, p=0.003). The proportion of patients with disease flares remained stable (<15%). There was no increase in mean values of the Bath Ankylosing Disease Activity Index, the Rheumatoid Arthritis Disease Activity Index-5 and the Patient Global Assessment in patients with axSpA, RA and PsA, respectively.

Conclusion A short interruption of in-person patient–rheumatologist interactions had no major detrimental impact on the disease course of axSpA, RA and PsA as assessed by patient-reported outcomes.

Key messages

What is already known about this subject?

- Partial or complete closure of rheumatology services was experienced in many countries as part of SARS-CoV-2 containment measures.
- Remote consultation strategies might partly compensate for lower number of face-to-face visits to prevent a postponement of treatment decisions for inflammatory rheumatic diseases.

What does this study add?

- In this real-life cohort study of patients with axial spondyloarthritis, rheumatoid arthritis and psoriatic arthritis with available patient-reported disease activity assessments during the first wave of the COVID-19 pandemic via a web-based application after a drop in face-to-face consultations no increase in disease activity could be observed.
- Although the proportion of patients with medication non-compliance slightly increased during the pandemic, the proportion of patients with disease flares remained stable.
- The patient population followed here used a smartphone app regularly and might be more invested in disease management. The results have to be interpreted in this light.

How might this impact on clinical practice or future developments?

- The lack of a major detrimental effect of a short interruption of physical consultations on the disease course of several inflammatory rheumatic diseases informs potential future measures of public lockdown.
- As patient-reported outcomes are insufficient to guide treat-to-target efforts, assessments of long-term outcomes are warranted.
- Future studies are needed to confirm the usefulness of remote strategies to regularly assess patient-reported outcomes.

INTRODUCTION

The ongoing COVID-19 pandemic remains an important healthcare challenge.[1] Data on the course of inflammatory rheumatic diseases during the pandemic are scarce.[2] Partial or complete closure of rheumatology services was experienced in many countries as part of virus containment measures and transient lockdown of public life.[3] It remains unclear, whether remote consultation strategies might partly compensate for lower numbers of face-to-face visits to prevent a postponement of treatment decisions.[4] Additional factors may also potentially contribute to disease worsening during the pandemic. Some patients may choose to preventively stop immunosuppression out of fear of complications.[2] Moreover, the psychological stress (anxiety about a new disease, economic pressure, less recreational opportunities and so on) encountered during the pandemic should not be underestimated.[5] The aim of this study was to assess the

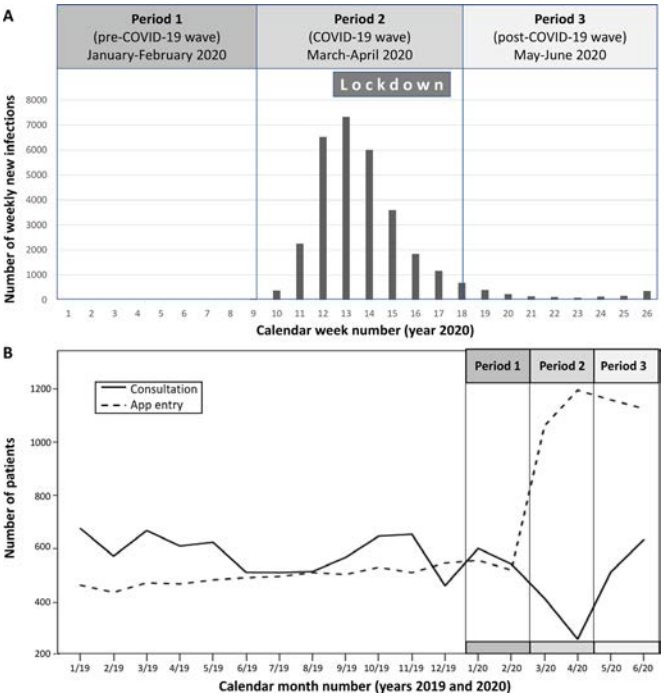

Figure 1 (A) Number of weekly new SARS-CoV-2 infections registered in Switzerland, defining three periods: a pre-COVID-19-wave phase from 1 January to29 February 2020; a COVID-19-wave phase from 1 March to 30 April 2020 and a post-COVID-19-wave phase from 1 May to 30 June 2020. The 5-week period of partial lockdown of public life imposed by the Swiss Federal Council is inscribed during the COVID-19-wave phase. (B) Monthly numbers of face-to-face consultations and remote app entries of patients with inflammatory rheumatic diseases followed in the Swiss Clinical Quality Management cohorts. The COVID-19-wave phases defined in (A) are indicated from January to June 2020.

course of self-reported disease activity and of drug adherence in patients with axial spondyloarthritis (axSpA), rheumatoid arthritis (RA) and psoriatic arthritis (PsA) before, during and after the initial COVID-19 wave in Switzerland.

METHODS

Choice of disease assessments periods

The specific COVID-19 situation in Switzerland in the first 6 months of 2020 is detailed in the supplementary appendix. According to the described longitudinal course of SARS-CoV-2 infection numbers, we defined three study periods of 2 months duration each: (1) a pre-COVID-19 wave phase from 1 January to 29 February 2020; (2) a COVID-19 wave phase from 1 March to 30 April 2020 and (3) a post-COVID-19 wave phase from 1 May to 30 June 2020 (figure 1A).

Study population

Patients diagnosed as having axSpA, RA or PsA in the Swiss Clinical Quality Management (SCQM) cohort[6–8] were included if at least one patient-reported disease activity measure was available in each of the study periods defined above, irrespective of whether the assessment was performed during consultations or remotely via a web-based application. All patients currently followed in SCQM, defined as patients with at least one visit during the last 18 months, served as control. The voluntary use of the app by the patients to monitor disease activity and drug compliance monthly started on 1 January 2019.[9] Additional information provided to patients during the pandemic is compiled in the supplementary appendix.

All patients gave informed consent prior to data collection. Ethical approval was given by the Geneva cantonal committee for research ethics (2020-01708).

Disease activity assessments

Patient-reported disease activity assessments included the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) in axSpA,[10] the Rheumatoid Arthritis Disease Activity Index-5 (RADAI-5) in RA[11] and the Patient Global Assessment (PGA) visual analogue scale for disease activity in PsA,[12] both during visits and for app entries. Disease activity measures were investigated for each 2-month period as previously defined. A clinically important worsening in individual patients from period 1 to 2 and from period 2 to 3 was defined as follows: BASDAI showed increase of 2 points in axSpA; RADAI-5 showed increase of 1.4 points in RA[11] and PGA showed increase of 1.2 points in PsA.[12]

Adherence to treatment

All other answers except 'yes' to the question 'Do you take the following medication regularly?' in the monthly app questionnaire were considered as non-compliance with prescribed medication (online supplemental information).

Statistical analyses

McNemar's test was used to compare the proportions of patients with drug non-compliance or experiencing a disease flare and the paired t-test was used to compare disease activity scores between two subsequent periods.

RESULTS

Number of visits and of APP entries over time

The monthly number of patients consulting rheumatologists and the monthly number of patients with app entries for 2019 and the three periods of interest in 2020 are depicted in figure 1B. The number of visits declined by 52% with the implementation of virus containment arrangements from n=543 in February to n=262 in April 2020. Given measures taken to motivate patients to use the app to enter disease activity and their willingness to contribute to shared decision making and research, this was paralleled by an increase in app entries (from 521 to 1195).

Adherence to DMARD therapy

Baseline characteristics of 287 axSpA, 248 RA and 131 PsA patients fulfilling the inclusion criteria are shown in table 1. The patients in the individual disease categories were comparable with the respective group of all SCQM patients currently followed in SCQM, with the exception of the subset of patients with RA, which was younger and had a slightly lower disease activity score at inclusion (online supplemental table S1). The low number of face-to-face visits precluded a comparison between patients with clinical visits and remote data entries. The majority of patients (>70%) were treated with a biological disease-modifying antirheumatic drug (bDMARD) at the study start with the proportion of patients on synthetic DMARDs depending on the underlying disease (table 1). The prepandemic proportion of patients with non-compliance to the prescribed medication was around 15%. There was a slight increase in the number of non-adherent patients during the pandemic, the difference to the pre-pandemic numbers reaching statistical significance in axSpA (table 1). Adherence returned to prepandemic levels in the post-COVID-19 phase.

Table 1 Baseline characteristics of patients, mean disease activity scores as well as number of disease flares and of drug non-compliance cases in the respective 2 months before, during and after the COVID-19 wave in Switzerland

	Axial spondyloarthritis (N=287)	Rheumatoid arthritis (N=248)	Psoriatic arthritis (N=131)
Male sex, n (%)	141 (49.1)	70 (28.2)	66 (50.4)
Age (years), mean (SD)	47.1 (11.8)	55.3 (13.2)	52.6 (10.7)
Disease duration, mean (SD)	17.4 (11.3)	14.0 (10.6)	15.7 (10.7)
Medication at start of period 1, n (%)			
Conventional-synthetic DMARDs	44 (15.3)	142 (57.3)	45 (34.4)
Targeted-synthetic DMARDs	3 (1.0)	39 (15.7)	8 (6.1)
Biologic DMARDs	203 (70.7)	176 (71.0)	101 (77.1)
Patient-reported disease activity, mean (SD)	BASDAI	RADAI-5	PGA
Period 1	3.40 (2.23)	2.46 (2.05)	3.43 (2.55)
Period 2	**3.23 (2.25)***	2.39 (2.03)	3.30 (2.33)
Period 3	3.29 (2.32)	2.47 (2.13)	3.44 (2.25)
Patients with disease flares at follow-up, n (%)			
Period 1	7 (2.4)	20 (11.0)	10 (9.8)
Period 2	7 (2.4)	27 (10.9)	19 (14.5)
Period 3	12 (4.2)	33 (13.3)	21 (16.0)
Patients with non-compliance with prescribed DMARD medication, n (%)			
Period 1	38 (13.2)	37 (14.9)	19 (14.5)
Period 2	**57 (19.9)***	55 (22.2)	25 (19.1)
Period 3	**29 (10.1)***	42 (16.9)	14 (10.7)
Patients with documented SARS-CoV-2 infection, n (%)	4 (1.4)	10 (4.0)	0 (0)

Period 1=pre-COVID-19-wave phase (1 January to 29 February 2020); period 2=COVID-19-wave phase (1 March to 30 April 2020); period 3=post-COVID-19-wave phase (1 May to 30 June 2020).
*Values in bold indicate a significant difference in comparison to the respective value in the previous period (p=0.02 for BASDAI in period 2 vs period 1; p=0.003 and p=0.006 for the proportion of patients with drug non-compliance in period 2 vs period 1 and in period 3 vs period 2, respectively).
BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; DMARD, disease-modifying antirheumatic drug; PGA, Patient Global Assessment of disease activity; RADAI-5, Rheumatoid Arthritis Disease Activity Index-5.

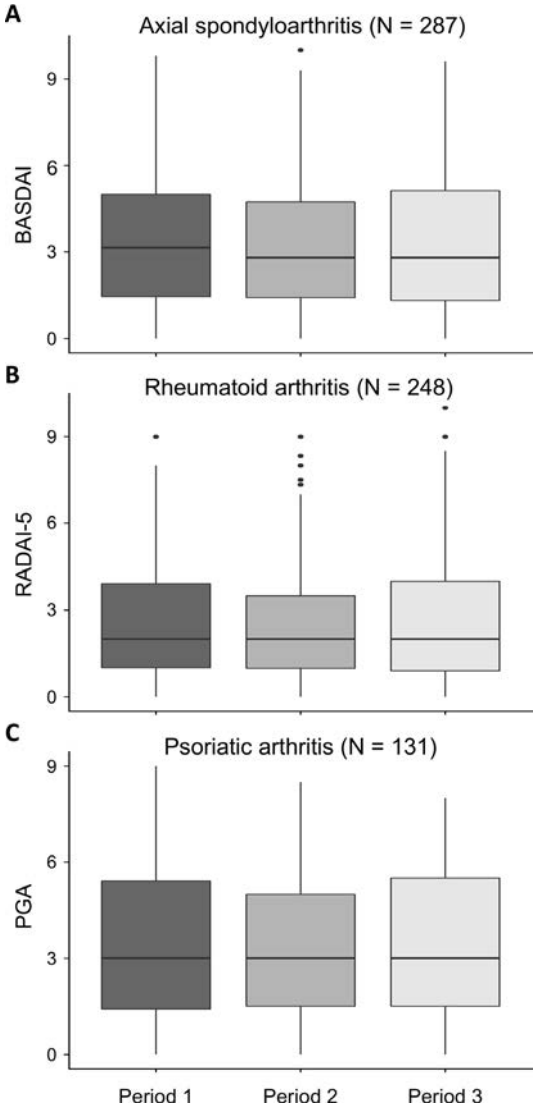

Figure 2 Disease activity values for the pre-COVID-19-wave phase (period 1), the COVID-19-wave phase (period 2) and the post-COVID-19-wave phase (period 3) in patients with axial spondyloarthritis (A), rheumatoid arthritis (B) and psoriatic arthritis (C). The horizontal line in the boxes represents the median value. BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; PGA, Patient Global Assessment of disease activity; RADAI-5, Rheumatoid Arthritis Disease Activity Index-5.

Course of disease and number of disease flares

Patient-reported disease activity outcomes were stable over the first 6 months of 2020 (figure 2 and table 1), with a slight decrease during the pandemic wave, reaching statistical significance in axSpA (mean (SD) BASDAI 3.40 (2.23) before the pandemic and 3.23 (2.25) during the pandemic, p=0.02). To put the disease activity scores in a broader perspective, monthly median values from all SCQM patients are shown separately for physical consultations and remote app entries from January 2019 to June 2020 in the online supplemental figures S1 and S2. The proportion of patients with a disease flare during the pandemic wave was <15% for all three diseases (table 1) and no statistical significance could be found when compared with the proportion with disease worsening in the pre-COVID-19 phase.

DISCUSSION

A web-based smartphone application had been implemented within the Swiss registry long before the current pandemic and allowed us to follow the course of inflammatory arthritides over the whole initial COVID-19 wave. We noted an acute drop in clinical encounters that was paralleled by an increase in app entries. Our study demonstrates that disease activity as assessed by the BASDAI in axSpA, the RADAI-5 in RA and PGA in PsA remained stable and even slightly decreased over the duration of the pandemic wave at the population level. Moreover, a disease flare occurred in <15% of patients, not statistically different from the pre-COVID-19 phase. Although cut-offs for a clinically important worsening exist for the patient-reported outcomes used here for RA and PsA, there is no consensus for a BASDAI cut-off in this regard. We have used a worsening by two points as its performance was comparable with the defined Ankylosing Spondylitis Disease Activity Score cut-off against the external standard 'patient-worsening'.[13] Patient-reported worsening was investigated in a recent observational study in patients with RA and patients with axSpA and was experienced by 29% of patients over a duration of 3 months.[14]

The results presented here can only be interpreted in the context of a rather short first COVID-19 pandemic wave as encountered

in Switzerland. A recent international survey in 35 EULAR (European League Against Rheumatism) countries found that a partial closure of rheumatology services of 5–8 weeks duration during the COVID-19 pandemic was reported by 81% of 1428 respondents,[3] underscoring the representativeness of our data.

Current guidelines based on preliminary data do not recommend the preventive cessation of immunosuppressive medication in the absence of infection.[15 16] To continue or to stop medication in individual situations during the COVID-19 pandemic ultimately is part of a shared decision-making process between the patient and his rheumatologist. We have therefore focused on patient-reported non-adherence to the medication entered in the database by the rheumatologist and not on actual drug changes. We hypothesise that the duration of the pandemic was too short for the documented transient decrease in drug adherence to be reflected in an increase in disease flares.

Regular assessments of disease activity is a key component of the treat-to-target principle in the management of rheumatic diseases. In addition to the voluntary reporting of disease activity by the patients, we assume an important increase in the number of remote patient–physician interactions (email and phone calls) during the pandemic. Although their actual figures remain unknown, the influence of telemedicine on the outcome presented here should not be underestimated.[4] We acknowledge the fact that patient-reported measures cannot replace clinical examination. Recent data have suggested that their exclusive use might be insufficient to guide treat-to-target efforts.[17] In the absence of alternatives in the context of suspended visits to physicians, their use is however warranted.

An important limitation of this work is that we could only evaluate patients with regular assessments of disease activity, which was mostly based on remote data entries during the pandemic. This subset using the smartphone app is probably more invested in disease management and the non-compliance figures might be under-represented.

In conclusion, a temporary interruption of in person consultations during the COVID-19 pandemic had no major detrimental impact on the disease course of patients with inflammatory rheumatic diseases as assessed through patient-reported outcomes.

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Patient consent for publication Not required.

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EPIDEMIOLOGICAL SCIENCE

Towards consensus in defining and handling contextual factors within rheumatology trials: an initial qualitative study from an OMERACT working group

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ABSTRACT

Objectives The Outcome Measures in Rheumatology Initiative established the Contextual Factors Working Group to guide the understanding, identification and handling of contextual factors for clinical trials. In clinical research, different uses of the term 'contextual factors' exist. This study explores the perspectives of researchers (including clinicians) and patients in defining 'contextual factor' and its related terminology, identifying such factors and accounting for them in trials across rheumatology.

Methods We conducted individual semistructured interviews with researchers (including clinicians) who have experience within the field of contextual factors in clinical trials or other potentially relevant areas, and small focus group interviews with patients with rheumatic conditions. We transcribed the interviews and applied qualitative content analysis.

Results We interviewed 12 researchers and 7 patients. Researcher's and patient's descriptions of contextual factors were categorised into two broad themes, each comprising two contextual factors types. The 'treatment effect' theme focused on factors explaining variations in treatment effects (A) among patients and (B) among studies. The 'outcome measurement' theme focused on factors that explain (C) variations in the measurement result itself (apart from actual changes/differences in the outcome) and (D) variations in the outcome itself (beside treatment of interest). Methods for identifying and handling contextual factors differed among these themes and types.

Conclusions Two main themes for contextual factors with four types of contextual factors were identified based on input from researchers and patients. This will guide operationalisation of contextual factors. Further research should refine our findings and establish consensus among relevant stakeholders.

Key messages

What is already known about this subject?

- Contextual factors should be considered when developing core outcome sets. Guidance and operationalisation of the current definition are needed to ensure consistency in understanding, approaching and identifying contextual factors.
- Within Outcome Measures in Rheumatology, the Contextual Factors Working Group was formed to develop guidance on how to address contextual factors in clinical trials.

What does this study add?

- This qualitative study, using semistructured interviews with researchers and small focus group interviews with patients, suggests that contextual factors can be grouped into two broad themes: 'treatment effect' and 'outcome measurement.' The 'treatment effect' theme comprises two types of contextual factors: (A) 'effect modifying' (pertaining to effect variations among patients) and (B) 'meta confounding' (pertaining to effect variations among studies). The 'outcome measurement' theme also comprises two types of contextual factors: (C) 'measurement affecting' (pertaining to variations in measurement results) and (D) 'outcome explaining' (pertaining to variations in the outcome itself).

INTRODUCTION

A 'core outcome measurement set' is a minimum consensus-based set of outcome domains and instruments that should be measured and reported in clinical trials for a specific health condition and/or intervention.[1] Since 1992, the Outcome Measures in Rheumatology (OMERACT) initiative has successfully developed core sets for many rheumatological conditions[2] and kept patients actively involved since 2002.[3]

In 2012, the concept of contextual factors was introduced in the OMERACT process. In clinical research, different uses of the term

Key messages

How might this impact on clinical practice or future developments?

- This study provides a foundation for developing a consensus-based operational definition of contextual factors, which may specify relevant contextual factor types and include guidance on how to identify such factors and take them into account to ensure proper interpretation of clinical trial findings.

'contextual factors' exist, describing different concepts.[4–7] Within OMERACT, a contextual factor is defined as a 'variable that is not an outcome of the study, but needs to be recognised (and measured) to understand the study results. This includes potential confounders and effect modifiers'.[3] Core set developers need to consider if there are contextual factors that should be measured in all trials. However, the research presented at the OMERACT meeting in 2014 revealed much heterogeneity in understanding, approaching and identifying contextual factors.[8] To address this, the Contextual Factors Working Group (CFWG) was formed to develop guidance on how to address contextual factors in clinical trials.[8]

In 2018, the CFWG presented a research plan: initially it would collect 'case scenarios' involving 'contextual factors' from OMERACT working groups; then develop an operational definition and guidance on how to address contextual factors in rheumatology trials when developing core outcome measurement sets; and ultimately develop a generic set of important contextual factors (ie, important across all rheumatic diseases) that should always be considered in rheumatology trials based on empirical evidence and consensus.[9] To operationalise the definition of contextual factors, an expert-driven approach, including qualitative data collection with a subsequent consensus process among important stakeholders, was proposed.

The objective of the current study is to explore the perspectives of researchers (including clinicians) and patients in defining 'contextual factor' and its related terminology, identifying such factors and accounting for them in trials across rheumatology (ie, across different OMERACT working groups).

METHODS

Design

In this qualitative study, we conducted semistructured interviews with researchers and small focus group interviews with 2–3 patients, and applied qualitative content analysis.[10 11] As a research method, qualitative content analysis aims 'to provide knowledge and understanding of the phenomenon under study.'[12] We published a protocol online prior to conducting any interviews (online supplemental file 1 and www.parkerinst.dk).

Participants and setting

Individually interviewed participants were required to be researchers (eg, statisticians, methodologists, trialists, including clinicians) who have experience in the field of contextual factors in clinical trials or other potentially relevant areas, such as predictive/prognostic factors, effect modification, subgroup effects, stratified analyses or equity efforts (ie, initiatives centred on factors of social inequity). We used purposive sampling to maximise variation of disciplines, sex and geographical representation, and expanded our sample by snowball sampling (ie, asking each participant to suggest additional researchers).[13] We initially identified participants among our co-authors, the OMERACT Executive board and authors of relevant empirical studies and known guidance documents. We selected patients from the patient research partners (PRPs) of the CFWG. The main interviewer (SMN) determined the sample size by theoretical saturation, defined as the size where subsequent interviews contribute no more new data.[14]

We approached potential participants by email invitation. On their acceptance of participation, we provided an overview of the interview content, the research protocol and case scenarios involving contextual factors previously collected from OMERACT working groups.[9]

Data collection

From April to July 2018, one investigator (SMN) interviewed the researchers individually (average 47 min) and interviewed the focus groups (2–3 patients) supported by 1–2 coinvestigators (TW and CF; average 1 hour and 21 min). We conducted the interviews online or face to face. We conducted all interviews in English, using a predefined semistructured interview guide (online supplemental file 1) and probing questions, allowing relevant statements to be explored in more depth. Patients were interviewed using an adapted interview guide (ie, reformulated

Table 1 Characteristics of the interviewed researchers and patients

	Researchers (n=12)	Patients (n=7)
Females	6	5
Age, years, mean (SD)	58 (8)*	55 (8)
Continent		
North America	3	2
Europe	8	3
Australia	1	2
Involved in OMERACT		
Currently involved	11	7
Never involved	1	0
Organisation		
Academic	11	–
Healthcare	1	–
Main role providing CF experience		
Rheumatologist	5	–
Statistician	2	–
Epidemiologist	2	–
Methodologist	1	–
Occupational therapist	1	–
ICF expert	1	–
Involved in patient care		
Currently	6	–
Previously	3	–
Never	3	–
Rheumatic condition		
Rheumatoid arthritis	–	4
Psoriatic arthritis	–	2
Bechet's syndrome	–	1
Research experience beside PRP role		
Yes	–	6
No	–	1

Values are number of participants, unless indicated otherwise.
*Data on age were missing for three researchers.
CF, contextual factor; ICF, The International Classification of Functioning, Disability and Health; OMERACT, Outcome Measures in Rheumatology; PRP, patient research partners.

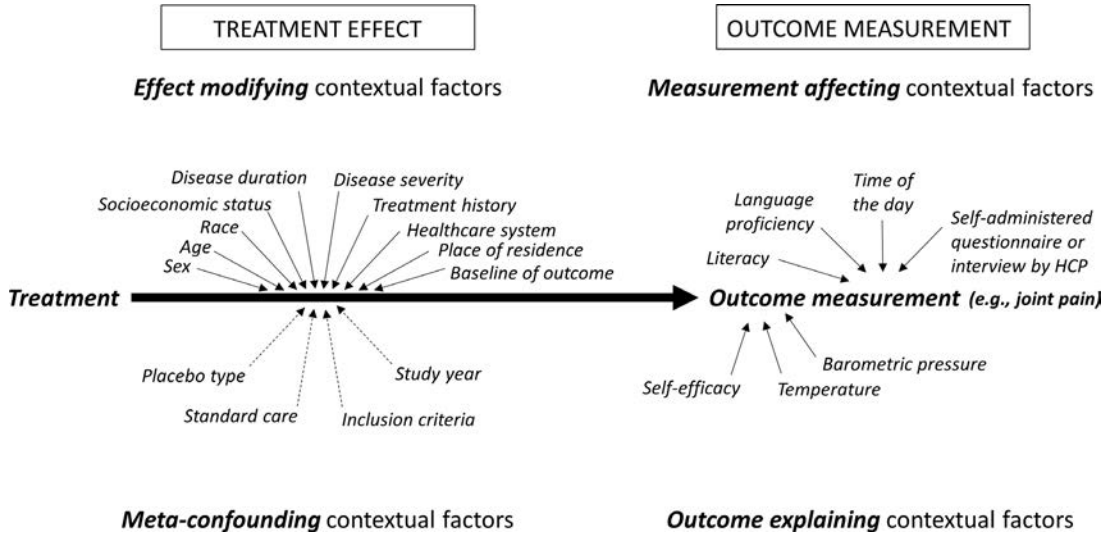

Figure 1 Illustration of the two themes for contextual factors, each describing two types of contextual factors. Specific examples of factors may fit within more than one contextual factor type. Meta-confounding contextual factors (marked with dotted lines) are factors that can be investigated only across trials (on trial level) and are therefore not relevant within individual trials. The meta-confounding factor, study year, may capture different important aspects to consider across trials, such as different therapeutic trends of the time and, hence, typical treatment history of patients, as well as trends in exclusion criteria (eg, tuberculosis screening). HCP, healthcare professional.

using lay terms in collaboration with a PRP, MdW). We audio recorded the interviews, transcribed verbatim, returned the transcripts to the participants for comments and/or corrections, and collected demographic data.

Data analysis

One investigator (SMN, supported by MUR) conducted qualitative content analysis[10 11] (investigator characteristics in online supplemental file 1) using NVIVO (V.12 Pro). We generated the coding frame by initially creating main categories in a concept-driven way based on the structure of the interview guide, and adding subcategories in an inductive, data-driven way with open coding based on 'successive summarising'. This method involved paraphrasing relevant passages while removing unnecessary parts. We revised the coding frame, added explanations and supporting quotes, and subsequently, conducted further data exploration to search for patterns and co-occurrences of selected categories.[10]

We ensured rigour and credibility by discussing key findings at CFWG meetings, and sharing a draft of the findings with some of the interviewees to ensure viewpoints were appropriately interpreted and the account made sense to other researchers and patients (ie, 'member checking').[15] We ensured comprehensive reporting by following the Consolidated Criteria for Reporting Qualitative Studies[16] and the Standards for Reporting Qualitative Research.[17]

Patient involvement

During the whole process, we involved two PRPs who are familiar with the research topic. These and five additional PRPs with experience of living with rheumatic conditions were involved as participants in the interviews.

RESULTS

Participant characteristics

A total of 16 researchers were invited; 4 (25%) did not respond and 12 (75%) agreed to participate. All seven (100%) invited patients agreed to participate. The researchers represented several stakeholder groups, and half were involved in patient care (table 1). The patients represented three rheumatic conditions.

Reflections on the current OMERACT definition

The current OMERACT definition describes a contextual factor as a 'variable that is not an outcome of the study, but needs to be recognised (and measured) to understand the study results. This includes potential confounders and effect modifiers'[3] Only a minority of the participants found the definition to be clear and understandable (A.1 in online supplemental table 1). Some thought the term 'contextual factor' was too broad and confusing. Some researchers discussed whether 'confounder' should be part of the definition because it may be less relevant in randomised trials. In contrast to the definition's first part, many considered the outcome itself at baseline to be a possible contextual factor (eg, the level of pain at baseline may be important when interpreting the changes in pain at follow-up in a trial). Overall, the patients had difficulty understanding the definition, mainly due to the terms used:

> I still find it quite difficult to understand. I think I have an idea of what a contextual factor is. I'm not sure that I know exactly the difference between a confounder or an effect modifier. Do we really need these terms? (…) I think, it's a definition for researchers, but it's not a definition for patient research partners. (Patient 3)

Participants' own description of contextual factors

The participants' own descriptions of contextual factors revealed two broad themes, each comprising two types of contextual factors. The first theme, 'treatment effect', focused on factors that explain variations in treatment effects (A) among patients (or groups of patients), and (B) among studies. The second theme, 'outcome measurement', focused on factors that explain (C) variations in the measurement result itself (apart from actual changes/differences in the outcome) and (D) variations in the outcome itself (apart from the treatment of interest). These four types may be termed 'effect modifying', 'meta-confounding', 'measurement affecting' and 'outcome explaining' contextual

Table 2 The two themes for contextual factors, each describing two types of contextual factors

Theme	Treatment effect theme	Outcome measurement theme
Description	Factors that influence (or are associated with or predict) the treatment effects.	Factors that influence the outcome measurement.
Rationale	To understand the study results in terms of for whom and/or in which settings a treatment shows an effect, and to assess the external validity/generalisability of a study, which relates to stratification/precision medicine.	To understand the study results in terms of what influences the outcome measurement (beside the treatment of interest), and to understand 'what is behind the numbers' of a measurement.
Types	A. Effect modifying factors are effect modifiers and explain the variability in treatment effect among patients according to characteristics, and may guide treatment decisions (stratified medicine). B. Meta-confounding factors relate to the interpretation of the results of a trial when comparing with other trials (eg, in meta-analysis), and explain inherent variations in treatment effects among trials according to trial-level characteristics.	C. Measurement affecting factors explain the variability in the measurement itself, and relate to the difficulty or inability to measure an outcome (validity/reliability), and may impact our ability to see a treatment response. D. Outcome explaining factors (besides treatment of interest) affect the outcome; they may be prognostic factors* and may explain different impact of symptoms or perceptions of a response, and may confound group trial results. Such factors may follow the ICF framework.[4]
Lay terms to a patient†	Factors that may predict how well you will benefit from a treatment. Factors that we need to know in a study to know whether the findings can be applied to a particular situation.	Clinicians and researchers need to know what affects your assessment (eg, of pain), so they can understand the numbers. When they ask you about your scores, you may say "Well, it depends (…)". Factors that influence your condition and your life with the condition, besides the treatment you are getting.
Examples of evidence	► Disease duration: Rheumatoid arthritis (RA) patients, with a history of responding inadequately to biologics, tend to have a higher chance of responding to Baricitinib compared with placebo if they had RA for ≥10 years.[34] (ie, disease duration modifies the effect of Baricitinib; example of CF type A). ► Study year (capturing disease severity): Over time, disease characteristics of RA patients in trials on TNFα inhibitors have generally become less severe. This may be due to a change in standard of care, trial site location, trends in inclusion criteria.[35] (ie, study year may capture inherent CFs that are important when interpreting study results, such as in a meta-analysis; example of CF type B).	► Literacy when assessing reliability of joint pain measurement instruments: In RA patients, VAS pain assessments are less reliable in illiterate patients compared with literate patients[36] (example of CF type C). ► Weather: In knee OA patients, reporting more severe pain (WOMAC pain) was associated with lower ambient temperature and higher change in barometric pressure[37] (example of CF type D). ► CFs for worker productivity: OMERACT members (incl. PRPs, HCPs, etc) were asked to propose and rank CFs affecting WP in arthritis patients. Key CFs identified were type of job, personal factors, disease status, financial need, societal incentive, and age, and should be considered when interpreting WP measurements (example of CF type D).
Suggested criteria for important CFs†	Strong suspicion until evidence exists, evidence for statistical interaction and important variability in effect across subgroups. For generic factors, criteria for strong and consistent evidence across rheumatology.	Factors that patients frequently consider important for interpreting outcome measurements, or for their condition/life with their condition. For generic factors, need to be relevant across countries and conditions.
Suggested methods for identifying important CFs†	Investigate CFs in existing data sets, request trialists to measure CFs and provide stratified analyses as supplement, conduct systematic review. Use existing guidelines on investigating subgroup effects. For generic factors, investigation of effect modifiers in IPD meta-analysis, literature review and/or seeking expert/stakeholder opinion, use CFs identified in OMERACT disease working groups.	Ask patients and/or clinicians directly or do a systematic review.

*Prognostic factors are factors predicting the outcome or course of a patient's condition, regardless of treatment.[38]
†Descriptions mainly relate to only two types of contextual factors (ie, the 'effect modifying'—and 'outcome explaining' contextual factors, respectively), due to lack of data on the two remaining types ('meta-confounding' and 'measurement affecting').
CFs, contextual factors; HCPs, healthcare professionals; ICF, The International Classification of Functioning, Disability and Health; IPD, individual patient data; OA, osteoarthritis; PRPs, patient research partners; RA, rheumatoid arthritis; TNF, tumour necrosis factor; VAS, Visual Analogue Scale; WOMAC pain, Western Ontario and McMaster Universities Arthritis Index pain subscale; WP, worker productivity.

factors, respectively (figure 1, table 2 and part A.2 in online supplemental table 1). Specific examples of factors may fit within more than one contextual factor type.

Few researchers recognised that both themes exist; most emphasised only one of them. The patients mostly focused on what (besides treatment) affects their condition, their lives with the condition, and how symptoms are perceived—which in turn also affects their lives (these considerations relate to the outcome measurement theme). Several patients emphasised that contextual factors are inherently patient centric:

> In terms of what you were doing here is patient centric, in terms of the contextual factors, because we're the only ones who really know what they are. (Patient 7)

Some researchers initially considered contextual factors to be measured at baseline (A.4 in online supplemental table 1), and hence, fixed, but later acknowledged that some may be time-varying:

> I have to admit that I usually think of contextual factors as being fixed, but I can't see why they can't be time-varying (Researcher 7)

However, allowing contextual factors to vary over time adds complexity, and several researchers recommended focusing only on contextual factors measured at baseline. One researcher termed time-varying contextual factors 'mediators', which may explain why a treatment works in terms of working mechanism, and the researcher mentioned adherence to a regimen and patient-therapist relationships as examples. However, the researcher pointed out that 'mediators' are not mentioned in the current definition.

Explaining contextual factors in lay terms to a patient

When researchers were asked how to explain contextual factors in lay terms to patients (A.3 in online supplemental table 1 and table 2), within the treatment effect theme, contextual factors were often explained as factors that may determine which

Table 3 Suggestions on how to take contextual factors into account in future research

Theme	Treatment effect theme*	Outcome measurement theme*
Designing	► Measure CFs according to evidence-based and/or consensus-based CF list available for investigators and regulators. ► Design trials so confounding is avoided (eg, by excluding specific types of patients). ► Ensure balance of CFs among the treatment groups. ► Ensure sufficient variation within CFs in the trial population. ► Require that some CFs be investigated in meta-research.	► Measure CFs relevant for outcome of interest. ► Allow flexibility to deviate from CF list. ► Avoid influence from CFs by measuring outcomes as consistently as possible (eg, at same time each day).
Analysing	► Adjust for CFs (for confounders). ► Stratify analyses for CFs (effect modifiers). ► Conduct proper analysis for effect modifiers (ie, test for interaction and present stratified results). ► Prespecify analyses in an analysis plan and specify whether they are exploratory or confirmatory (most trials are not powered to detect subgroup effects). ► Aggregate data from several trials and stratify.	► Conceptually adjust the outcome measurements for relevant/influential CFs.
Reporting	► Require stringent reporting of CF data (measure of variability, amount of missing data, how it was measured, and how well it was measured). ► Require CFs be in reporting guideline for rheumatology trials. ► Report CFs (prognostic factors) as part of extensive baseline table. ► Stratify results by CFs (predictive factors) (eg, as appendix). ► Account for CFs when interpreting results (with respect to generalisability, differing results according to levels of CFs, or explaining skewed results from imbalances among groups). ► Ask stakeholders how they prefer CFs to be reported.	► Account for CFs when interpreting results (in terms of what the numbers mean) and co-report relevant/influential CFs.

*Suggestions within these themes mainly relate to only two types of contextual factors (ie, the 'effect modifying'—and 'outcome explaining' contextual factors, respectively), due to lack of data on the two remaining types.
CFs, contextual factors.

patients experience an effect. Within the outcome measurement theme, one researcher suggested explaining contextual factors within the International Classification of Functioning, Disability and Health (ICF) framework and providing examples for specific outcomes. The patients themselves repeatedly expressed that the terms 'confounder' and 'effect modifier' were problematic and that examples are needed:

> (…) it would be good if you could find an example within rheumatology (…) And I think that would be very helpful if you also could find an example of a contextual factor that has been studied, and for which we have some data to show how it influences. (Patient 3)

Terminology

For the treatment effect theme, researchers often considered contextual factors to be related with the terms 'effect modifiers' (ie, factors modifying the effects of a treatment), and 'predictive factors' (ie, factors predicting the effects of a treatment) and used terms such as 'baseline covariate', 'table 1 factors', 'subgroup effects' and 'baseline covariance'. For the outcome measurement theme, one researcher explained that the contextual factors are not required to predict treatment response (A.5 in online supplemental table 1).

> (…) all of these are contextual factors, irrespective of their role as a predictive factor or not. (Researcher 3)

Examples of contextual factors

Examples of contextual factors mentioned by at least five participants were age, sex, place of residence, socioeconomic status, disease duration, healthcare system, adherence and support, (online supplemental figure 1, and part A.6 in online supplemental table 1). These were mostly considered 'effect modifying' contextual factors as most of the interviews concerned those. Some factors were sometimes considered specific to disease, outcome or treatment. Within the outcome measurement theme, the contextual factors mentioned related often to specific outcomes, such as joint pain (figure 1). Consistent with the ICF, some researchers only considered two categories of factors (ie, personal and environmental factors). Examples of factor categories that some researchers intuitively did not consider contextual factors included disease-related, intervention-related and measurement-related factors (eg, how the questionnaire was administered), baseline status of outcome of interest, and factors relating to study design.

Identifying important contextual factors

For considering contextual factors to be important (B.1 in online supplemental table 1) within the treatment effect theme, a researcher suggested that a strong suspicion—based on expert consensus—be required until evidence exists of a statistically significant interaction between the contextual factor and intervention, with important effect size (ie, important variability in effect size among subgroups or settings). For generic (across diseases) contextual factors, researchers suggested that sufficient (meaning strong and convincing) and consistent evidence across rheumatological conditions should be present. It was further emphasised that the criteria need to be strict and that there should be consensus about them:

> There should be some very, very strict criteria, before we as OMERACT, can say, this is core and we mandate everybody to measure this always. (…) and then you'd have to have some sort of consensus exercise to say, well, we're only going to name it 'core' if we can show in at least three rheumatology conditions that it makes a difference, something like that. (Researcher 1)

Researchers suggested several different methods for identifying important contextual factors (table 2).

Contextual factors in future research

Within the treatment effect theme, researchers provided many different suggestions on how future trials can take contextual factors into account in their design, analysis and reporting (table 3 and part B.2 in online supplemental table 1). Participants emphasised that a list of important contextual factors should be available when designing trials. The suggested analysis methods and reporting depended to some extent on the participant's discipline and on the terms (eg, confounders, prognostic factors, effect modifiers) with which they associated contextual factors. Several participants suggested that the analyses had to

Box 1 Efforts potentially related to contextual factors

- Recommendations on subgroup effects, including investigating,[18–20] reporting[19 21] and evaluating the credibility[22] of subgroup effects in trials, but also in systematic reviews[23–25] from various research groups as well as regulators, such as European Medicines Agency (EMA)[27] and US Food and Drug Administration (FDA).[28–30]
- Efforts aimed at equity,[39–43] centred on factors of social inequity, represented by the acronym PROGRESS-Plus (ie, place of residence, race/ethnicity/culture/language, occupation, sex/gender, religion, education, socioeconomic status, social capital and other characteristics, such as age, disability, sexual orientation, time-dependent situations and relationships).
- The Prognostic Research Strategy framework,[20 38] including guidelines for prognostic factors and factors predictive of treatment effect.
- The Context and Implementation of Complex Interventions framework,[44] including context separated into seven domains (ie, geographical, epidemiological, sociocultural, socioeconomic, ethical, legal, political).
- The International Consortium for Health Outcomes Measurement,[45 46] including so-called 'case mix variables' (ie, risk-adjustment variables) for the outcome set developed.
- The COnsensus-based Standards for the selection of health Measurement INstruments,[47 48] including guidelines for assessing 'cross-cultural validity' and 'inconstancy' in systematic reviews of patient-reported outcome measures.
- The International Classification of Functioning, Disability and Health framework,[4] including so-called personal and environmental contextual factors.
- The Grading of Recommendations, Assessment, Development and Evaluations,[49] including recommendations for assessing inconsistency and applicability in systematic reviews.
- The Cochrane Collaboration's revised tool for assessing risk of bias in randomised trials (V.2.0),[50] including risk of bias in measurement of the outcome.
- Efforts on estimands and sensitivity analysis in clinical trials by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use,[31] FDA[32] and EMA,[33] including descriptions of 'intercurrent events'.
- Efforts investigating placebo effects,[5 6] using the terms 'contextual effect' or 'context effect' (and 'context factors' and 'contextual factors').
- Efforts investigating the active use of the patients' context in patient care,[7] using the term 'contextualisation' of patient care referring to the process of identifying the context (circumstances) of individual patients and, if necessary, adapting the plan of care.

be prespecified and that stratified results according to contextual factors should be presented. Within the outcome measurement theme, fewer and less statistical approaches were suggested.

Further comments and suggestions

The participants acknowledged the importance of the effort of the OMERACT CFWG (B.3 in online supplemental table 1) but raised concerns on several potential issues: whether a generic set of contextual factors can be developed; how to deal with factors that are not feasible to measure (eg, due to cost or causing delays in trials); and how to decide that something is so important that everybody needs to measure it, when robust evidence is lacking to make that call.

One researcher advocated that OMERACT should focus on factors within the outcome measurement theme and argued that this should be the niche of OMERACT, as others are already looking into factors within the treatment effect theme:

> I think those are maybe of primary importance to OMERACT, the ones that are influencing the very meaning of the results of what those numbers mean, how we should be interpreting these numbers. (…) but does OMERACT need to have a special little niche where it talks about the outcomes and what you need to do to measure outcomes well, which nobody else is doing? Nobody else is picking up the contextual factors that you need to be able to perfect your outcome measurements. (Researcher 11)

Other suggestions included: to ensure the operational definition can be understood both by people who are familiar with statistics and those who are not; to pass measures of contextual factors through the OMERACT instrument filter; and to use the term 'important contextual factors' rather than 'core contextual factors' until sufficient evidence is present. Two researchers even suggested not using the term 'contextual factors' altogether. Further, comments included that differences between sexes are neglected in trials, and study year and type of placebo are neglected in systematic reviews. Also, a researcher commented that contextual factors may be population, intervention, comparison, outcome and time specific.

DISCUSSION

This study found that contextual factors overall may be described within two broad themes: those relating to the 'treatment effect' and those relating to the 'outcome measurement.' Each theme, in turn, comprised two types of contextual factors, thus making four types of contextual factors. The descriptions of the contextual factor types should not be considered final, but rather the first step in approaching a complex concept. It is intended to engender debates regarding improving interpretation of trial results, and eventually lead to a consensus-based operational definition.

Most participants in this study recognised only one type of contextual factor, indicating that efforts are needed to facilitate understanding of all four types when describing contextual factors. This finding may explain the heterogeneity in understanding and identifying contextual factors within (and outside) OMERACT. This study provides a foundation for designing a Delphi study to reach consensus on an operational definition of contextual factors. As OMERACT mainly focuses on clinical trials, 'meta-confounding' contextual factors may be considered outside the scope of such effort.

Operationalising contextual factors will include refining the descriptions of each contextual factor type and developing guidance for each of them (ie, how to identify and account for them in trials). Guidance for 'effect modifying' contextual factors may already exist, related to investigating,[18–20] reporting[19 21] and evaluating the credibility[22] of subgroup effects in trials, and for systematic reviews.[23–25] For sex/gender specifically, the Sex and Gender Equity in Research guideline[26] recommends that results are presented disaggregated by sex. Guidance from regulators, such as European Medicines Agency[27] and US Food and Drug Administration also exists.[28–30] The 'outcome explaining' contextual factors may relate to so-called 'intercurrent events'.[31–33] Many potentially relevant efforts may provide inspiration when developing guidance (box 1).

One limitation of the study is the absence of investigator triangulation (ie, corroboration of key findings through analysis by several investigators and subsequent consensus). Member checking (ie, sharing a draft of the findings and inquiring whether viewpoints

were faithfully interpreted)[15] was conducted for only some of the participants. As we used purposive sampling, the participants may not be representative of all relevant experts. The term 'contextual factor' has been used to describe different concepts in clinical research.[4–7] These other concepts might potentially have received more emphasis in the interviews if a different sample of experts had been included. Most participants focused on 'effect modifying' or 'outcome explaining' contextual factors; little data was available for the two other types, making the findings less conclusive and leaving more to be clarified during a subsequent consensus process. Finally, this study did not address how to measure contextual factors.

To conclude, this qualitative study found that contextual factors overall may be described in two broad themes, 'treatment effect' and 'outcome measurement', with each theme comprising two types of contextual factors. The methods for identifying and handling contextual factors differ between the types, so an operational definition of contextual factors may need to specify these types, and include guidance on how to identify such factors and take them into account. Further research should refine our findings and establish consensus.

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TRANSLATIONAL SCIENCE

Local steroid activation is a critical mediator of the anti-inflammatory actions of therapeutic glucocorticoids

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ABSTRACT

Objectives The enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) plays a well-characterised role in the metabolism and activation of endogenous glucocorticoids (GCs). However, despite its potent upregulation at sites of inflammation, its role in peripheral metabolism and action of therapeutic GCs remains poorly understood. We investigated the contribution of 11β-HSD1 to the anti-inflammatory properties of the active GC corticosterone, administered at therapeutic doses in murine models of polyarthritis.

Methods Using the tumour necrosis factor-tg and K/BxN serum-induced models of polyarthritis, we examined the anti-inflammatory properties of oral administration of corticosterone in animals with global, myeloid and mesenchymal targeted transgenic deletion of 11β-HSD1. Disease activity and joint inflammation were scored daily. Joint destruction and measures of local and systemic inflammation were determined by histology, micro-CT, quantitative RT-PCR, fluorescence activated cell sorting and ELISA.

Results Global deletion of 11β-HSD1 resulted in a profound GC resistance in animals receiving corticosterone, characterised by persistent synovitis, joint destruction and inflammatory leucocyte infiltration. This was partially reproduced with myeloid, but not mesenchymal 11β-HSD1 deletion, where paracrine GC signalling between cell populations was shown to overcome targeted deletion of 11β-HSD1.

Conclusions We identify an entirely novel component of therapeutic GC action, whereby following their systemic metabolism, they require peripheral reactivation and amplification by 11β-HSD1 at sites of inflammation to deliver their anti-inflammatory therapeutic effects. This study provides a novel mechanistic understanding of the anti-inflammatory properties of therapeutic GCs and their targeting to sites of inflammation in polyarthritis.

Key messages

What is already known about this subject?

- Potent anti-inflammatory glucocorticoids such as prednisolone are rapidly metabolised, and circulate in both their active and inactive (prednisone) forms.
- Enzymes such as 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which is potently upregulated at sites of inflammation, reactivates inactive glucocorticoids such as prednisone.

What does this study add?

- This study demonstrates that following their oral delivery and systemic metabolism, the anti-inflammatory properties of active glucocorticoids are completely dependent on their peripheral reactivation by 11β-HSD type 1.
- The global deletion of 11β-HSD type 1 results in profound therapeutic glucocorticoid resistance.

How might this impact on clinical practice or future developments?

- This study provides a novel mechanistic understanding of the anti-inflammatory properties of therapeutic glucocorticoids and their targeting to sites of inflammation.

INTRODUCTION

Due to their anti-inflammatory actions, therapeutic glucocorticoids (GCs) have been widely used in the management of inflammation. However, despite their continuing widespread use, several critical aspects of their therapeutic action remain unclear.[1] The enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) plays a well characterised role in the hepatic activation of structurally inactive GCs (such as cortisone and prednisone), converting them to their active counterparts (such as hydrocortisone and prednisolone).[2 3] However, the role of 11β-HSD1 in mediating the anti-inflammatory, disease-modifying actions of therapeutic GCs remains poorly understood. This represents a significant barrier to our understanding of the mechanisms of action of therapeutic GC action in vivo and to the development of GCs with an enhanced benefit:risk ratio.

We explore the contribution of pre-receptor steroid metabolism by the enzyme 11β-HSD1 to the anti-inflammatory actions of GCs using in vivo models of chronic polyarthritis. We demonstrate a fundamental role for the peripheral re-activation of GCs in mediating their anti-inflammatory properties, with mice with global 11β-HSD1 deletion showing a complete resistance to their therapeutic effects of orally administered GCs in their active form. These findings change our understanding of

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how many structurally active therapeutic GCs elicit their anti-inflammatory effects, requiring peripheral reactivation by the enzyme 11β-HSD1, after their initial systemic inactivation, to mediate their beneficial immune-modulatory effects.

MATERIALS AND METHODS

Models of polyarthritis

The tumour necrosis factor (TNF)-tg model of chronic inflammatory polyarthritis, obtained courtesy of Professor George Kollias (BSRC Fleming, Athens), was maintained on a C57BL/6 background and compared with WT littermates.[4] At day 32 of age, at the first onset of measurable polyarthritis, male TNF-tg mice received drinking water supplemented with either corticosterone (Cort) (100 µg/mL, 0.66% ethanol), or vehicle (0.66% ethanol) for 3 weeks. Mice were scored as previously described.[5 6] At day 53, serum was collected by cardiac puncture and tissues excised for analysis. Serum transfer-induced arthritis (STIA) was induced by intravenous injection of 100 µL arthritogenic serum from KRN mice (K/BxN).[7] Ankle or wrist joint thickness was monitored using callipers and reported as the change from baseline.

Targeted deletion of 11β-HSD1

11β-HSD1 KO animals with global 11β-HSD1 deletion were crossed with TNF-tg animals to generate TNF-tg$^{11\beta KO}$ animals as previously described.[8] Mesenchymal 11β-HSD1 KO animals were generated by crossing flx/flx-HSD11B1 mice with Twist2-cre mice to generate 11βHSD1flx/flx/Twist2cre animals, which were paired with TNF-tg animals to produce TNF-tg$^{11\beta HSD1flx/flx/Twist2cre}$ (TNF-tg$^{11\beta flx/tw2cre}$).[9–11] Myeloid targeted 11β-HSD1 KO animals were generated by crossing flx/flx-HSD11B1 mice with LysM-cre mice to generate 11βHSD1flx/flx/LysMcre animals, which were paired with TNF-tg animals to produce TNF-tg$^{11\beta HSD1flx/flx/LysMcre}$ (TNF-tg$^{11\beta flx/LysMcre}$).[12]

11β-HSD1 activity

11β-HSD1 activity was determined by thin-layer chromatography as previously reported.[8 13] Briefly, ex vivo tissue biopsies and in vitro cultures were incubated with 100 nmol/L of 11-dehydrocorticosterone (11-DHC) and tritiated [^{3}H] tracer. Steroid conversion was measured using a Bioscan imager (Bioscan, Washington, District of Columbia, USA) and fractional conversion calculated.

Primary fibroblast-like synoviocytes and macrophage culture

Primary fibroblast-like synoviocytes (FLS) were isolated from combined hind legs and front paws from mice following dissection and cleaning of tissue as previously reported.[13] Briefly, joints were digested in RPMI containing 2% fetal calf serum (FCS), 2.5 mg/mL collagenase D (Roche) and 20 µg/mL DNase (Sigma-Aldrich) for 45 min at 37°C with agitation. After filtering, cells were cultured in RPMI containing 10% FCS and 1% pen-strep and cultured to passage 3 before use. Primary murine peritoneal macrophages were isolated by CD11b+ve selection with CD11b MicroBeads (Miltenyi Biotec, Surrey, UK) following peritoneal lavage in phosphate-buffered saline, and maintained in Dulbecco's Modified Eagle Medium containing 10% FCS and 1% pen-strep and maintained for up to 48 hours.

Gene expression analysis

Gene expression was assessed by TaqMan Gene Expression Assays (ThermoFisher Scientific) following mRNA isolation by innuPREP RNA Mini Kit (Analytikjena, Cambridge) and reverse transcription (Multiscribe, ThermoFisher Scientific) as per the manufacturer's guidelines. Ccl2, cxcl2, Cxcl10, Tnfα, il1β, Il6 and gilz were determined using species-specific probe sets by real-time PCR on an ABI7500 system (Applied Biosystems, Warrington, UK). mRNA abundance was normalised to either 18S or Gapdh. Data, obtained as Ct values and ΔCt determined (Ct target–Ct 18S/GAPDH), were expressed as arbitrary units (AU) using the following transformation: (arbitrary units (AU)$=1000\times(2^{-\Delta ct})$).

ELISA analysis

Serum interleukin (IL)-6 and corticosterone (R&D Systems, Abingdon, UK) were determined using commercially available ELISA assays in accordance with the manufacturer's instructions.

Histological analysis of joints

Histochemistry was performed on paraffin-embedded 10 µm sections. Pannus size at the humerus/ulna joint interface and osteoclast numbers on the bone surface pannus (following tartrate-resistant acid phosphatase (TRAP) staining) were determined using ImageJ software as previously reported.[5 14] For quantification, the mean of three adjacent 10 µm sections cut from the centre of the joint from six animals were assessed.

MicroCT morphometry analysis

Front paws from mice were imaged using a Skyscan 1172 micro-CT scanner (Bruker) using X-ray beam settings of 60 kV/167 μA with a 0.5 mm aluminium filter. Projections were taken every 0.45° at 580 ms exposure. Image volumes were reconstructed using the Feldkamp algorithm (NRecon V.1.6.1.5, Bruker) having applied beam hardening correction. Front paws were reconstructed and MeshLab V.1.3.2 was used to generate meshes which could then be scored for bone erosions as described previously.[5]

Serum steroid measurements

Serum samples were collected by cardiac bleeds to assess systemic metabolism between groups; 200 µL of serum was spiked with 0.2 ng of internal standard (corticosterone-d8 and cortisol-d4; purchased from Sigma-Aldrich, UK). Steroids were extracted via liquid-liquid extraction with 2 mL of tert-methyl butyl ether (MTBE). MTBE was evaporated to dryness under nitrogen at 55°C. Samples were reconstituted in 125 µL of 50/50 methanol/water for liquid chromatography tandem mass spectrometry analysis.[15 16] Samples were measured on a Waters Xevo-XS mass spectrometer coupled to an Acquity uPLC with an electrospray ionisation source in positive ionisation mode. Steroids were identified by comparison to authentic reference standards, (Sigma-Aldrich), with matching retention time and identical mass transitions and quantified relative to a calibration series. Concentrations were calculated relative to internal standard corticosterone to corticosterone-d8 and 11-DHC and its isomer metabolite to cortisol-d4.

Tissue digestion and flow cytometric analysis of synoviocytes

One hind leg and one front paw per mouse was dissected and cleaned of tissue as previously reported.[8] Briefly, joints were digested in RPMI containing 2% FCS, 2.5 mg/mL collagenase D (Roche) and 20 µg/mL DNase (Sigma-Aldrich) for 45 min at 37°C with agitation. After filtering, cells were centrifuged, red cells lysed and cells counted before being filtered through 40 µm cell strainer, incubated with anti-CD16/CD32 blocking antibody (1:200; eBioscience) for 10 min at RT, followed by staining with antibody cocktail at 4°C. Antibodies for membrane

staining are outlined in online supplemental table 1). Data were acquired using a BD LSR Fortessa X20 and analysed using FlowJo software (FlowJo). The following gating strategy was used for myeloid cells: live cells were gated on CD45+CD11b+ cells. Neutrophils identified as Ly6g+, macrophages were Ly6g−, F4/80+ and inflammatory activated M1-like macrophages were F4/80+MHC class II+. T cells were identified as live CD45+CD3+. CD3+ cells were then stratified as CD4+ or CD8+ T cells. B cells were identified as CD45+CD3 and CD19+.

Statistical analysis

Statistical significance was defined as $p<0.05$ using either an unpaired Student's t-test or two-way analysis of variance with Tukey post hoc analysis where a Gaussian distribution was identified.

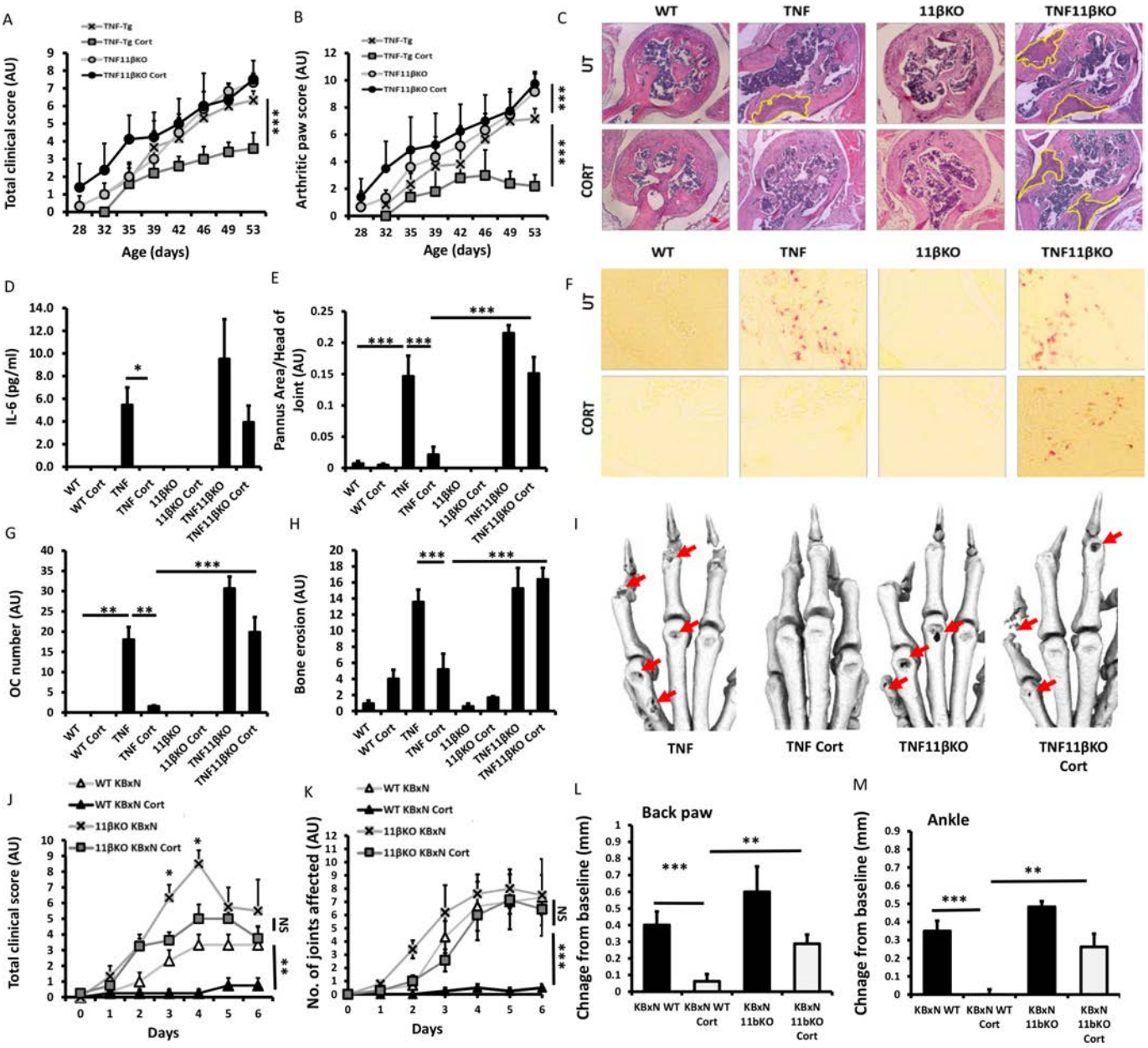

Figure 1 (A) Total clinical scores (arbitrary units (AU)), (B) arthritic paw scores (AU) and (C) representative images of synovitis at the ulna/humerus joint interface of wild-type (WT), tumour necrosis factor (TNF)-tg, 11βKO and TNF-tg$^{11\beta KO}$ animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. (B) of TNF-tg and TNF-tg$^{11\beta KO}$ animals receiving vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. (D) Serum levels of interleukin (IL)-6 determined by ELISA, (E) histological scoring of synovitis (AU), (F) representative images of TRAP stained osteoclasts at the ulna/humerus joint interface, (G) osteoclast number (AU) at the ulna/humerus joint interface, (H) quantification of bone erosion (AU) in the wrist, metacarpals and phalanges of WT, TNF-tg, 11βKO and TNF-tg$^{11\beta KO}$ animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. (I) representative images of three-dimensional reconstructions of front paws of TNF-tg and TNF-tg$^{11\beta KO}$ animals receiving vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks, red arrows indicate erosions. (J) Total clinical scores (AU), (K) arthritic paw scores (AU) and swelling (mm) of (L) back paws and (M) ankles of WT and 11βKO animals after induction of arthritis with K/BxN serum receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 1 week. Values are expressed as mean±SE, n=6 per group for all TNF-tg experiments and n=5 (K/BxN), n=6 (K/BxN/Cort), n=6 (K/BxN$^{11\beta KO}$) and n=5 (K/BxN$^{11\beta KO}$/Cort). Statistical significance was determined using two-way analysis of variance with Tukey post hoc analysis. *P<0.05, **p<0.005, ***p<0.001.

RESULTS

11β-HSD1 KO animals are resistant to therapeutic GCs

We crossed the TNF-tg murine model of chronic polyarthritis onto the 11βKO background to generate TNF-tg animals with deletion of 11β-HSD1 (TNF-tg$^{11\beta KO}$). Wild-type (WT), TNF-tg, 11βKO and TNF-tg$^{11\beta KO}$ animals received either vehicle or corticosterone in drinking water at 50 and 100 µg/mL as previously reported.[2 14 17] At 50 µg/mL, no significant change in disease activity or joint inflammation were apparent in TNF-tg animals and was discontinued from the study (online supplemental figure 1A–C). At 100 µg/mL corticosterone resulted in a significant reduction in clinical scores and joint inflammation in TNF-tg animals (figure 1A,B). In contrast, anti-inflammatory effects of corticosterone were absent in TNF-tg$^{11\beta KO}$ animals. Similarly, serum IL-6 was reduced in TNF-tg mice receiving corticosterone ($p<0.05$), which was absent in TNF-tg$^{11\beta KO}$ counterparts (figure 1D). TNF-tg mice receiving corticosterone showed a marked reduction in pannus invasion and osteoclast numbers at the pannus bone interface, which was entirely absent in TNF-tg$^{11\beta KO}$ animals (figure 1C–G). Micro-CT analysis of juxta-articular erosions confirmed that corticosterone significantly reduced joint destruction in TNF-tg mice, but not TNF-tg$^{11\beta KO}$ animals (figure 1H,I). In the KBxN serum induction model of polyarthritis, similar patterns were observed with total clinical scores and joint inflammation scores being reduced by corticosterone in WT, but not in 11βKO animals at day 6 (figure 1J–M). Here, at early time points (days 3–4) GCs were able to partially suppress clinical scores of disease, including weight loss and lethargy in 11βKO animals, without impacting on measures of joint inflammation and swelling. These data demonstrate that GC activation by the enzyme 11β-HSD1 is a necessary step in mediating the anti-inflammatory actions of the GC corticosterone in the joints of animals with polyarthritis.

Oral corticosterone generates circulating 11-DHC substrate for 11β-HSD1 activation

Metabolism and inactivation of therapeutic GCs by renal 11β-HSD2 creates a circulating pool of inactive GC (corticosterone to 11-DHC in mice) available for peripheral activation by 11β-HSD1. To assess the systemic metabolism of oral administered corticosterone, we measured serum levels of the corticosterone and its inactive derivative 11-DHC. No differences were observed in daily intake of corticosterone between groups, determined by quantifying daily drinking water intake per mouse, with an average exposure of 22.5+1.44 µg/g of body weight. Here, serum corticosterone and 11-DHC were detected and significantly increased following administration of corticosterone, with exposure comparable across groups (figure 2A,B). In all groups, exposure to corticosterone resulted in a significant reduction in adrenal weights relative to vehicle (figure 2C). Analysis of corticosterone inactivation by the 11β-HSD enzymes within the synovium was assessed by thin layer chromatography in WT, TNF-tg, 11βKO, TNF-tg anNFtg$^{11\beta KO}$ animals and showed no significant variation between groups (online supplemental figure 2). These data confirm a comparable increase in serum corticosterone and inactive 11-DHC in TNF-tg and TNF-tg$^{11\beta KO}$ animals receiving oral corticosterone.

Effects of oral corticosterone on leucocyte recruitment are dependent on 11β-HSD1

We examined infiltrating leucocytes and inflammatory mediators in synovial tissue digests to assess their regulation by corticosterone. Here, while corticosterone resulted in a significant decrease in total leucocytes, neutrophils, macrophages, CD8+ and CD19+, but not CD3+ and CD4+ populations in TNF-tg mice, TNF-tg$^{11\beta KO}$ animals were resistant to the actions of corticosterone on many of these parameters, with no apparent reduction in total leucocytes, macrophages and neutrophils (figure 3A–D). TNF-tg$^{11\beta KO}$ animals receiving corticosterone also possessed significantly higher numbers of total leucocytes, neutrophils, macrophages, CD3+ ($p<0.01$) and CD4+ cell populations relative to TNF-tg counterparts receiving corticosterone (figure 3A–E). Here, corticosterone skewed macrophage polarisation, with reduced numbers of inflammatory activated M1-like polarised or macrophages relative to total macrophages, TNF-tg animals, while TNF-tg$^{11\beta KO}$ animals showed complete resistance to this effect (figure 3H). However, TNF-tg$^{11\beta KO}$ animals retained an effective suppression of both CD8+ and CD19+ cell populations in response to corticosterone (figure 3F–H). Analysis of gene expression in synovial tissue digests revealed a significant reduction in the chemokines Ccl2, *Cxcl10* and cytokine *Il-1b*, and an increased expression of

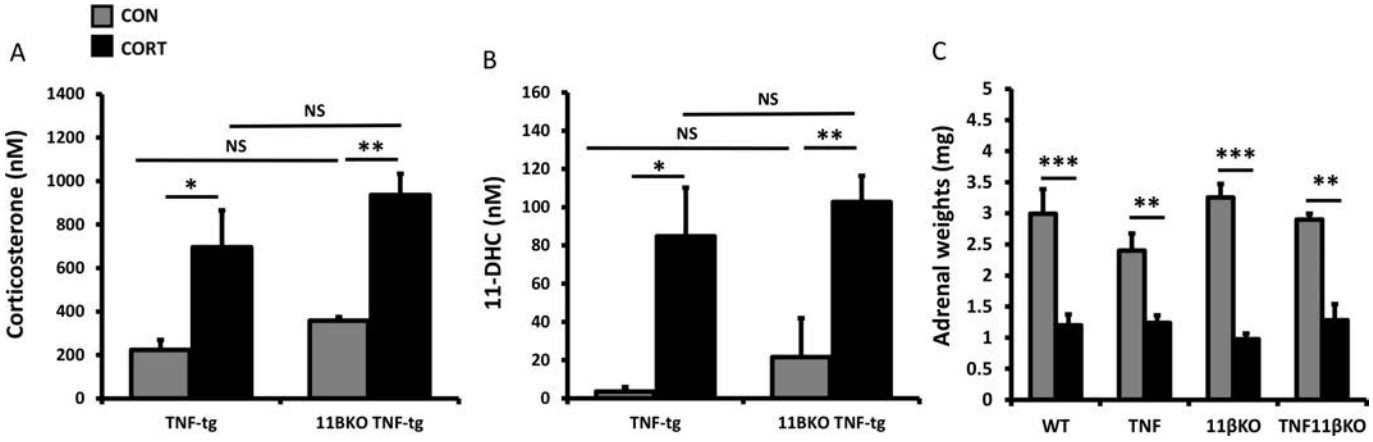

Figure 2 (A, B) Serum corticosterone and 11-dehydrocorticosterone (11-DHC) determined by liquid chromatography tandem mass spectrometry (LCMS) from tumour necrosis factor (TNF)-tg, 11βKO and TNF-tg11βKO animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. (C) Adrenal weights in wild-type (WT), TNF-tg, 11βKO and TNF-tg11βKO animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. Values are expressed as mean±SE, n=6 per group for ELISA and adrenal weights and n=3 per group for LCMS. Statistical significance was determined using two-way analysis of variance with Tukey post hoc analysis. *P<0.05, **p<0.005, ***p<0.001. NS, not significant.

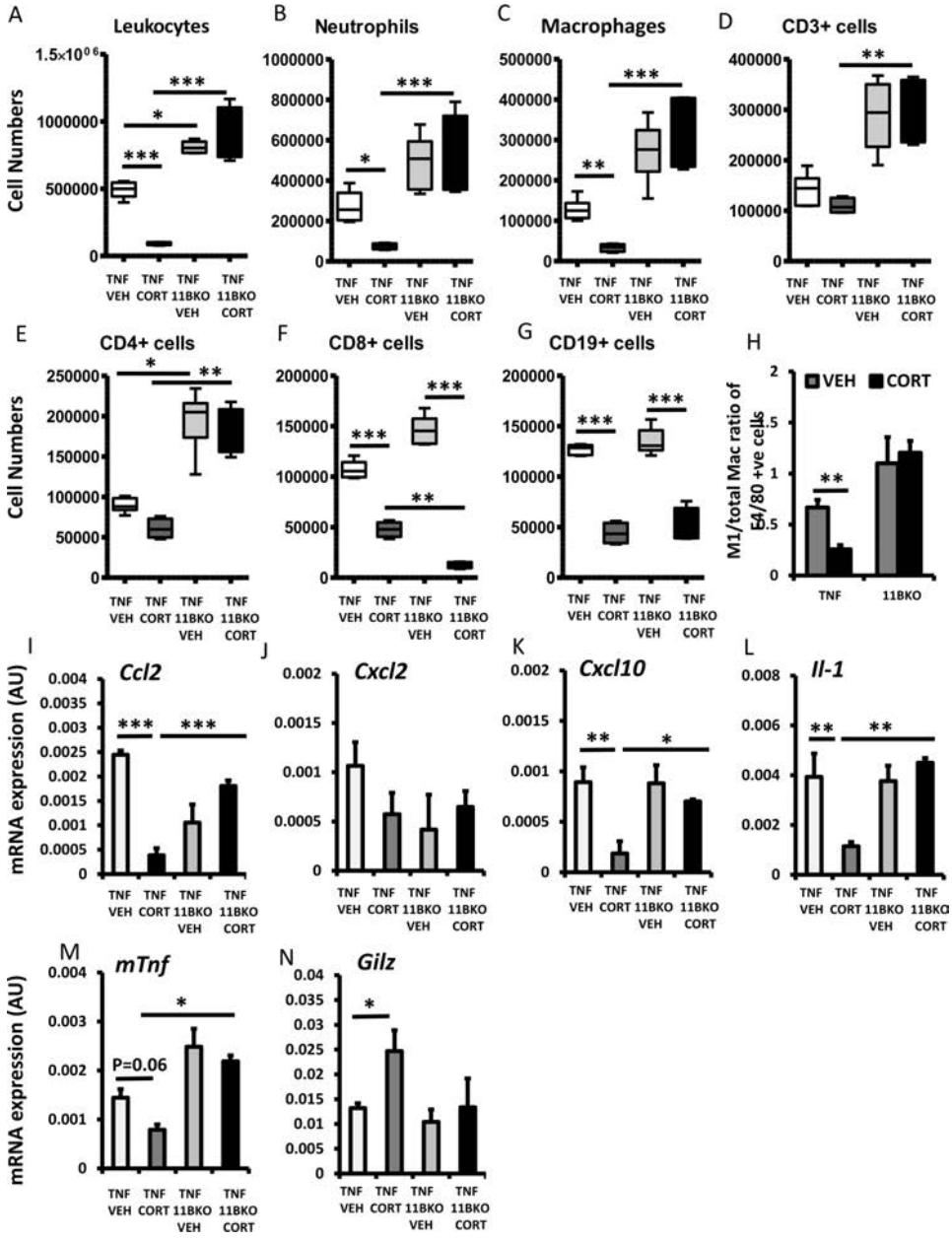

Figure 3 Cell numbers of (A) total leucocytes, (B) neutrophils, (C) macrophages, (D) CD3+ populations, (E) CD4+ populations, (F) CD8+ populations, (G) CD19+ populations and (H) the M1-like/total macrophage ratio determined by flow cytometry in tumour necrosis factor (TNF)-tg and TNF-tg$^{11\beta KO}$ animals receiving vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. Gene expression (AU) of (I) Ccl2, (J) Cxcl2, (K) Cxcl10, (L) Il-1, (M) mTnf and (N) Gilz determined by quantitative PCR in tibia isolated from TNF-tg and TNF-tg$^{11\beta KO}$ animals receiving vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. Values are expressed as mean±SE, n=6 per group. Statistical significance was determined using two-way analysis of variance with Tukey post hoc analysis. *P<0.05, **p<0.005, ***p<0.001.

anti-inflammatory *Gilz* expression in TNF-tg animals receiving corticosterone, which was entirely absent in TNF-tg$^{11\beta KO}$ animals (figure 3I–N). These data reveal that TNF-tg$^{11\beta KO}$ animals show marked resistance to the anti-inflammatory properties of therapeutic GCs on leucocyte recruitment and on regulation of local inflammatory mediators.

Mice with stromal deletion of 11β-HSD1 retain anti-inflammatory responses to GCs

Given the stromal upregulation of stromal 11β-HSD1 sites of inflammation, we wished to delineate its specific contribution to GC resistance in the TNF-tg$^{11\beta flx/tw2cre}$ mouse relative to TNF-tg littermates, where we have previously reported effective mesenchymal deletion.[3 8] A significant reduction in 11β-HSD1 activity was apparent in primary fibroblasts and osteoblasts isolated from TNF-tg$^{11\beta flx/tw2cre}$ animals, while activity was retained in non mesenchyme derived tissues such as livers and spleen (figure 4A,B). Suppression of adrenal weights was apparent across all groups in response to corticosterone (figure 4C). Corticosterone significantly reduced clinical scores and measures of joint inflammation in both TNF-tg$^{11\beta flx/tw2cre}$ and TNF-tg littermates (figure 4D,E). While circulating levels of the acute response cytokine IL-6, remained elevated in TNF-tg$^{11\beta flx/tw2cre}$ receiving corticosterone, analysis of pannus invasion, osteoclast numbers and joint destruction by micro-CT, indicated that corticosterone was equally effective at suppressing disease activity

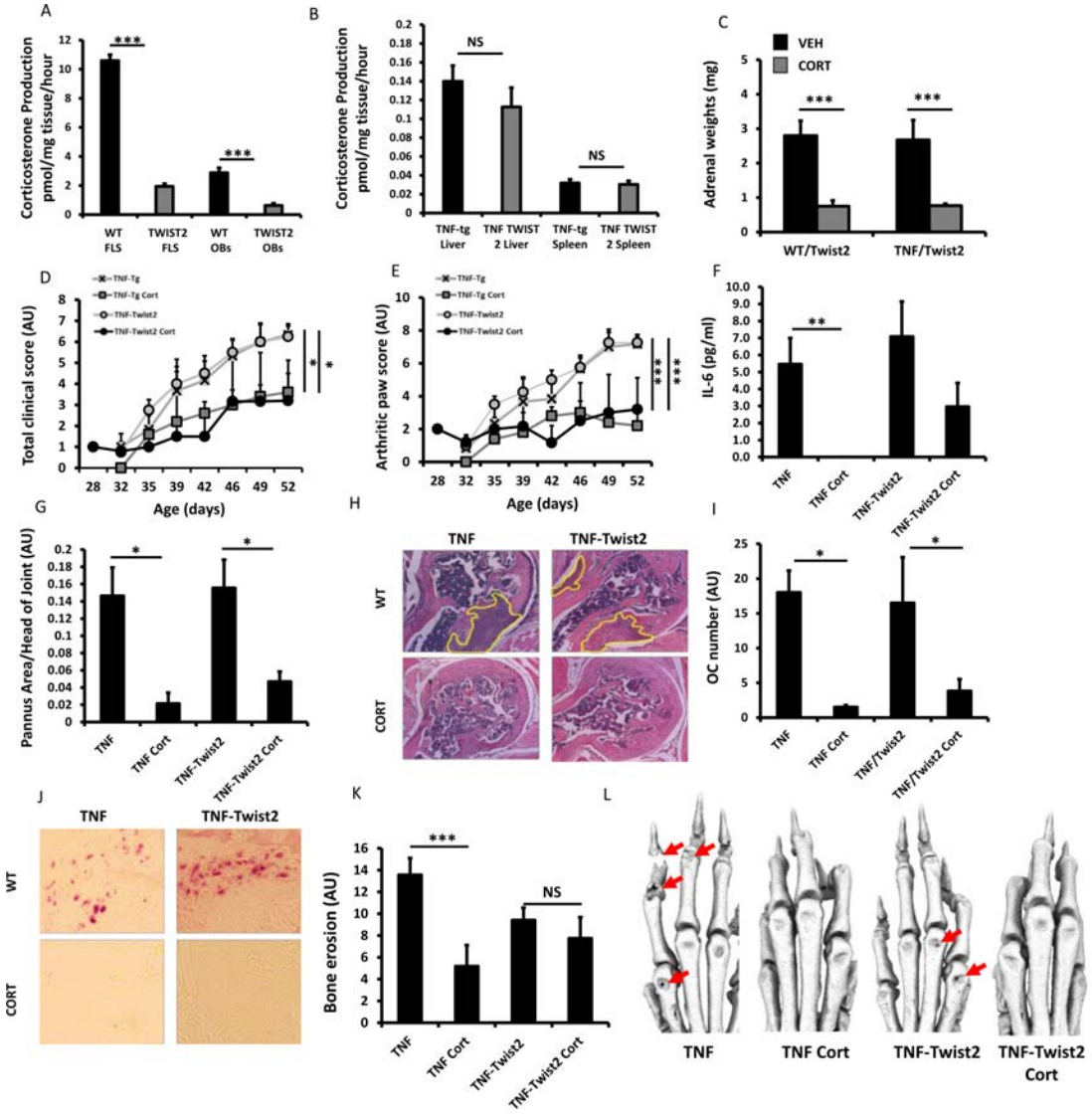

Figure 4 Corticosterone production (pmol/mg tissue/hour) in (A) fibroblast-like synoviocytes (FLS) and osteoblasts (OBs) cultures and (B) liver and spleen ex vivo biopsies isolated from tumour necrosis factor (TNF)-tg and TNF-tg$^{11\beta flx/tw2cre}$ mice determined by scanning thin layer chromatography. (C) Adrenal weights (mg), (D) total clinical scores (AU), (E) arthritic paw scores (arbitrary units (AU)), (F) serum IL-6 determined by ELISA, (G) histological scoring (AU) and (H) representative images of synovitis at the ulna/humerus joint interface, (I) histological scoring (AU) and (J) representative images of TRAP stained osteoclast numbers at the ulna/humerus joint interface, (K) quantification of bone erosion (AU) in the wrist, metacarpals and phalanges and (L) representative images of three-dimensional reconstructions of front paws in TNF-tg and TNF-tg TNF-tg$^{11\beta flx/tw2cre}$ animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. Values are expressed as mean±SE, n=6 per group. Statistical significance was determined using two-way analysis of variance with Tukey post hoc analysis. *P<0.05, **p<0.005, ***p<0.001.

in both TNF-tg and TNF-tg$^{11\beta flx/tw2cre}$ animals (figure 4C,G–L). Consequently, despite effective deletion of 11β-HSD1 in the mesenchymal compartment, TNF-tg$^{11\beta flx/tw2cre}$ animals retain a robust anti-inflammatory response to corticosterone.

Partial GCs resistance with myeloid deletion of 11β-HSD1

At sites of inflammation, 11β-HSD1 is highly expressed in macrophages and is implicated in regulating their anti-inflammatory properties.[3 18 19] We used the LysMCre mouse (targeted towards neutrophils, macrophages and granulocytes) to generate tg$^{11\beta flx/LysMcre}$ animals with a deletion of 11β-HSD1 in the myeloid compartment.[12] 11β-HSD1 activity was significantly reduced in both peripheral blood mononuclear cell and peritoneal macrophages relative to WT counterparts (figure 5A). In contrast, normal 11β-HSD1 activity was apparent in tissues such as muscle, fat and liver (figure 5A,B). Corticosterone significantly reduced adrenal weights in both TNF-tg$^{11\beta flx/LysMcre}$ animals and TNF-tg littermate controls of (figure 5C). TNF-tg$^{11\beta flx/LysMcre}$ mice receiving corticosterone showed a significant reduction in joint inflammation scores but not in total clinical scores, while serum IL-6 levels were similarly decreased in both TNF-tg and TNF-tg$^{11\beta flx/LysMcre}$ (figure 4D–F). A, significant reductions in both pannus size and osteoclast numbers were apparent in both TNF-tg and TNF-tg$^{11\beta flx/LysMcre}$ animals receiving corticosterone (figure 5G–J). However, evidence of residual pannus and osteoclast numbers in TNF-tg$^{11\beta flx/LysMcre}$ animals was supported by a greater incidence of juxta-articular joint destruction determined by micro-CT relative to TNF-tg counterparts ($p<0.05$) (figure 5K,L). These data demonstrate that mice with a myeloid targeted deletion of 11β-HSD1 retain the capacity to respond to therapeutic GCs.

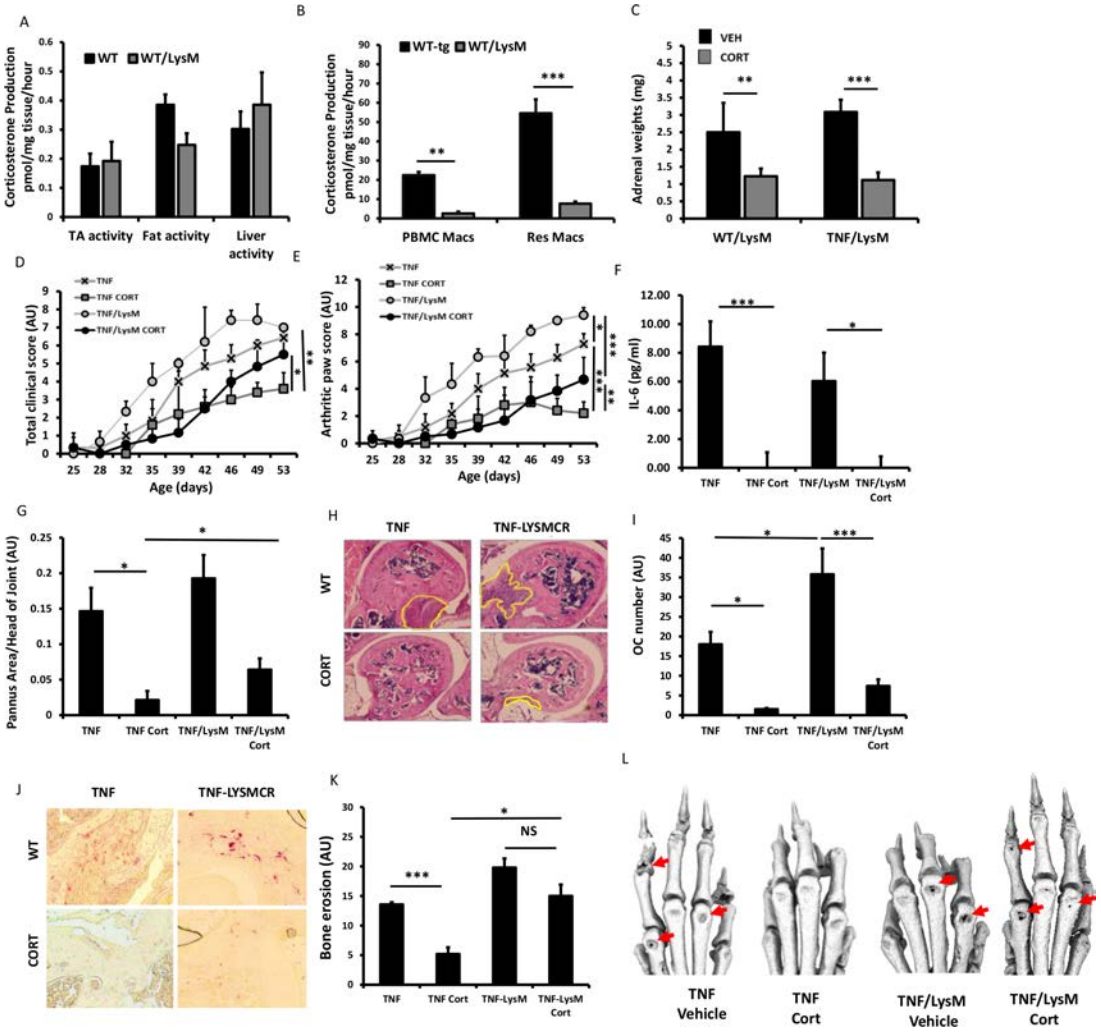

Figure 5 Corticosterone production (pmol/mg tissue/hour) in (A) tibialis anterior (TA) muscle, fat and liver ex vivo biopsies and (B) monocyte, peripheral blood mononuclear cell (PBMC)-derived macrophages and resident macrophages isolated from tumour necrosis factor (TNF)-tg and TNF-tg$^{11\beta flx/LysMcre}$ mice determined by scanning thin layer chromatography. (C) Adrenal weights (mg), (D) total clinical scores (arbitrary units (AU)), (E) arthritic paw scores (AU), (F) serum interleukin (IL)-6 determined by ELISA, (G) histological scoring (AU) and (H) representative images of synovitis at the ulna/humerus joint interface, (I) histological scoring (AU) and (J) representative images of TRAP stained osteoclast numbers at the ulna/humerus joint interface, (K) quantification of bone erosion (AU) in the wrist, metacarpals and phalanges and (L) representative images of three-dimensional reconstructions of front paws in TNF-tg and TNF-tg$^{11\beta flx/LysMcre}$ animals receiving either vehicle or corticosterone (100 µg/mL) in the drinking water for 3 weeks. Values are expressed as mean±SE, n=6 per group. Statistical significance was determined using two-way analysis of variance with Tukey post hoc analysis. *P<0.05, **p<0.005, ***p<0.001.

Paracrine GC signalling compensates for cell-specific 11β-HSD1 deletion

Given our findings in the stromal and myeloid targeted models, we performed co-culture experiments in FLS and macrophage to determine if GCs activated in one cell population could influence the other by paracrine signalling. We generated conditioned media by exposing WT and 11β-HSD1 KO FLS to the inactive GC 11-DHC for 24 hours, which was then placed on 11β-HSD1 KO macrophages for a further 24 hours prior to measuring GC response genes (figure 6A). Here, 11β-HSD1 KO macrophages responded to conditioned media from WT FLS exposed to 11-DHC (increasing Gilz and suppressing IL-6), but not conditioned media from 11β-HSD1 KO FLS (figure 6B,C). Conditioned media from WT and 11β-HSD1 KO macrophages conditioned with 11-DHC were then placed on 11β-HSD1 KO FLS for 24 hours and GC responsive gene analysed (figure 6D). 11β-HSD1 KO FLS responded to conditioned media from WT macrophages exposed to 11-DHC (increasing Gilz and suppressing IL-6), but did not respond to conditioned media generated in 11β-HSD1 KO macrophages (figure 6E,F). Similarly, IL-6 production in 11β-HSD1 KO FLS was suppressed in response to media from WT macrophages exposed to 11-DHC, but not from 11β-HSD1 KO macrophages exposed to 11-DHC (figure 6G). These data confirm that 11β-HSD1 can mediates paracrine GC signalling between distinct cell populations present at sight of inflammation, including macrophages and FLS.

DISCUSSION

Despite the potent upregulation of 11β-HSD1 at sites of inflammation, its roles in mediating the effects of active therapeutic GCs have remained poorly understood.[19–22] Here, studies by Schmidt *et al* and Hardy *et al* reported increasing levels of 11β-HSD1 within FLS and synovial macrophages that correlated with inflammation. Using murine models of polyarthritis, we have identified an entirely novel and, until now, unrecognised

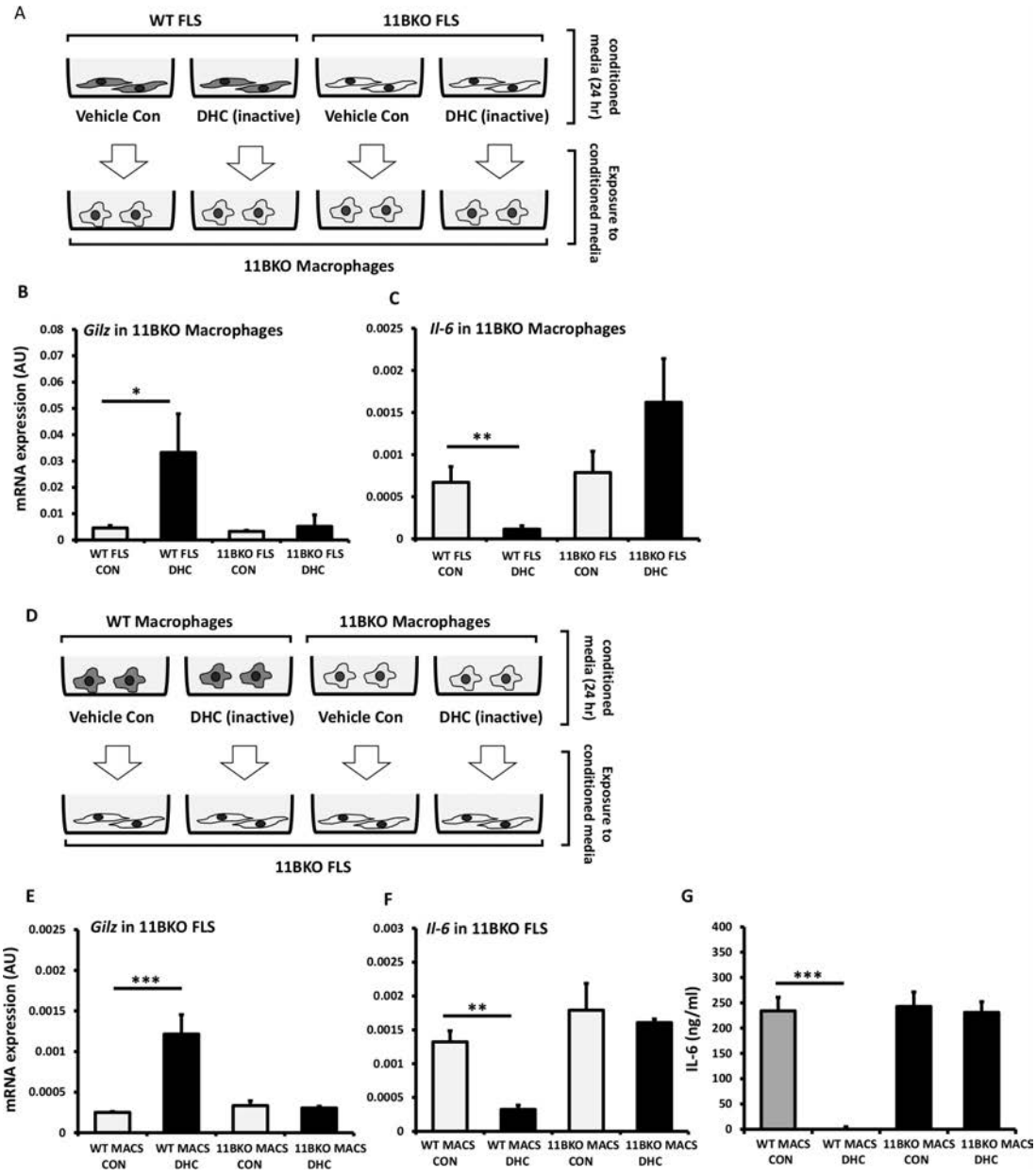

Figure 6 (A) Schematic representation of conditioned media experiments in which media from wild-type (WT) and 11βKO fibroblast-like synoviocytes (FLS) treated with either vehicle or dehydrocorticosterone (DHC) is used to treat 11βKO macrophages. Gene expression (arbitrary units (AU)) of (B) Gilz and (C) Il-6 in 11βKO macrophages treated with conditioned media from WT and 11βKO FLS determined by quantitative PCR (qPCR). (D) Schematic representation of conditioned media experiments in which media from WT and 11βKO macrophages treated with either vehicle or DHC is used to treat 11βKO FLS. Gene expression (AU) of (E) Gilz and (F) Il-6 in 11βKO FLS treated with conditioned media from WT and 11βKO macrophages determined by qPCR. (G) Protein levels of IL-6 (ng/mL) in the media of 11βKO FLS treated with conditioned media from WT and 11βKO macrophages determined by ELISA. Values are expressed as mean±SE, n=3 per group. Statistical significance was determined using one-way analysis of variance with Tukey post hoc analysis. *P<0.05, **p<0.005, ***p<0.001.

component of therapeutic GC action, whereby they require peripheral reactivation by 11β-HSD1 at sites of inflammation to deliver anti-inflammatory effects (figure 7). Here, the global transgenic deletion of 11β-HSD1 prevents this critical step, resulting in severe GC resistance in both TNF-tg and K/BxN models of polyarthritis.

The importance of endogenous GC metabolism by 11β-HSD1 in the pathophysiology of inflammatory polyarthritis are well established.[8 18 23] We used oral corticosterone to suppress disease activity and joint inflammation in murine models of polyarthritis.[2 14 24] We observed effective suppression at 100 µg/mL in the drinking water, where daily intake of corticosterone was 22.5 µg/g in the mice, and would be estimated to equate to administration of 40 mg hydrocortisone or 10 mg of prednisolone per day in an adult. Here, total active corticosterone and inactive 11-DHC, increased in TNF-tg and TNF-tg$^{11\beta KO}$ mice, with the reduced transcortin binding affinity of 11-DC predicted to further elevate its circulating free levels.[25] In the models of polyarthritis, disease activity, synovitis and joint destruction were markedly suppressed in animals receiving corticosterone. However, we observed a profound GC resistance in 11β-HSD1 KO animals, despite equivalent serum exposure to corticosterone and 11-DHC. While these animals with deletion of 11β-HSD1 retained a capacity to respond to

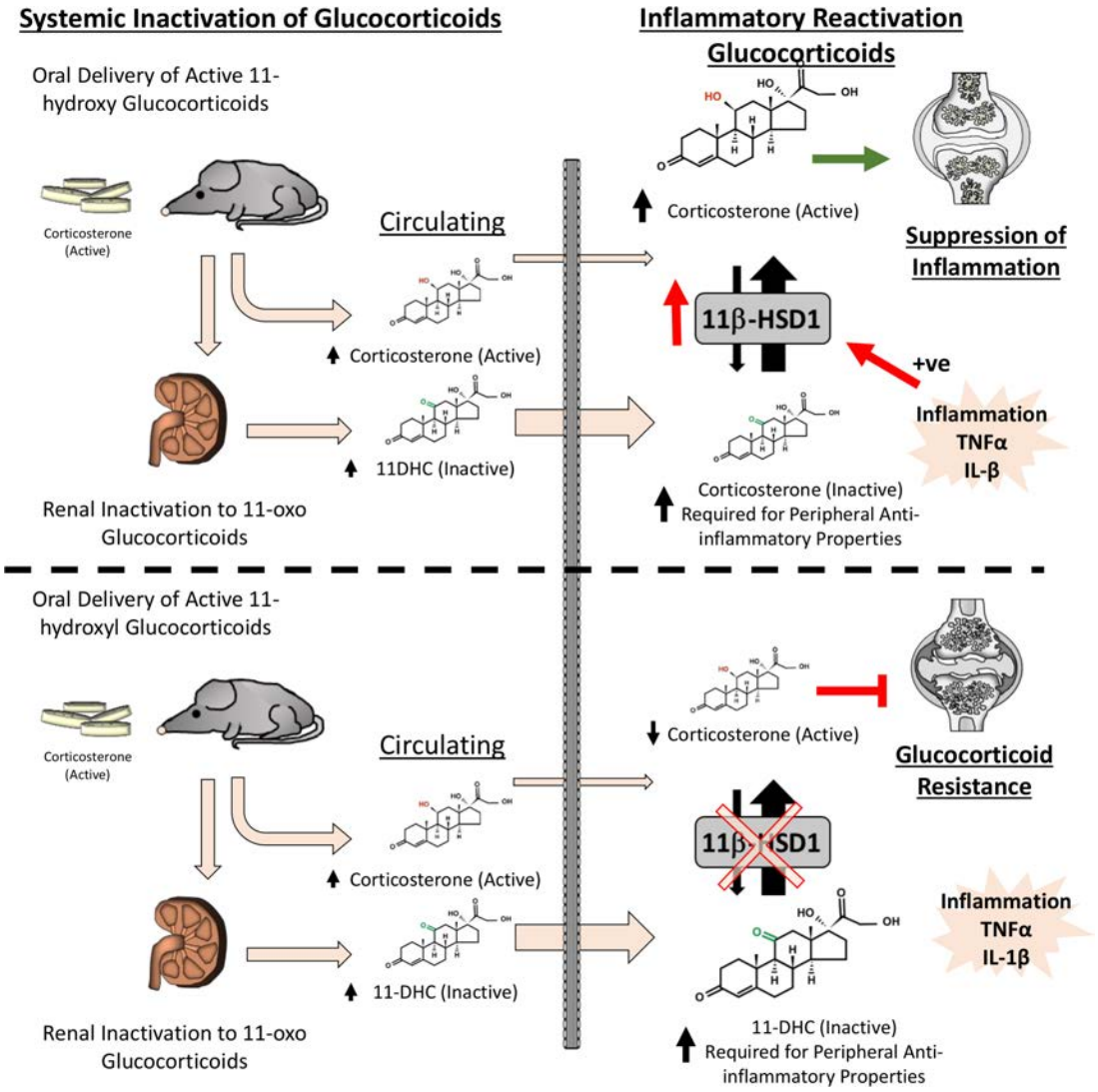

Figure 7 At therapeutic doses, the glucocorticoid corticosterone requires peripheral reactivation by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) at sites of inflammation, which is potently upregulated by pro-inflammatory factors such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β, to enable their anti-inflammatory effects. The deletion of 11β-HSD1 (shown below the dashed line) prevents this critical step, resulting in severe GC resistance.

oral corticosterone, with evidence of a GC-mediated adrenal suppression and limited improvements in body weight and pain behaviour in the K/BxN model, these levels were insufficient to mediate anti-inflammatory actions within the joint. This indicates that the peripheral metabolism and activation of GCs such as corticosterone by 11β-HSD1 are required to mediate their anti-inflammatory properties. In this study, we used the GC corticosterone in our models as, within mice, it possesses equivalent action and metabolism as the steroid hydrocortisone in humans. Further research is now required to examine how synthetic GCs such as prednisolone and prednisone are metabolised by 11β-HSD1 at sites of inflammation in murine models and in human disease cohorts. This is of particular interest in human inflammatory disease, with Schmidt *et al* reporting shifts towards reduced synovial GC activation in synoviocytes in rheumatoid arthritis (RA) relative to osteoarthritis.[20] This appeared to occur secondary to a shift in the 11β-HSD1/11β-HSD2 ratio, favouring steroid inactivation and was potentially attributed to a loss of sympathetic nerve fibre signalling to the RA joint. Analysis of synovial tissue shed light on the mechanism of GC resistance in the TNF-tg$^{11\beta KO}$ mouse. A key mechanism of action of therapeutic GC in the inflamed synovium is the suppression of leucocyte recruitment and reduction in pro-inflammatory cytokines and chemokines through suppression of pro-inflammatory pathways.[26–28] In this feature of anti-inflammatory GC action, there remains ongoing debate in relation to the relative contributions of transactivation and transrepression as mechanism underlying the anti-inflammatory effects of GCs.[29] In this study, we were unable to assess whether the anti-inflammatory properties of GC metabolism by 11β-HSD1 were predominantly mediated by transrepression or transactivation, which remains a prominent area of interest and the focus of prominent reviews in the field.[1] In TNF-tg$^{11\beta KO}$ animals, expression of pro-inflammatory mediators persisted at sites of inflammation in response to corticosterone, with increased inflammatory activated M1-like polarisation, revealing a critical role for therapeutic GC metabolism by 11β-HSD1 in this process. These data suggest that in response to therapeutic GCs, 11β-HSD1 may mediates a shift from inflammatory activated M1-like macrophages to M2-like polarisation. However, the precise nature of these changes in this setting remains complex and is the subject, requiring more detailed characterisation, which in itself is the feature of

a notable systematic review by Tardito *et al*.[30] Of interest, we observed that several leucocyte population, including CD8 T cells and CD19 B cells within the synovium of 11β-HSD1 KO animals retained responsiveness to oral corticosterone and were suppressed to a similar degree as WT counterparts. These data indicate that certain leucocyte populations retain the capacity to respond to circulating levels of active corticosterone, present even in the absence of 11β-HSD1, suggesting they possess a lower GC receptor activation threshold that is independent of 11β-HSD1.

Given that FLS and macrophages highly express 11β-HSD1 in inflammatory environments, we examined whether targeted deletion of 11β-HSD1 within mesenchymal derived FLS and myeloid-derived macrophages could recapitulate global steroid resistance. Here, despite effective 11β-HSD1 deletion in FLS, TNF-tg$^{11\beta flx/tw2cre}$ animals showed an entirely normal anti-inflammatory response to oral corticosterone, suggesting that GC reactivation within this subset alone was not critical to the anti-inflammatory properties of corticosterone. Similar findings were evident in myeloid-targeted TNF-tg$^{11\beta flx/LysMcre}$. Here despite effective deletion of 11β-HSD1 within macrophages, TNF-tg$^{11\beta flx/LysMcre}$ animals retained the capacity to respond to corticosterone. However, their response did appear to be muted, with disease activity scores being greater than TNF-tg counterparts, and with evidence of persistent joint destruction despite exposure to therapeutic GCs. The contribution of 11β-HSD1 within further leucocyte populations such as T cells to corticosterone resistance in the global KO deserve further scrutiny in this context but went beyond the scope of this study.

However, these data may suggest that the autocrine amplification of GCs by 11β-HSD1 within fibroblasts or macrophages alone is insufficient to mediate the anti-inflammatory actions of therapeutic GCs in vivo, as occurs with cell-targeted GC receptor KO studies.[31–33] Instead, we explored whether paracrine signalling between macrophages and FLS might overcome cell-targeted deletion of 11β-HSD1 using in vitro models. These experiments revealed that metabolism of 11-DHC by 11β-HSD1 could mediate paracrine GC signalling between FLS and macrophages, compensating for cell-targeted deletion of 11β-HSD1 and reversing steroid resistance. Consequently, it may be that targeting any one cell population is insufficient to reproduce the phenotype observed with global 11β-HSD1 deletion. Ultimately, it is important to note that results observed in animal models are not always replicated in human disease, and these findings now require robust validation in patients with inflammatory disease.

In this study, we demonstrate a profound and previously unrecognised role for pre-receptor metabolism and activation of GCs by the enzyme 11β-HSD1 in mediating the anti-inflammatory therapeutic actions of oral GCs. Consequently, this study adds significant insight into our mechanistic understanding of therapeutic GC action. Here, a greater awareness of the how 11β-HSD1 targets the anti-inflammatory actions of therapeutic GCs at sites of inflammation may be able to inform the development of better-tolerated steroids that possess enhanced kinetics and activation efficiency by 11β-HSD1 to improve targeting and dosing efficacy, as well as informing ongoing studies examining the application of therapeutic 11β-HSD1 inhibitors.

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Ethics approval Procedures were performed under guidelines by the Animal (Scientific Procedures) Act 1986 in accordance with the project licence (P51102987) and approved by the Birmingham Ethical Review Subcommittee (BERSC).

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information. All data relevant to the study are included in the article or uploaded as online supplementary information. Data are available on reasonable request from Dr Hardy. Reuse of data is not permitted by a third party without authorisation.

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CLINICAL SCIENCE

Criterion validity of ultrasound in the identification of calcium pyrophosphate crystal deposits at the knee: an OMERACT ultrasound study

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ABSTRACT

Objective To evaluate the discriminatory ability of ultrasound in calcium pyrophosphate deposition disease (CPPD), using microscopic analysis of menisci and knee hyaline cartilage (HC) as reference standard.

Methods Consecutive patients scheduled for knee replacement surgery, due to osteoarthritis (OA), were enrolled. Each patient underwent ultrasound examination of the menisci and HC of the knee, scoring each site for presence/absence of CPPD. Ultrasound signs of inflammation (effusion, synovial proliferation and power Doppler) were assessed semiquantitatively (0–3). The menisci and condyles, retrieved during surgery, were examined microscopically by optical light microscopy and by compensated polarised microscopy. CPPs were scored as present/absent in six different samples from the surface and from the internal part of menisci and cartilage. Ultrasound and microscopic analysis were performed by different operators, blinded to each other's findings.

Results 11 researchers from seven countries participated in the study. Of 101 enrolled patients, 68 were included in the analysis. In 38 patients, the surgical specimens were insufficient. The overall diagnostic accuracy of ultrasound for CPPD was of 75%—sensitivity of 91% (range 71%–87% in single sites) and specificity of 59% (range 68%–92%). The best sensitivity and specificity were obtained by assessing in combination by ultrasound the medial meniscus and the medial condyle HC (88% and 76%, respectively). No differences were found between patients with and without CPPD regarding ultrasound signs of inflammation.

Conclusion Ultrasound demonstrated to be an accurate tool for discriminating CPPD. No differences were found between patents with OA alone and CPPD plus OA regarding inflammation.

Key messages

What is already known about this subject?

- Previous monocentric studies have suggested a high diagnostic accuracy of US for identifying calcium pyrophosphate deposition disease (CPPD).
- Studies were carried out using different definitions and reference standards making the results not comparable.

What does this study add?

- This study is the first international multicentric study, carried out according to the Outcome Measures in Rheumatology methodology for validation of imaging as an outcome for CPPD and using validated definitions.
- Histology has been used as reference test instead of synovial fluid examination or conventional radiology that has demonstrated to have low-sensitivity values for identifying CPPD.

How might this impact on clinical practice or future developments?

- Ultrasound is the most validated examination for identifying CPPD. This makes of ultrasound the first choice for CPPD diagnosis both in clinical practice and for studies.

INTRODUCTION

Calcium pyrophosphate deposition disease (CPPD) is an umbrella term used to describe all instances of calcium pyrophosphate crystals (CPP) occurrence in tissues.[1] The actual prevalence and incidence of the disease are not known, but it is considered to be

one of the most common causes of arthritis[2] with a prevalence increasing with ageing.[3]

Diagnosis of CPPD is one of the most challenging since the disease can present with many different clinical subsets.[1 4] In addition, since its prevalence increases with age, its presentation can frequently be associated with other diseases prevalent in the same range of age such as osteoarthritis (OA) or polymyalgia rheumatica making the differential diagnosis and the attribution of symptoms to CPPD or to the other disease really difficult. This is particularly true for the association with OA that appears to be very frequent and has been described by the European League Against Rheumatism (EULAR) taskforce as one of the clinical manifestations of CPPD.[1]

According to the EULAR recommendations for the terminology and diagnosis of CPP deposition,[1] definite CPPD joint involvement is considered established when synovial fluid analysis (SFA) with polarised microscopy detects CPP in affected joints. However, even if the specificity of SFA is very high (100%), the sensitivity is approximately 70%[5] which is suboptimal as approximately one-third of the patients could be missed. Further, an invasive manoeuvre (ie, arthrocentesis) has to be performed, which is not always possible either due to comorbidities or to the use of some medications. In addition, technically not in all cases, aspirations of synovial fluid from affected joints are successful. Finally, some not yet solved issues have been raised in the past regarding the reliability of SFA.[6]

Given the limitations of SFA for detecting CPPD, the need to establish alternative methods for diagnosis appears relevant for clinical practice. It has been established that CPP crystals are formed directly in the hyaline cartilage (HC) and fibrocartilage and then shed in the synovial space following different stimuli such as cartilage degeneration or damage. Free crystals in the joint cavity may then create an inflammatory response, depending on their inflammatory potential (size, type, surface charge and protein coating).[7] This pathogenetic mechanism makes it clear why in some cases SFA could be negative (ie, low number of crystal shedding in the joint space from the cartilage and fibrocartilage) and suggests that imaging could have a role in the identification of CPP in the tissues even before their shedding in the joint cavity.

Conventional radiography (CR) has demonstrated an overall low diagnostic accuracy in CPPD[5] and as reported in the EULAR recommendations, the absence of CPP by X-rays does not exclude CPPD, while on the other hand, the presence of opacities suggestive of calcifications is not necessarily due to CPP crystals.[1] Dual energy CT has been used only in the most recent years and has demonstrated to be able to identify accurately CPPD and to distinguish it from other calcium crystal deposition in joints but has to be still validated for use in clinical practice.[8] MRI has been considered not able to detect CPP so far[9] but in the recent years, new MRI techniques have shown promising results in the identification of CPPD.[10]

Ultrasound has been applied in CPPD for the first time in 1995[11] and since then many studies addressed its role in CPPD diagnosis.[12–16] However, most of them adopted different definitions for ultrasound identification of CPPD and different reference standards, making studies not comparable.[17] For these reasons, the Outcome Measures in Rheumatology (OMERACT) Ultrasound Working Group created a subgroup to specifically address issues related to the development of ultrasound as an outcome measurement tool in CPPD. Following the OMERACT Ultrasound stepwise methodology,[18] the group developed a new set of definitions to identify the CPP aggregates at the level of fibrocartilage, HC, tendons and synovial fluid.[19] Then, reliability studies to test these definitions on static images and on patients were performed, demonstrating a good inter-reader reliability at the level of the knee and wrist joints.[19 20]

In the present study, we aimed to evaluate the validity of ultrasound by addressing the truth (as expressed by criterion validity) of the definitions through the identification of CPP depositions at the level of menisci and knee HC, using tissues microscopic analysis as reference standard[7] in patients affected by OA and OA plus CPPD. As a secondary aim, inflammation changes were also assessed by ultrasound in order to highlight eventual differences between patients with late stage OA alone and patients with CPPD.

PATIENTS AND METHODS

All members of the OMERACT Ultrasound in CPPD subgroup were invited to participate in this multi-centre, cross-sectional study. Each centre enrolled consecutive patients on the waiting list for total knee replacement surgery (TKR) due to OA, referred to the local Orthopaedic Department. Recruitment was developed in a competitive manner from January to September 2019. All patients underwent an ultrasound examination of the affected knee before surgery. At the day of surgery, both menisci and the HC of the femoral trochlea were retrieved for analysis. Patients who had a positive history of current primary inflammatory joint disease (ie, rheumatoid arthritis, psoriatic arthritis, etc) or who were unable to sign an informed consent form were excluded from the study. All patients signed an informed consent. This study is reported according to the Standards for Reporting Diagnostic accuracy studies guidelines.[21]

Ultrasound assessment

Within 2 weeks before surgery, each patient underwent an ultrasound examination of the knee candidate to TKR. The ultrasound examination was performed by experienced sonographers, all members of the OMERACT Ultrasound in CPPD group. At each centre, all ultrasound examinations were performed by a local ultrasonographer who was blind to the clinical history of the patient.

For each knee examined, the sonographer evaluated both lateral and medial menisci and the HC of the femoral trochlea, giving a dichotomous score about the presence/absence of CPP deposits, based on the OMERACT definitions[20] (figure 1). Effusion and synovitis were assessed following previously published scoring systems.[22] The reliability of the sonographers was tested previously and the results have already been published.[19 20]

Ultrasound examination was performed using the following scanning protocol: the knee menisci were examined with the knee in complete extension and in semiflexed position (30°–45°) sliding with the probe (without lifting it) over the structure

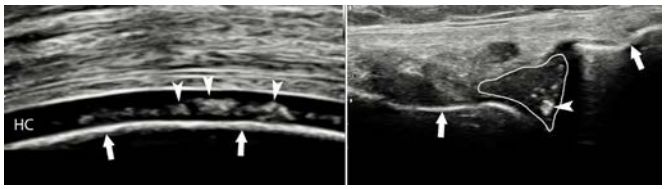

Figure 1 Typical appearance of calcium pyrophosphate deposition disease (CPPD) in the hyaline cartilage (HC) of the knee (on the left panel) and in the meniscus (right panel). Arrowheads: calcium pyrophosphate (CPP) crystal deposits, arrows: bone profile, HC corresponding to the anechoic (black) layer above the bone profile, continuous white line on the right panel: the meniscal fibrocartilage.

under examination from the anterior to the posterior horn. Both transverse and longitudinal scans were used if considered necessary. HC of the femoral trochlea was scanned with the knee in maximum flexion with both transverse and longitudinal suprapatellar scans. The suprapatellar, medial and lateral parapatellar recesses were examined with longitudinal scans in order to assess effusion and synovitis.

Being a multicentre study with different ultrasound machines used, no general setting was created a priori, but the grey-scale parameters were set by the sonographers in order to optimise images for identifying CPP within cartilage and fibrocartilage tissues in the first patient. Once the setting was optimised, it was saved as a preset and was used throughout the study. Gain, probe frequency and the dynamic contrast parameters could be changed during the examination, starting from the saved preset, in order to further optimise image quality at the characteristics of each patient. Power Doppler parameters also were set independently in each machine by the sonographers in order to achieve maximal sensitivity according to the previously published guidelines for power Doppler setting for inflammatory blood flow.[23 24]

Menisci and cartilage retrieval, conservation and shipment

Transverse sections of the femoral condyles and both menisci were collected from the patients and washed with phosphate-buffered saline or physiological saline solution to remove blood as it has been demonstrated that such solutions do not dissolve CPP.[25] Wash fluid was discarded, and each sample was stored in separate sterile cups and immediately frozen at −80°C. The frozen samples were shipped in dry ice to the University of Padua, Italy or processed at the collecting centre (where possible) for the microscopic analysis according to a prespecified protocol. An adhesive ticket with the unique ID code of the patient and the type of sample was attached to each cup.

Menisci and cartilage microscopic analysis

Menisci were transversally sectioned into 10 segments with approximately the same dimensions using a scalpel and each resulting transverse surface obtained was vigorously scraped with a clean curette or spatula. Incisions perpendicular to the free surface were performed with scalpel in 10 different regions of hyaline femoral cartilage. Clean curette or spatula were used to penetrate the inner layer of the cartilage at the site of incision and scrape the tissue. Tools to cut and scrapers were changed for each specimen. The material removed by the scraping was placed directly on a microscope slide previously rinsed with 70% Ethanol Solution, followed by a drop of water and a cover slip, and observed at 400× magnification using compensated polarised light microscopy. The observation was focused on the detection of CPP crystals by morphology and birefringence, defined as present/absent. CPP crystals were defined as small, rod-shaped or rhomboid-shaped, that polarise light and are positively birefringent (crystals aligned with the compensator filter are blue, whereas those lying perpendicular are yellow). Patients were considered positive for CPPD if at least one of their tissue specimens revealed the presence of CPP crystals. All examiners were blind to US findings.

Power calculation, statistical analysis, patient involvement and ethics

In order to calculate the necessary number of patients for reliable results, an assumed prevalence of CPP deposits at the meniscus fibrocartilage of 30% with an expected sensitivity of 85%, and specificity of 95% was considered, yielding a total number of 50 patients to be sufficient to estimate both sensitivity and specificity with a precision of 15%–20%, setting alpha to 5%. The association between ultrasound variables (grade of effusion, synovial hyperplasia and power Doppler) and CPPD/OA was evaluated by the two-sample Wilcoxon rank-sum (Mann-Whitney) test. Only patients with full ultrasound and microscopic data regarding the primary objective of the study were included in the final analysis. Stata V.14 software was used for statistical analysis. The research protocol was done without patient involvement. Patients were not invited to comment on the study design and were not consulted to develop patient-relevant outcomes or interpret the results. Patients were not invited to contribute to the writing or editing of this document for readability or accuracy. The study was approved by the local Ethics Committee of the University of Ferrara (principal investigator site, study number 171190 approved on 17 December 2017) and subsequently by local ethics committees of all the participating centres.

RESULTS

Eleven researchers participated in the study, coming from seven countries (France 1 centre 3 pts, Italy 3 centres 12, 12 and 25 pats, Mexico 1 centre 8 pts, Romania 2 centres 7 and 4 pts, Spain 1 centre 12 pts, Switzerland 1 centre 11 pts, USA 1 centre 7 pts). Being members of the OMERACT US group, all participants were experienced sonographers and used high-end machines (Samsung RS080A, GE Logiq E9, Esaote SpA MyLab Class C) for this study equipped with linear high frequency probes (from 12 MHz to 18 MHz). From the 101 consecutive patients who were enrolled in the study only 68 were included in the analysis as for 38 patients not all anatomical pieces were retrieved during surgery and therefore were excluded. The mean age of the patients included in the study was of 71 years old (±), 44 were women. All patients had OA of the knee of grade 3 or 4 according to Kellgren-Lawrence scoring.[26]

The microscopic analysis of the specimens, that was also the reference standard, was carried out on site (according to the above reported protocol) in three centres (Mexico, Romania–Bucharest, USA) while all other researchers shipped the anatomical pieces to the central laboratory in Padua for analysis. The longest time between retrieval of pieces and histological analysis was of approximately 3 months (due to accumulation of all cases before the unique shipment of the pieces) but in all cases, there was no interruption of the cold chain. The prevalence of CPPD in the cohort of patients, meaning at least one of the examined tissues of each knee positive at microscopy, was of 50% (34/68). Thirty-three patients were positive at the medial meniscus and 31 at the lateral for a total of 34 patients positive for meniscus as most of them were positive in both. Regarding the HC, 30 knees were positive at the medial condyle and 24 at the lateral one for a total of 33 knees positive at the HC. All knees that were positive at the HC were also positive at the meniscus (at least one) while one knee was positive at the meniscus level without presenting deposition at the HC. In figure 2, pictures of a meniscus with and without CPPD are represented.

The overall diagnostic accuracy of ultrasound for CPPD at the knee (all tissues included) was of 75% with a sensitivity of 91% and specificity of 59%. However, values of sensitivity and specificity were quite different depending on the tissue under examination, yielding the higher sensitivity values at the level of the medial meniscus (87%) and the higher specificity at the level of the HC of the medial condyle (92%). By combining the different sites under examination, the best combination for sensitivity and specificity

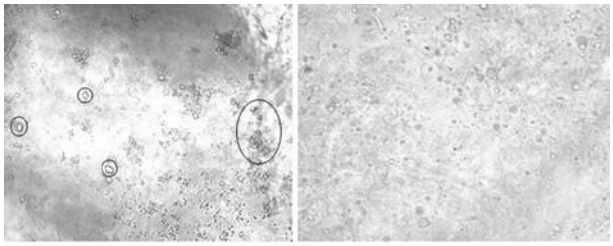

Figure 2 Tissue fragment from meniscus with calcium pyrophosphate (CPP) crystals (left frame, typical rod-shaped or rhomboid-shaped crystals compatible with CPP are highlighted in the black circles) and tissue fragment from meniscus without crystals (right frame).

values was obtained by assessing the medial meniscus in combination with the HC of the medial condyle. Regarding predictive values, global assessment of the knee yielded a positive predictive value (PPV) of 69% and a negative predictive value (NPV) of 87%. Depending on the site under examination, PPV ranged from 68% to 88% while NPV presented a lower variability ranging from 83% to 88%. Ultrasound results are reported in table 1.

Regarding the ultrasound signs of inflammation (ultrasound data on inflammation not available for two patients, both of them in the OA group), 61 patients presented effusion (mean grade 1,65±1, median 2), 48 synovitis (mean 1.3±1, median 1) and 19 positive power Doppler signal (mean 0.36±0.65, median 0). Group values are reported in figure 3. No statistically significant differences were found regarding ultrasound inflammation signs between knees affected by OA alone and OA plus CPPD.

DISCUSSION

Even if CPPD appears to be a prevalent disease in the elderly and may cause acute or chronic arthritis,[1] the diagnosis is still challenging and no specific treatment is available. In a recent study on a cohort of patients diagnosed as early seronegative RA, it was found that 7% of patients that were older than 60 were affected also by misdiagnosed CPPD that could explain the symptoms.[27] The G-Can (Gout, Hyperuricemia and Crystal Associated Disease Network) performed a study in 2018 aimed to identify the unmet needs and propose research priorities in CPPD.[28] According to the results of that study, development of advanced imaging modalities for improved detection of CPPD was the fourth more important research priority. A feasible, reliable and accurate examination for CPPD detection could not only improve the diagnostic process but also be used to perform epidemiologic studies, contribute to the understanding of the natural evolution of the disease, improve the follow-up of patients and (hopefully in the near future) monitor the effect of crystal dissolution treatments.

The OMERACT 'Ultrasound in CPPD subtask force' was created in November 2014 with the aim to validate ultrasound in the assessment of the disease. As emerged by a systematic literature review and a meta-analysis,[17] one of the main issues regarding the use of ultrasound in CPPD was the lack of standardised, universally accepted definitions for CPPD detection. In the first step, ultrasound definitions were created and then tested in various sites in order to test the reproducibility of the examination.[19 20] These first studies demonstrated that the OMERACT definitions were reliable when applicated to the knees and wrists. The next step at that point, according to the OMERACT US methodology, was to assess the criterion validity discrimination ability of the definitions, meaning the ability of the technique outcome measurement instrument to detect the truth according to an agreed gold standard.[18]

All previous studies dealing with the discrimination ability of ultrasound in CPPD were carried out in one or maximum two centres and did not use validated definitions for ultrasound CPPD identification.[17] Further, only one previous study used as reference standard the microscopic analysis of tissues.[5] In that study, only one centre with high level expertise in the USA in CPPD was involved and the results were better than in this study. The definitions used in that study were the ones created in 2005 by the same group, and the results probably reflect the high confidence of the sonographer in their application. In this OMERACT study, the definitions were created only some months before this study and not all participants had the same confidence with them. Probably in the next future, growing experience on the use if the OMERACT definitions could allow to reach better results both regarding reliability and accuracy aspects. This is the first international multicentre study that is using validated definitions for CPPD identification. Thus, the results do not depend on local expertise on CPPD or characteristics of a specific population but can be considered as universally obtainable.

Ultrasound demonstrated some differences in the accuracy of the detection (also called diagnostic accuracy) in different sites of the knee. The highest sensitivity was observed in the medial meniscus (87%) and the lower at the lateral HC (71%), while the highest specificity was observed at the medial cartilage (92%) and the lower at the lateral meniscus (68%). These differences could be due to some technical issues regarding ultrasound in advanced OA, which was the case of our cohort of patients. A common disorder in these patients is a varus knee alignment[29] that generally determines a medial meniscus extrusion from the joint line.[30] In this situation, ultrasound can access a larger portion of the meniscus allowing a better identification of CPP crystals. On the other hand, the high degree of the degeneration of the menisci in this condition could lead to some hyperechoic areas within the fibrocartilage that could be due to fibrosis[31 32] and could create some false positives decreasing specificity, especially in the lateral meniscus that is more difficult to assess being placed deeper in the joint rim. Regarding the cartilage, the varus alignment of the knee is associated with patellofemoral OA especially of the lateral side[33] determining a loss of width of the HC and consequently technical difficulties in identifying CPPD at this level. On the other hand, the presence of typical ultrasound

Table 1 Results of the ultrasound diagnostic accuracy for detection of CPPD at meniscus and hyaline cartilage of the knee

	Diagnostic accuracy	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Global	0.75	0.91	0.59	0.69	0.87
Medial meniscus	0.82	0.87	0.77	0.77	0.87
Lateral meniscus	0.75	0.83	0.68	0.68	0.83
Medial cartilage	0.86	0.79	0.92	0.88	0.85
Lateral cartilage	0.82	0.71	0.88	0.77	0.84
Medial side (combined cartilage and meniscus)	0.82	0.88	0.76	0.79	0.87
Lateral side (combined cartilage and meniscus)	0.78	0.88	0.69	0.73	0.86

CPPD, calcium pyrophosphate deposition disease.

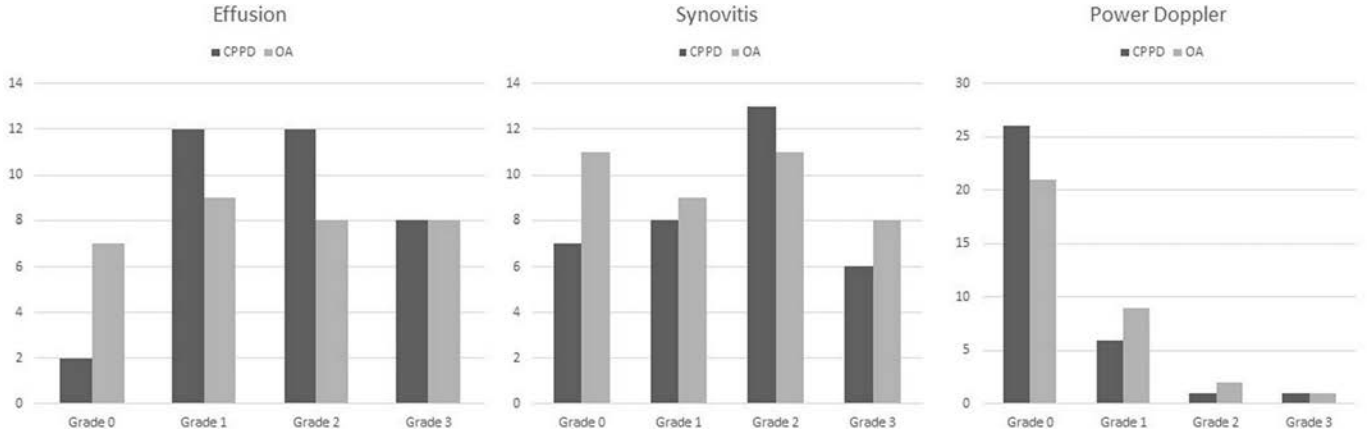

Figure 3 Distribution of patients with by grade of ultrasound effusion (left), synovitis (centre) and power Doppler (right). CPPD,calcium pyrophosphate deposition disease; OA, osteoarthritis.

signs at the HC is quite specific for CPPD as there have not been described until now common conditions that could mimic CPPD at this level.

An important result of this study is the high percentage of both PPV and NPV of ultrasound being, respectively, 79% and 87%. In contrast to sensitivity and specificity that indicate the effectiveness of a test with respect to a trusted 'outside' referent, PPV and NPV indicate the effectiveness of a test for categorising people as having or not having a target condition. This means that ultrasound can be used also in clinical practice for diagnosing the disease. In case of clinical trials where high PPVs are necessary in order to be sure to include patients, specific sites can be used in order to increase this possibility. For example, the HC of the medial condyle provides a PPV of 88%. The predictive values are also used in order to identify examinations that can be suitable for screening purposes. For diseases with a high rate of asymptomatic patients, like CPPD appears to be, high NPVs are important for a screening test.[34] In this context, ultrasound could be proposed as a screening test for CPPD.

Ultrasound inflammatory changes at our cohort were common in both groups and there was no statistically significant difference neither for effusion nor for synovitis/power Doppler values. In a recent study carried out on patients with knee pain and effusion that could be due to CPPD or OA but not in advanced stages was observed a statistically significant difference in the total volume of effusion both in terms of ultrasound and also after complete aspiration of the synovial fluid, being more abundant in patients with CPPD and in the white blood cells found in the synovial fluid, being higher in CPPD.[35] Probably, the advanced stage of the disease in this cohort of patients was responsible for the larger amount of effusion in patients with OA alone as demonstrated in previous studies,[36] making the difference between the two groups not significant. Further, SFA was not carried out in this study so qualitative changes in the SF of the two groups are not known. So, this result should not be interpreted like CPPD does not cause inflammation but rather that in both groups high volume of SF can be found but probably for different reasons and that this is not discriminatory for one or the other diagnosis. Further studies are needed in order to better establish this aspect.

This study presents some limitations. The research for basic calcium phosphate (BCP) crystals in the tissues was not made. In previous studies that used high-sensitivity imaging techniques for the identification of calcium crystals in knee cartilage of patients with late stage OA, it was demonstrated that 100% of these patients presented BCP calcifications[37] so it could be expected that also in our cohort, 100% of patients would present BCP crystals. Further, the identification of BCP crystals is challenging with normal microscopy due to the small size and the lack of birefringence even when the Alizarin Red staining is used.[38] This is a very important point for this study, as in ordinary polarised microscopy examination, BCP crystals cannot be identified on the contrary of CPPD that can be easily distinguished. This means that our reference standard for CPPD did not present any misclassification bias and consequently also the index test (ultrasound) was really tested for discrimination of CPPD. Further, heterogeneity of machines and not standardised setting could be considered as a limitation. However, from our point of view, this is one of the strong points of the study and means that the results are reflecting a real-world use of ultrasound for the detection of CPPD at the knee by expert sonographers. Finally, some researchers performed the reference test on site and not at the central lab in Padua and this could raise reliability issues for the reference test. Unfortunately, there are no reliability tests on this technique and we did not have the opportunity to test it in this study for technical reasons. However, we tried to minimise this risk by inviting all participants to share doubtful images with the central lab and take a collegial decision for the presence of CPPD.

In conclusion, this international multicentre study developed by the OMERACT 'Ultrasound in CPPD subtask force' demonstrated the ability of ultrasound to discriminate CPPD in the knee. The best site for CPPD identification is the medial aspect of the knee considering both menisci and HC of the medial condyle that provide the highest accuracy. To this point and to the best of our knowledge, ultrasound appears to be the better-validated imaging technique for CPPD identification. Future studies will aim to assess the sensitivity to change of ultrasound in CPPD, also for assessing inflammation/joint damage, for a multifaceted disease like CPPD.

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SARS CoV-2 infection among patients using immunomodulatory therapies

The risk of coronavirus disease 2019 (COVID-19) and disease progression among patients using immunomodulatory therapy is unclear. Accordingly, we implemented an active surveillance project with USA/Canada Infectious Disease specialists via the Emerging Infections Network (EIN) to identify COVID-19 cases occurring in patients who use immunomodulatory therapy and to describe their clinical outcomes.

EIN listserv members include 2396 infectious disease physicians in the USA/Canada linked via a moderated listserv. On 8 April via listserv, we requested reports of COVID-19 cases among patients receiving immunomodulatory therapy. Two weekly reminders were later sent and case reports were collected until 22 May. We collected information regarding patient demographics, COVID-19 test results, symptoms, hospitalisation details, complications, treatment, pre-existing conditions, concomitant therapies, and patient outcomes. We conducted descriptive analyses of these patient factors and compared differences between survivors and non-survivors. We grouped immunosuppressive therapies by class (table 1).

Thirty-eight physicians screened over 2500 COVID-19 cases from which 77 (3%) were identified using immunomodulatory drugs. Of these, 52% were female, median age of 60 years (range, 16–84) and 83.1% had autoimmune disease (rheumatoid arthritis (19, 24.7%), ulcerative colitis (5, 6.5%) and sarcoidosis (5, 6.5%) were most common). Comorbidities included hypertension (26, 33.8%), diabetes (19, 24.7%), underlying chronic kidney disease (11, 14.3%) and others. All patients had PCR-confirmed COVID-19. Symptoms included dyspnoea (70.1%), fever (68.8%) and cough (64.9%). At time of COVID-19 diagnosis, 31 (40%) were using biologic therapies including anti tumor necrosis factor (anti-TNF) therapies (n=16), rituximab (n=6), abatacept (n=2), tocilizumab (n=2) and other (n=5). Among those using non-biologics at baseline (46, 60%), the following therapies were in use: janus kinase (JAK) inhibitors (3, 6.5%), non-biologic disease-modifying antirheumatic drugs (DMARDs) (11, 24%), prednisone alone (5, 11%) or other (27, 59%). Among those who received anti-COVID-19 treatment (n=41), the most common treatment regimens included hydroxychloroquine (n=27), azithromycin (n=10) and/or tocilizumab (n=10). Overall, 63 (81.8%) patients were hospitalised, 27 (35.1%) required mechanical ventilation, 37 (48.1%) required ICU care and 9 (11.7%) died. Patients who died were slightly older (median 68 years vs 58

Table 1 COVID-19 outcomes among autoimmune patients receiving immunomodulatory therapy

	Autoimmune cohort							
	Anti-TNF* biologic with/ without DMARDs† and/ or corticosteroids (n=16)	Biologic‡ (non-TNF) with/ without DMARDs and/or corticosteroids (n=15)	Non-biologic DMARDs alone (n=11)	Non-biologic DMARDs and corticosteroids (n=3)	Corticosteroids§ alone (n=9)	JAK inhibitor¶ (n=3)	Other**immune-modulatory therapy with/ without DMARDs and/or corticosteroids (n=11)	Post solid organ transplant (n=13)
Female (%)	56.3	66.7	81.8	66.7	33.3	66.7	72.7	15.4
Median age, years	59 (27–81)	54 (26–79)	70 (38–84)	62 (52–68)	54 (34–62)	63 (49–63)	62 (16–71)	58 (46–74)
Comorbidities (%)								
Hypertension	37.5	20.0	36.4	66.7	11.1	0.0	36.4	61.5
Diabetes	12.5	6.7	36.4	0.0	11.1	0.0	27.3	69.2
Indication for immunosuppressive (%)								
Rheumatoid arthritis	56.3	20.0	54.6	33.3	0.0	33.3	9.1	N/A
IBD††	31.3	0.0	36.4	33.3	22.2	66.7	9.1	N/A
Sarcoidosis	6.3	0.0	0.0	33.3	33.3	0.0	9.1	N/A
COVID-19 Treatment‡‡ (%)								
Azithromycin	0.0	6.7	9.1	0.0	0.0	33.3	27.3	30.8
Hydroxychloroquine	6.3	46.7	18.2§§	66.7	22.2	33.3	45.5	61.5
Tocilizumab	0.0	20.0	9.1	0.0	44.4	0.0	9.1	15.4
Baseline immunomodulatory treatment (%)								
Unchanged	18.8	60.0	45.5	33.3	100.0	66.7	45.5	53.9
Modified	75.0	40.0	54.6¶¶	33.3	0.0	33.3	45.5	46.2
Unknown	6.3	0.0	0.0	33.3	0.0	0.0	9.1	0.0
Outcome (%)								
Hospitalised	50.0	73.3	90.9	100.0	100.0	66.7	81.8	100.0
Intensive care unit	6.3	53.3	27.3	66.7	77.8	33.3	63.6	61.5
Ventilator support	0.0	40.0	9.1	66.7	66.7	33.3	45.5	46.2
Deceased***	0.0	13.3	18.2	33.3	11.1	0.0	18.2	7.7

*Anti tumour necrosis factor (TNF) therapy includes (n): adalimumab (5), certolizumab (1), etanercept (7), golimumab (1), infliximab (2). Adalimumab, etanercept and infliximab biosimilars included.
†Non-biologic disease-modifying antirheumatic drugs (DMARDs) with/without immunomodulatory therapy (n): balsalazide (2), hydroxychloroquine (5), leflunomide (1), mesalamine (1), methotrexate (12) or sulfasalazine (2).
‡Non-TNF biologic therapy includes (n): abatacept (2), anakinra (1), dupilumab (1), ocrelizumab (1), omalizumab (1), rituximab (6), secukinumab (1) or tocilizumab (2).
§Corticosteroids include (n): prednisone (5) or inhaled steroids (4); n=26 for total prednisone use with/without immunomodulatory therapy, median daily dose 15 mg (2–60 mg).
¶Janus kinase (JAK) inhibitors include (n): tofacitinib (2) and upadacitinib (1); both tofacitinib patients continued dosing as prescribed.
**Other immunomodulatory therapy includes (n): azathioprine (2), cyclosporine (1), cyclophosphamide (1), fingolimod (2), interferon beta-1b (1), mycophenolate (3) and tacrolimus (2) includes Crohn's disease and ulcerative colitis.
††Inflammatory bowel disease (IBD) includes Crohn's disease and ulcerative colitis.
‡‡Treatment groups are not mutually exclusive
§§Mutually exclusive from patients with baseline hydroxychloroquine use
¶¶Among those on methotrexate alone (n=5), 80% modified treatment
***Deceased by drug (n): azathioprine (1), balsalazide (1), cyclosporine and prednisone (1), rituximab and prednisone (2), methotrexate alone (1), prednisone alone (1), methotrexate and prednisone (1), and mycophenolate, tacrolimus and prednisone (1).

years) and similar with regard to comorbidities as those who survived. No patients taking anti-TNF therapy at baseline died (table 1).

Like other early reports, our surveillance effort yielded few biologic or JAK inhibitor using patients severely ill with COVID-19. Certainly, a lower risk of exposure could help explain this (ie, those patients perceiving high risk are social distancing), but it is also possible these therapies are protective against severe outcomes. A rheumatology registry of over 600 COVID-19 patients with autoimmune disease observed that those using biologics, in particular anti-TNF therapy, were less likely to be hospitalised.[1]

While the overall proportion of patients who died in this case series is higher than reported in the US general population,[2] this would be expected given the likelihood that most COVID-19 cases being consulted on by ID physicians would be within the inpatient setting. While we identified only a small number of anti-TNF users, none of them died. TNF blockers could hypothetically inhibit innate antiviral responses with COVID-19 or predispose to secondary bacterial infection, although in animal models of viral pneumonia they can be protective, and among inflammatory bowel disease (IBD) COVID-19 patients, the clinical outcomes of those using TNF blockers have been observed to be comparable or better to those using non-biologic DMARDs.[3 4] While JAK inhibitors decrease innate viral immunity and might potentially increase the risk of viral progression, we found only two tofacitinib and one upadicitinib patients and all three had complete recovery. This and other studies involve small numbers of patients, making further population-based studies necessary to understand the risk of DMARDs with COVID-19.

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Coronavirus disease 19 (Covid-19) and non-steroidal anti-inflammatory drugs (NSAID)

We have read with interest the report by Monti *et al*[1] concerning the clinical course of coronavirus disease (Covid-19) in patients with chronic inflammatory arthritis. However, we could not find data about the use of non-steroidal anti-inflammatory drugs (NSAID) among their patients.

Whether concomitant NSAID treatment may be harmful or safe in patients with Covid-19 is unknown. However, anti-inflammatory therapies might prevent fatal cytokine storm of Covid-19. Ibuprofen, a commonly prescribed NSAID, was found to reduce interleukin-6 (IL-6) in human tissues,[2] and in sputum.[3] Accordingly, several clinical trials of anti-IL-6 therapies for the treatment of severe Covid-19 are actively recruiting.

NSAIDs are still broadly used for the treatment of chronic inflammatory arthritides such as rheumatoid arthritis and spondyloarthritis. The European League Against Rheumatism recommends NSAIDs as effective symptomatic therapies in early arthritis, under the condition to be used at the minimum effective dose for the shortest time possible, and after evaluation of gastrointestinal, renal and cardiovascular risks.[4] Importantly, it is established that uncontrolled inflammation due to active arthritis is associated with an increased risk of infection.[5]

We believe it is important to report the use of NSAIDs in clinical studies of Covid-19 as there have been inappropriate warnings against the use of these drugs, and consequent confusion in the general audience and medical community. The WHO declared to press that there is no evidence of an increased risk of death with the use of NSAIDs in Covid-19. Until more evidence is available, we are recommending our patients with chronic inflammatory arthritis not to discontinue the NSAIDs they are already taking as a regular prescription.

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Non-steroidal anti-inflammatory treatment during covid-19: friend or foe? Response to: 'Coronavirus disease 19 (Covid-19) and non-steroidal anti-inflammatory drugs (NSAID)' by Giollo *et al*

We thank Dr Giollo *et al*[1] for their interest and comment on our published paper on the outcome of covid-19 in patients with rheumatic diseases.[2] The authors highlighted the importance of reporting on the use of non-steroidal anti-inflammatory drugs (NSAIDS) commonly prescribed to patients with chronic arthritis. The effect of NSAIDS on the course of covid-19 is still unknown. It can be speculated that NSAIDs might have a beneficial role in the relief of symptoms resulting from prostaglandin and proinflammatory cytokines, including interleukin-6 overproduction, with potential strict interactions with the cytokine-release syndrome believed to occur in some patients during covid-19. However, the role of NSAIDs during viral infections is controversial,[3] and the selective inhibition of interferon-gamma production by natural killer and T cells has been described to be associated with a worsening of clinical outcome during some viral infections.[4] Given the unknown effects that the use of NSAIDs in patients with rheumatic diseases might have on covid-19, we agree with Dr Giollo *et al*[1] that data on this class of drugs should be collected. Nevertheless, the extensive, often unreported use of NSAIDs through self-medication over-the-counter practice[5] limits the reliability of observational data on this aspect. We suggest that specific, controlled studies on the use of NSAIDs could be informative, especially for patients with rheumatic diseases, who frequently require NSAIDs as complementary drugs to control their chronic condition.

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COVID-19 pneumonia in a large cohort of patients treated with biological and targeted synthetic antirheumatic drugs

We read with interest the article by Monti *et al*,[1] who evidenced, in a cohort of subjects affected by COVID-19, a low prevalence of patients treated with biological disease-modifying antirheumatic drugs (bDMARDs) and targeted synthetic disease-modifying antirheumatic drugs (tsDMARDs).

Despite a notable heterogeneity in different countries and even in different regions of the same country, a high lethality is reported among elderly patients with several comorbidities.[2]

National and international registers have been created to collect patients affected by rheumatic diseases, as well as patients with interstitial lung disorders.[3]

Since 20 February to 7 April 2020, we collected clinical data of 859 patients affected by different rheumatic diseases and sarcoidosis, treated with stable and full dosage of bDMARDs or tsDMARDs at Siena Rheumatology Unit and Siena Regional Referral Centre for Sarcoidosis.

All patients underwent a telephone survey in order to establish their clinical status, the appearance of signs and symptoms of COVID-19 and the presence of nasal–pharyngeal swab positivity. Patients were predominantly from central and southern regions of Italy. During telephone assessment, the patient's health status and chronic disease therapy during the pandemic period were evaluated. Clinical and pharmacological data of our population are summarised in table 1.

Only two patients were diagnosed with COVID-19. The first one, a 50-year-old woman affected by rheumatoid arthritis and treated with rituximab since 2016, presented bilateral diffuse interstitial pneumonia at chest X-ray; she was hospitalised, treated with lopinavir–ritonavir and discharged after 3 days.

The second patient was an 87-year-old woman affected by diabetes mellitus and in treatment with tocilizumab for 9 months for giant cell arteritis. She lived in a retirement home where COVID-19 outbreak was reported, leading to several intensive care unit (ICU) hospitalisations among the other inmates. In this context, she underwent nasal–pharyngeal swab with a positive result; she remained fully asymptomatic, without interrupting biological therapy.

Our findings may suggest that a limited number of patients affected by immune–inflammatory diseases and treated with biological therapies were diagnosed with COVID-19 during the 45-day period of pandemic in Italy. None of our patients developed a severe COVID-19 infection. Notably, one of them was asymptomatic, despite living in a small cluster with a high incidence of COVID-19. This severe impaired patient was in treatment with tocilizumab, a drug recently proposed for COVID-19 in phase II and III clinical trials.

COVID-19 led to concerns for the increased risk of severe respiratory complications in patients treated with bDMARDs and tsDMARDs.

However, our preliminary survey shows that patients treated with bDMARDs or tsDMARDs did not develop life-threatening complications from COVID-19.

This apparently surprising finding can better be explained through the comprehension of the pathological mechanisms leading to acute respiratory distress syndrome, in which overexpression of inflammatory mediators plays a crucial role.[4]

An immune dysregulation is reported in patients affected by COVID-19 with an imbalance in T cells,[5] high serum levels of interleukin (IL)-6, IL-1 and tumour necrosis factor alpha, particularly in those subjects requiring hospitalisation and ICU admission,[6] suggesting an intriguing role of bDMARDs in the treatment of COVID-19.[7]

Since bDMARDs significantly modify and impair circulating inflammatory cytokines involved in both rheumatic diseases and acute respiratory distress syndrome, we may postulate that our patients lack the immune triggers responsible of the most severe clinical features.

In line with Monti *et al*,[1] our survey can support clinicians for the management of this kind of patients, not suggesting a preventive interruption of bDMARDs and tsDMARDs in relation to COVID-19 pandemic. Nevertheless, our findings should not lead to enthusiastic conclusion on a protective role of bDMARDs: our patients are fully aware of their increased invective risk and during the very first phases of the pandemic adopted all protective measures. Finally, we may hypothesise that some of our patients were misdiagnosed due to an oligoasymptomatic course of the disease.

Table 1 Patients features

Drug	Patients	Mean exposure to drug (years)	Mean age (years)	Disease (n)
Adalimumab	91	4.9	57.47	AS: 28 PsA: 42 RA: 15 Takayasu: 1 Sarcoidosis: 5
Etanercept	94	5.3	61.3	AS: 22 PsA: 27 RA: 45
Infliximab	90	4.5	57.97	AS: 47 PsA: 26 EA: 2 RA: 5 BD: 5 Takayasu: 1 SAPHO: 1 Sarcoidosis: 3
Certolizumab	41	1.5	52.41	AS: 6 PsA: 25 RA: 10
Golimumab	44	3.1	54.68	AS: 8 PsA: 31 RA: 4 EA: 1
Rituximab	225	2.9	61.96	RA: 158 SSc: 54 AAV: 8 IIM: 5 SLE: 6 SS: 3 Sweet: 1
Tocilizumab	38	3.5	62.9	RA: 27 GCA: 11
Sarilumab	12	1.1	61.25	RA: 12
Ustekinumab	7	2.1	59.71	PsA: 7
Secukinumab	75	2.1	55.16	AS: 20 PsA: 55
Ixekizumab	6	0.5	57.16	PsA: 6
Canakinumab	1	1.0	50.0	Still: 1
Abatacept	55	2.7	62.2	RA: 55
Baricitinib	68	1.9	60.46	RA: 68
Tofacitinib	12	1.2	61.18	RA: 12

AAV, ANCA-associated vasculitis; AS, ankylosing spondylitis; BD, Behçet disease; EA, enteropathic arthritis; GCA, giant cell arteritis; IIM, idiopathic inflammatory myopathies; PsA, psoriathic arthritis; RA, rheumatoid arthritis; SAPHO, synovitis, acne, pustulosis, hyperostosis, osteitis; SLE, systemic lupus erythematous; SS, Sjogren's syndrome; SSc, systemic sclerosis.

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Prevalence of COVID-19 among patients with rheumatic diseases: the need to await results from large collaborative studies. Response to: 'COVID-19 pneumonia in a large cohort of patients treated with biological and targeted synthetic antirheumatic drugs' by Conticini *et al*

We thank Dr Conticini *et al*[1] for their comment on our previously published paper describing the course of COVID-19 in a cohort of patients treated with biologic and targeted synthetic disease-modifying anti-rheumatic drugs (b/tsDMARDs).[2] The authors commented on the low prevalence of subjects treated with bDMARDs in our cohort of patients affected by COVID-19; however, our paper actually described the course of severe acute respiratory coronavirus-2 (SARS-CoV-2) infection in the cohort of patients attending our biologic clinic rather than the opposite. Nonetheless, assessing the prevalence of COVID-19 in patients treated with b/tsDMARDs was out of the scope of our study.[2] The main message that can be drawn by observational studies on our and other smaller cohorts of patients with rheumatic diseases such as the one reported by Conticini *et al*[1] is that, in our limited series of patients, SARS-CoV-2 infection did not seem to have a worse course or outcome compared with the general population. We have previously highlighted how a high degree of caution should be applied when interpreting these results and when assessing an immunocompromised patient with COVID-19. Large, multicentre, national and international cohorts have been launched to actively recruit patients such as the Italian Society of Rheumatology–sponsored registry (COVID-19-RMD) or the European EULAR-COVID-19 Database.[3] The results from these large cohorts are awaited to properly assess the incidence and prevalence of COVID-19 among rheumatological patients and the clinical implications on this susceptible population. The evaluation of the epidemiology of COVID-19 among patients with rheumatic diseases will need to take into account the potential impact that the use of immunomodulatory drugs may have both on the course of the infection and on the careful preventive behavioural changes that our patients affected by chronic conditions might have adopted to protect themselves during the pandemic. The geographical differences of COVID-19 distribution among different Italian regions and in Europe should also be considered when evaluating the impact of the infection on rheumatological diseases populations.

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Rheumatic diseases in intensive care unit patients with COVID-19

Patients with autoimmune rheumatic diseases have an increased risk of viral infections that can be attributed to the underlying immunological abnormalities, comorbidities and immunosuppressive therapy. Moreover, immunocompromised patients with influenza had more severe disease, longer viral shedding and more antiviral resistance while demonstrating less clinical symptoms and signs.[1] COVID-19 in patients with autoimmune rheumatic diseases, particularly treated with immunosuppressive agents, is also likely to follow the deleterious course previously reported in the other respiratory viral infections.[2]

Surprisingly, little is known about the association of COVID-19 and inflammatory rheumatic diseases, although the number of patients infected with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has already exceeded two millions and continues to rise in many countries, including Russia. In a recent article, Monti *et al* suggested that patients with chronic arthritis treated with biological and targeted synthetic disease modifying antirheumatic drugs (DMARDs) do not seem to be at increased risk of respiratory or life-threatening complications from COVID-19 compared with the general population.[3] However, more data about the prevalence, severity and outcomes of COVID-19 in patients with rheumatic disease are urgently needed to identify patients at higher risk and to inform management guidelines.[4]

In a retrospective nationwide study, we evaluated the prevalence of autoimmune rheumatic diseases among 902 intensive care unit (ICU) patients with SARS-CoV-2 pneumonia who required noninvasive or invasive lung ventilation with or without inotropic support. According to the government's decision, medical records were submitted via Internet by all local COVID-19 hospitals across Russia to the Federal Center at the Sechenov University (Moscow) that provided advice on the antiviral therapy and critical care management. Diagnosis of the SARS-CoV-2 pneumonia suspected clinically was confirmed both by PCR and CT. In patients with inconclusive or pending results of PCR, SARS-CoV-2 pneumonia was defined as severe acute respiratory infection with typical CT findings (bilateral multilobar ground-glass opacification with a peripheral or posterior distribution, or multifocal consolidative opacities superimposed on ground-glass opacification, septal thickening and development of a crazy paving pattern)[5] and no other obvious aetiology.

Four hundred and fifty records (49.9%) were received from the Moscow hospitals, 123 records (13.6%) from the Moscow province and 329 records (36.5%) from the hospitals located in fifty two geographical regions of the Russian Federation. Autoimmune rheumatic diseases, including rheumatoid arthritis, systemic sclerosis, psoriatic arthritis, systemic lupus erythematosus and ankylosing spondylitis, as reported by the local physicians, were identified in 10 (1.1%) of 902 ICU patients with COVID-19 (table 1). Diagnoses were reported by the local physicians and could not be definitely ascertained. As expected, rheumatoid arthritis was the most common rheumatic disorder, given its higher prevalence in the general population. Six patients were older than 60 years of age. Seven patients had concurrent cardiovascular diseases (arterial hypertension, atrial fibrillation, previous myocardial infarction or stroke), and three patients had type 2 or steroid diabetes. Five patients (50%) died.

We assumed that immunocompomised patients with autoimmune rheumatic diseases were likely to present with a more severe course of COVID-19 requiring oxygen support and admission to ICU. However, the total prevalence of inflammatory rheumatic diseases among ICU patients with SARS-CoV-2 pneumonia was low (1.1%) and did not exceed that in the general population (1%–2%). Moreover, most critically ill patients with rheumatic disease had predictors of unfavourable outcomes of SARS-CoV-2 pneumonia, such as cardiovascular diseases, diabetes mellitus and obesity, that could contribute to development of acute respiratory distress syndrome (ARDS). Apparently, we cannot make any definite conclusions regarding the risk of severe COVID-19 in rheumatic patients, since we do not know the total number of infected patients with autoimmune rheumatic diseases in Russia. Of note, patients with any chronic diseases are probably at increased risk of contracting respiratory infections due to more frequent visits to outpatient clinics or hospitals where they can have contacts with infected individuals.

Table 1 Characteristics of ICU patients with rheumatic diseases and COVID-19

Diagnosis	Age/gender	Additional risk factors	Outcome
RA	78/F	Type 2 diabetes, arterial hypertension	ICU
RA	70/F	Arterial hypertension, atrial fibrillation	Recovered
RA	68/M	Type 2 diabetes, arterial hypertension, history of stroke and myocardial infarction	Death
RA	58/F	Arterial hypertension	ICU
RA	45/F	Bronchial asthma	Death
PsA	65/F	Arterial hypertension	Recovered
Systemic sclerosis	66/F	Multiple myeloma	Death
Systemic sclerosis	64/F	Atrial fibrillation, chronic kidney disease	Death
SLE	57/F	Type 2 diabetes, obesity, arterial hypertension	ICU
SpA	39/M	Steroid diabetes	Death

One patient with systemic sclerosis had generalised disease with skin, gastrointestinal tract and lung involvement, whereas the other patient presented with typical skin lesions and Raynaud syndrome. Detailed information about patient with SLE is not available.
F, female; ICU, intensive care unit; M, male; PsA, psoriatic arthritis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SpA, spondyloarthritis.

Accumulating evidence suggests that a proportion of patients with severe SARS-CoV-2 pneumonia have a cytokine release syndrome or cytokine storm, underlying ARDS that is the leading cause of mortality.[6] Various anti-inflammatory agents improve cytokine profile, suppress hyperinflammation and therefore may prevent virus-induced ARDS in patients with SARS-CoV-2 infection.[2] Several antirheumatic medications, that is, hydroxychloroquine/chloroquine, tocilizumab, sarilumab, anakinra, colchicine, are currently under investigation in patients with COVID-19. Tocilizumab, a humanised monoclonal antibody against the interleukin-6 receptor, seems to be the most promising agent for prevention and treatment of SARS-CoV-2-induced ARDS, given its established efficacy in patients with cytokine release syndrome caused by overactive immune response to chimeric antigen T-cell therapy for cancer. However, it cannot be stated if certain drugs are helpful, not helpful or aggravating for COVID-19 due to the lack of controlled trials.

In summary, our findings suggested that patients with autoimmune rheumatic diseases were not over-represented in the large cohort of ICU patients with SARS-CoV-2 pneumonia who developed ARDS. These data indirectly support the current recommendation not to interrupt therapies used in rheumatic patients to avoid flares of autoimmune disease.[7]

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Comorbidities and rheumatological diseases at the time of COVID-19. Response to: 'Rheumatic diseases in intensive care unit patients with COVID-19' by Moiseev *et al*

We thank Dr Moiseev *et al*[1] for their comment on our previous paper on the clinical course of severe acute respiratory syndrome Coronavirus-2 (SARS-CoV-2) disease 2019 (COVID-19) in patients treated with biologic and targeted synthetic disease-modifying anti-rheumatic drugs.[2] The authors assessed the outcome of patients with inflammatory rheumatic diseases among patients admitted to the intensive care unit (ICU) and reported findings in line with our cohort of outpatients.[2] Admission to the ICU due to COVID-19 in patients with rheumatic diseases did not exceed those expected in the general population. Nevertheless, the mortality of these patients was 50%. Interestingly, Moiseev *et al*[1] reported that the majority of rheumatologic patients admitted to the ICU had concurrent conditions including hypertension, previous cardiovascular events or diabetes mellitus. These same comorbidities had been previously identified as risk factors associated with worse outcome also in the general population affected by COVID-19.[3] This comment confirms the cautious impression that patients with rheumatologic diseases might not have a worse prognosis during COVID-19 and that underlying concomitant cardiovascular comorbidities might influence the severity of the infection. Nevertheless, the mortality rate of patients admitted to the ICU is still relevant, and patients with a significant comorbidity burden might be particularly at risk. Comorbidities are frequent in patients with rheumatologic diseases, even at a younger age compared with control populations,[4 5] and these might expose our patients to an excessive risk in case of severe SARS-CoV-2 infection.

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What is the true incidence of COVID-19 in patients with rheumatic diseases?

After its emergence in December 2019 in Wuhan, China, the COVID-19 outbreak has now one of its main epicentres in Lombardy (Italy), with more than 50 000 confirmed cases and 9000 deaths. As rheumatologists operating in the same pandemic area (Milan), we read with great interest the letter published by Monti and colleagues[1] about the description of COVID-19 among patients with rheumatic diseases treated with biologic disease-modifying drugs (bDMARDs). Certainly, the quantification of the risk of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection and its evolution towards severe interstitial pneumonia leading to acute respiratory distress syndrome (ARDS) is crucial in such a population of fragile patients. To fill this gap, in the same period of health emergency between 25 February and 2 April 2020, we collected data from patients treated with bDMARDs afferent to the Research Center for Adult and Pediatric Rheumatic Diseases of the ASST Gaetano Pini-CTO in Milan, by using a survey investigating the impact of COVID-19. The survey was administered face-to-face to all patients who underwent an outpatient visit or by telephone in those who missed a scheduled visit during the period under review. The final study population included 530 patients (372 women, mean age 50.1 years), affected by rheumatoid arthritis (49.6%), spondyloarthritis/psoriatic arthritis (SpA/PsA, 36.8%), connective tissue diseases (3.3%), sarcoidosis (one patient only) or juvenile idiopathic arthritis (10.3%). Most patients were treated with antitumour necrosis factor agents (53.7%), 39.3% with other bDMARDs (mainly interleukin (IL)-6 blockers (11.5%) and abatacept (10%)) and 7% with JAK inhibitors.

We recorded only three patients with mild COVID-19 confirmed by positive nasopharyngeal swab. Of these, only a 56-year-old man with sarcoidosis treated with adalimumab required hospitalisation with oxygen therapy, whereas a 40-year-old man with axial SpA receiving infliximab and a 68-year-old woman with PsA treated with secukinumab were both managed at home without any respiratory complication. None of the 10 patients who reported contact with established cases of COVID-19 developed symptoms of infection. Along with the results reported by Monti and colleagues,[1] our findings could provide further reassurance about the incidence of life-threatening COVID-19 in patients with rheumatic diseases receiving bDMARDs. Pathogenetically, ARDS complicating the more severe cases of SARS-CoV-2 pneumonia is associated with a massive but late immune response resulting in a cytokine release syndrome (CRS) orchestrated mainly by IL-6, which is currently the only considered target to treat most serious COVID-19.[2] The role of drugs targeted on alternative pathways in the management of CRS and consequently in the potential prevention of ARDS in patients with rheumatic diseases still needs to be clarified.[3]

However, it should also be noted that about 90% of our patients declared that they had adopted a preventive strategy against COVID-19 based on social distancing and use of personal protective equipment such as gloves and masks since the beginning of the epidemic. This stringent approach, which is likely to arise from patients' awareness of an additional risk due to rheumatic disease may introduce a bias that would lead to underestimating the real incidence of COVID-19. On the other hand, severe cases of COVID-19 are only the tip of the iceberg, as the vast majority of cases are asymptomatic or oligosymptomatic.[4] For this reason, in our survey we extended the evaluation to the reporting of even mild symptoms of viral infection, which have been recorded in 81 (15.2%) patients, suggesting that the real overall incidence rate of COVID-19 in our population might be significantly higher.

Finally, in comparison with Monti *et al*'s cohort,[1] ours also included a portion of paediatric patients (n=54), in which no cases of COVID-19 positivity were reported. However, we observed a frequency of patients carrying mild symptoms of potential infection consistent with the adult subgroup (14.8%) as possible confirmation of the already described tendency of children to get a less aggressive subset of COVID-19.[5]

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SHORT COMMUNICATION

A STIMULUS PARADIGM FOR ANALYSIS OF NEAR-FIELD HYDRODYNAMIC SENSITIVITY IN CRUSTACEANS

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We present several relatively simple procedures for studying the physiology of near-field mechanoreceptors in crustaceans which extend previous measures of sensitivity. The advantages include the quantitative analysis of range fractionation and directionality of receptors and interneurons in the sensory hierarchy of the central nervous system (CNS), based on a stimulus paradigm that is reproducible and easy to use. The technical considerations for quantitative fluid-coupled stimulation addressed by this paper are the complexity of dipole flow fields, reflected interference from traveling waves, and the underlying stimulus wave form. The techniques described here offer corresponding advantages for physiological experiments using other aquatic organisms.

In electrophysiological experiments, crustacean preparations are typically placed in an experimental chamber filled with water or saline solution. For studies on near-field sensory receptors, i.e. those responding to flow fields in the aquatic medium, a dipole or vibrating sphere is frequently used to generate stimulus waves (Tautz *et al.* 1981; Wiese and Wollnik, 1983; Ebina and Wiese, 1984; Hatt, 1986; Heinisch and Wiese, 1987; Tautz, 1987; Wiese and Marschall, 1990; Killian and Page, 1992*b*). A dipole stimulator is easily constructed by attaching a spherical probe to an electromechanical device such as a loudspeaker, pen motor or piezo crystal. A periodic signal fed to the transducer generates the oscillating dipole movements. With the sphere immersed in the bathing medium, dipole flow fields are generated (see Kalmijn, 1988, for further discussion of dipole sources), whereas dipole oscillations introduced at the air–saline interface generate traveling surface waves.

Numerous additional devices and techniques have been used to stimulate crustacean receptors. Several involve wave motion introduced from one end of the chamber by diaphragms or paddles (Laverack, 1962*b*, 1963; Flood and Wilkens, 1978), by cylindrical dippers (Wilkens and Larimer, 1972; Wiese *et al.* 1976; Wiese and Schultz, 1982; Reichert *et al.* 1983) or by water drops (Laverack, 1962*b*; Strandburg and Krasne, 1985).

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Key words: Crustacea, aquatic, hydrodynamic, mechanosensory, dipole, vertical stimulus orientation, directional sensitivity.

Another form of fluid-coupled stimulation involves small jets of saline (Laverack, 1963; Tautz, 1990; Schmitz, 1992). In other studies, receptor hairs have been stimulated directly by using a stylus in place of the dipole, e.g. a needle or glass capillary in contact with the hair (Laverack, 1962*a*; Killian and Page, 1992*a*; Yen *et al.* 1992; Nagayama and Sato, 1993) or a miniature wire loop or capillary tube placed over the hair shaft (Mellon, 1963; Wiese, 1976; Tautz *et al.* 1981; Killian and Page, 1992*b*).

Each of these techniques introduces complications for quantifying the stimulus–response properties of near-field receptors and/or their postsynaptic interneurons. For example, a local dipole field is highly non-uniform, with off-axis flows in many directions (Kalmijn, 1988). Whereas this type of stimulus may be representative of some biologically relevant signals, and is an appropriate stimulus in the context of behavior, it has limitations for physiological studies at the cellular level, particularly for directional sensitivity. Dipole fields and other forms of traveling waves contribute significantly to response latencies as noted by Ebina and Wiese (1984), suffer from distortion imposed by the walls of the experimental chamber, and are subject to reflected interference except at the chamber's natural harmonic frequency. Wave reflection persists owing to the high inertia of water, and both interference and distortion are compounded by the fact that experimental chambers typically represent only a small fraction of the animal's natural near-field aquatic environment. Water jets are also complex stimuli with variability in associated eddy currents (L. A. Wilkens and J. K. Douglass, personal observations). Direct-coupled stimulation, a valuable technique for describing the structure and function of sensory hairs (e.g. Mellon, 1963; Wiese, 1976), is nonetheless not completely satisfactory in the sense that sensitivity cannot be directly equated with a natural stimulus which is defined not only by stimulus frequency, displacement, velocity or acceleration, but also by the coupling factors of sensillum morphology, hinge stiffness and boundary conditions. For example, threshold velocity measured with direct-coupled stimulation would underestimate threshold values based on the velocities of a fluid-coupled stimulus. In addition, direct coupling is impractical for characterizing interneuronal sensitivity, i.e. where responses are based on broad receptive fields and/or contrast-enhancing mechanisms.

In contrast with aquatic organisms, natural stimulus fields can be created more easily for quantitative physiological studies involving terrestrial organisms. An extensive body of literature exists in which carefully controlled stimulus currents have been generated in the form of wind puffs (e.g. Westin *et al.* 1977; Boyan and Ball, 1989; Kondoh *et al.* 1991) or wind tunnel currents (Kanou and Shimozawa, 1984; Shimozawa and Kanou, 1984), especially for the cercal systems of insects. The study of terrestrial organisms is facilitated by the fact that experimental environments are not constrained dimension-wise by the need to 'hold' small quantities of air, thereby greatly reducing interference and maintaining accessibility.

Previous methods devised to quantify hydrodynamic stimulus currents and eliminate reflections from traveling waves have made use of a horizontal sound field chamber. In a study of vibration sensitivity, Tautz and Sandeman (1980) inserted crayfish chelae into a long (100 cm) tube fitted at the closed end with a rubber diaphragm/loudspeaker and with the open end curved upwards to permit fluid displacements without reflection. In a related

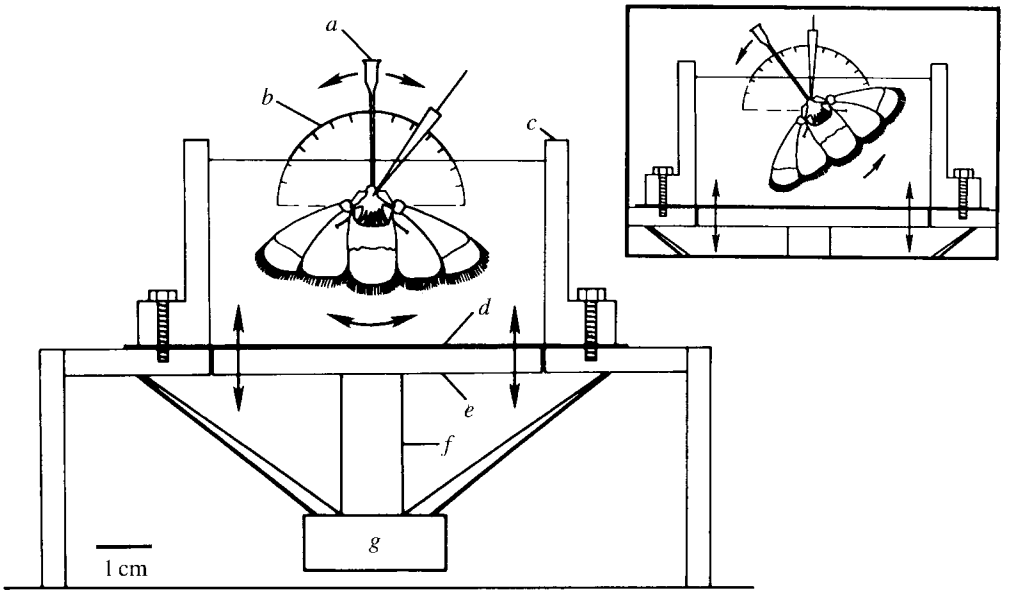

Fig. 1. Diagram of experimental chamber for studying hydrodynamic near-field receptors. The floor of the cylindrical chamber moves freely vertically and is supported by the cone of a loudspeaker mounted on the underside of the platform; a thin rubber membrane prevents loss of fluid. The saline bath therefore moves up and down (vertical arrows) as a uniform laminar stimulus current. The crayfish tailfan, also oriented vertically, is pinned to a pivoting stage (not shown) for rotation of the preparation relative to the vertical stimulus current (see inset). *a*, lever for rotation of preparation; *b*, miniature compass; *c*, Plexiglas chamber cylinder; *d*, rubber membrane; *e*, chamber floor; *f*, support rod for attaching chamber floor to speaker cone; *g*, speaker magnet.

study of frequency sensitivity, Plummer *et al.* (1986) introduced the crayfish tailfan into the top of a cylindrical chamber in which both ends were attached to a loudspeaker, with movement facilitated by rubber membranes. In each of these methods, chamber size is a potential limiting factor since the mass and inertia of water will attenuate displacement amplitudes and require attention to phase lag except at low frequencies.

Our experimental paradigm features stimulus displacements in the vertical plane which can be generated without setting up traveling waves, thereby eliminating or minimizing interference. The preparation is also oriented vertically, instead of the usual horizontal position of test subjects. We have used various permutations of the 'vertical paradigm', each being adaptable for testing most parameters of receptor sensitivity. For example, using a vertical cylinder in which the preparation is suspended from a platform extending from the side wall, the chamber floor acts as a piston and causes the bath solution to move up and down (Fig. 1). With the preparation centered in the bath, at least 1.5 cm from the perimeter, boundary conditions have no effect (Tautz, 1979) and hydrodynamic receptors, in our studies the filiform receptors on the crayfish tailfan, are subjected to unidirectional laminar stimulus currents in response to floor displacements. Alternatively, the entire chamber can be oscillated vertically with the preparation remaining stationary independent of the chamber. An example of this experimental design is illustrated in a

previous study (see Fig. 1 of Schultz and Wilkens, 1988). In this case, potential turbulence along the chamber walls is eliminated since the solution moves with the chamber. Results obtained using each method are comparable.

The volume and inertia of vertical chambers, as with horizontal sound-field chambers, will limit the range of stimulus frequencies that can be reproduced quantifiably. Continuous high-frequency stimulation may create trailing vortices as the preparation vibrates in the viscous aquatic medium (Kalmijn, 1988), resulting in unidirectional current flows superimposed on the periodic stimulus, a phenomenon observed in preliminary studies (T. Shimozawa and L. A. Wilkens, unpublished results). In addition, surface cavitation occurs at higher frequencies or larger displacement amplitudes. Quantitative stimulus–response analyses of receptor sensitivity involving fluid coupling at high frequencies (>50–60 Hz) therefore require careful attention to stimulus calibration.

In view of the constraints for quantifying water movements at high stimulus frequencies, it is helpful to reverse the stimulus paradigm, inducing movements of the preparation relative to a stationary test environment. This is accomplished by attaching the preparation to the electromechanical device. This arrangement has the advantage of increasing the range of stimulus frequencies over which receptor sensitivity can be evaluated, since the mass of the *in vitro* preparation, which may constitute only a small part of the animal, is small compared with that of a water-filled chamber. We have used this technique to analyze crayfish mechanoreceptors on the telson and uropods (Douglass and Wilkens, 1991; Douglass *et al.* 1993). The apparatus is illustrated in Fig. 2 and incorporates a mechanical vibrator as the electromechanical transducer (Pasco, model SF-9324; designed for wave motion experiments in physics laboratories). The vibrator features a built-in mounting post and twin diaphragms and has input–output specifications exceeding those of small loudspeakers, thereby providing wobble-free movements of the preparation at frequencies up to 150 Hz. Attached to the vibrator platform is a saline-filled chamber sealed around the movable post by a thin rubber membrane and stopcock grease. Afferent activity is recorded by suction electrode from the flexible nerve roots of individual tailfan appendages (telson and uropods).

The stimulus wave form is also an important consideration for the analysis of near-field sensitivity. Historically, single or continuous sinusoidal or triangular wave forms have been used as a stimulus source. Periodic signals are often gated for a prescribed number of cycles, and the amplitude may be modulated at onset and offset, to examine frequency-dependent response properties (e.g. Wiese and Wollnik, 1983). Whereas continuous periodic signals are the essence of acoustic (far-field) stimulation in terrestrial as well as aquatic organisms, they present drawbacks (such as cavitation and unidirectional flow, see above) for the analysis of near-field sensitivity, particularly with respect to directionality. For example, with a periodic function, the resulting stimulus movements will be inherently bidirectional. Response profiles for directionally sensitive receptors or interneurons will therefore be compounded by dual, perhaps interfering, stimulus displacements. Response profiles at high stimulus frequencies are especially difficult to interpret in that stimulus transduction and conduction delays may exceed the cycle period. A single sinusoidal or triangular wave form can also constitute a complex

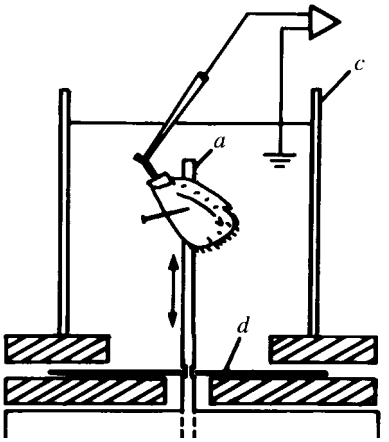

Fig. 2. Diagram of experimental chamber for vertical stimulation in which the bath is stationary and the preparation moves up and down (double-headed arrow). Here, the post of the electromechanical transducer supports the preparation, which again can be rotated in the vertical plane. In principle, an experimental preparation attached directly to the transducer could be inserted into a stationary water bath from above, or obliquely, and the stimulus paradigm would be equivalent. However, resting the transducer on the recording table provides greater stability and does not interfere with electrode placement from above. *a*, transducer post with Sylgard insert (not shown) for pinning the preparation; *b*, Pasco vibrator base; *c*, test chamber (7.5 cm×5 cm×5 cm) made from glass slides glued together with silicone cement; *d*, rubber membrane (exploded diagram).

stimulus owing to the sudden discontinuities of frequency and velocity at onset and offset. Thus, it may be unclear whether receptors are responding to a particular stimulus frequency or velocity or to the onset/offset stimulus transients (Plummer *et al.* 1986).

To avoid these complications, we drive the transducer with a modified sinusoid whose leading and trailing edges are half-cycle cosine waves (Fig. 3). The one-cycle cosine wave, which begins with a particle velocity of zero and thereby minimizes mechanical transients (Kanou and Shimozawa, 1984), is delivered from a function generator (Tektronix, FG 501) triggered by an external rate generator. The cosine wave is arrested at mid-cycle by a large, negative gate pulse (−10 V) fed to the voltage-controlled frequency (VCF) input of the function generator. The VCF gate pulse is delivered from a pulse generator triggered by the rate generator after an appropriate delay. This wave form creates unidirectional stimulus currents in which the repetition rate, the interval separating directional components, the amplitude and the underlying sinusoidal frequency can be controlled independently. Each directional component of the stimulus consists of an approximately constant-velocity current (e.g. where log velocity varies by less than ±15 % of V_{max} for the time interval spanning $\pm\pi/4$ radians on either side of the peak velocity), with gradual displacement onset and offset at the trough and peak. Thus,

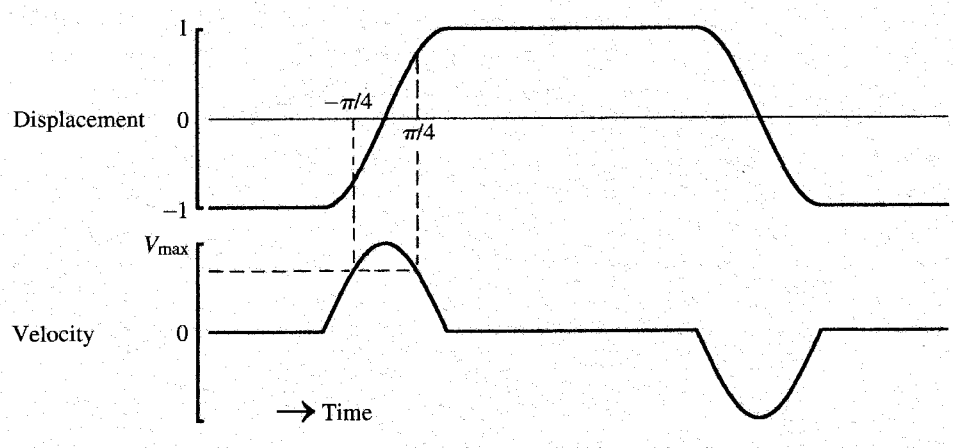

Fig. 3. Wave form for quantitative unidirectional stimulation of mechanosensory receptors. Upper curve represents displacements generated by a modified cosine wave. See text for description of wave form. The output of the function generator passes through 10 and 1 dB step attenuators in series with a power amplifier for driving the transducer. Lower curve illustrates the corresponding stimulus velocity. Dashed lines bracket the quarter cycle of the rising cosine wave where velocity is maximal, i.e. where log stimulus velocity varies by less than ±15 % of V_{max} (a cut-off point approximately 71 % of V_{max} as shown here on a linear scale).

by varying the cycle frequency (typically between 0.5 and 50 Hz) or the amplitude (by series attenuators, Hewlett-Packard models 355C and 355D), displacement- and velocity-sensitivity to unidirectional stimulus currents can be examined quantitatively. The actual displacements are monitored directly by photodetectors to ensure quantitative analyses of receptor sensitivity. Using a laser beam deflected by a mirror whose angle of orientation was altered by the transducer post, hair-cell responses have been detected for displacements of at least down to 0.1 μm.

The vertical unidirectional stimulus techniques presented here are especially well-suited for analyzing directional sensitivity in hydrodynamic receptors. Preparations mounted vertically are stimulated by alternating rostral and caudal displacements. By incorporating a pivot in the mounting platform to allow rotation in the vertical plane, the preparation can be stimulated with equivalent currents from other directions (see inset, Fig. 1). Rotation of the preparation from 90° clockwise to 90° counterclockwise simulates the full range of stimulus directions in two dimensions. Suction electrodes attached to the platform permit continuous *en passant* recording as the preparation is rotated, and highly reproducible results have been obtained for individual receptors (Douglass and Wilkens, 1991) and for interneurons. Fig. 4 illustrates the directional sensitivity of the caudal photoreceptor (CPR) interneuron, based on input from the intact tailfan; reproducibility is indicated by the narrow range of average vector angles in results from seven different animals (inset, Fig. 4). Similar results, including information on the underlying synaptic events, have been obtained by recording intracellularly from sixth ganglion interneurons with the ganglion pinned to a separate platform (T. Shimozawa and L. A. Wilkens, unpublished results).

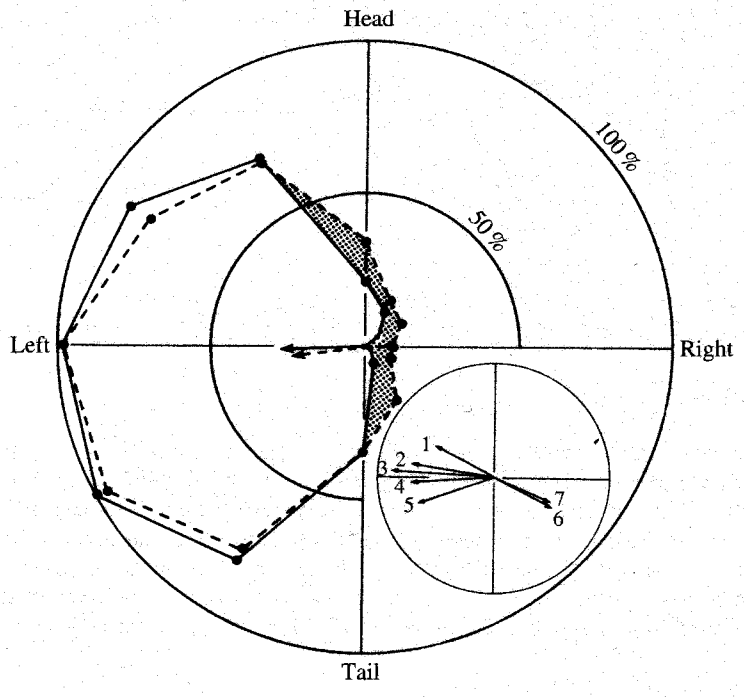

Fig. 4. Polar diagram plotting sensitivity of the left caudal photoreceptor (CPR) to near-field current stimulation in the chamber illustrated in Fig. 1 (reproduced with permission from Wilkens, 1988). Data are normalized relative to the maximum response. Solid line represents response of intact tailfan–sixth ganglion preparation; dashed line is response after section of contralateral sensory roots and illustrates the contribution of inhibitory input (hatched section). Arrows are corresponding vector averages for each of the 12 test directions. Inset lower right shows mean vectors representing CPR sensitivity in seven cells, 1–5 for the left CPR, 6 and 7 for the right CPR. The CPR exhibits peak near-field sensitivity to mediolateral currents from the ipsilateral direction (with corresponding bilateral sensitivity for the paired interneuron).

Relatively little is known about the directional sensitivity of mechanosensory systems in crustaceans. Excluding the statocysts, where directionality is the result of gravitational or inertial forces associated with posture or movement (Sandeman and Okajima, 1972; Ozeki *et al.* 1978; Takahata and Hisada, 1979, 1982), there exists for crustaceans no information concerning the partitioning of directional sensitivity among receptors or interneurons comparable to that of the cercal apparatus of orthopteroid insects (Boyan and Ball, 1990). This is due undoubtedly to the previous difficulties of stimulating hydrodynamic receptors equally along more than one directional axis. Relatively few studies, apart from those on the crayfish CPR (Fig. 4), have investigated directionality in a comprehensive fashion. Wiese and Wollnik (1983) examined the directionality of circumesophageal neurons in crayfish in response to a periodic dipole stimulus rotated around the animal. A similar study has been reported for interneurons in the abdominal CNS of the grass shrimp *Crangon crangon* (Heinisch and Wiese, 1987). The directional response profiles of these cells were in general relatively broad, with most crayfish

interneurons (90%) responding to all directions presented. Whether most crustacean interneurons receive multidirectional input, or whether dipole stimulation excites receptors somewhat non-selectively, remains to be determined.

The crustacean literature nonetheless contains numerous references to directionality in mechanosensory interneurons, beginning with the initial description of dual-innervated hair receptors (Mellon, 1963) and directional sensitivity in the CPR interneuron (Wilkens and Larimer, 1972). More recently, Sigvardt *et al.* (1982) and a review by Wine (1984) categorize identified interneurons as headward, tailward or bidirectionally sensitive. With the exception of data for the CPR and additional unidentified interneurons in crayfish and shrimp (Wiese and Wollnik, 1983; Heinisch and Wiese, 1987), these results are based on partial directional analyses, i.e. they are limited to the rostrocaudal axis. Although individual cells may respond preferentially to headward or tailward stimulation, the sensitivity to either of these directions in fact may be small relative to that for the preferred direction, as determined by testing at regular intervals from around the preparation (cf. CPR results from Flood and Wilkens, 1978, and Wilkens, 1988).

Such partial analyses of interneuron sensitivity, and the predominant rostrocaudal sensitivity of fourth-root telson mechanoreceptors (Wiese, 1976) and their postsynaptic interneurons (Wiese *et al.* 1976), have contributed to an inadvertent bias in the literature suggesting that mechanosensitivity is predominantly rostrocaudal. A recent behavioral study (Schmitz, 1992) indicates that crayfish are also sensitive to transverse stimuli directed at the abdomen. Using the techniques introduced here, a clearer picture of directional hydrodynamic sensitivity in crustacean mechanoreceptors is possible.

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Response to 'Is there a future for hydroxychloroquine/chloroquine in prevention of SARS-CoV-2 infection (COVID-19)?' by Moiseev *et al*

We thank Sergei Moiseev and colleagues for their comment in response to our letter 'To consider or not antimalarials as a prophylactic intervention in the SARS-CoV-2 (Covid-19) pandemic'.[1 2]

Antimalarial drugs hydroxychloroquine (HCQ) and chloroquine have been largely used for treating patients with systemic lupus erythematosus and other autoimmune rheumatic diseases (ARDs) for decades, and they are safe and well tolerated in such patients.[3] Conversely, there is still little evidence on their effectiveness in patients with Covid-19. As Moiseev and colleagues have pointed out, more data have been published after the submission of our letter; therefore, we welcome the opportunity to give an update. The results of five studies are now available: three open-label and two randomised controlled trials[4–8] (table 1). All studies have small sample sizes and enrolled non-critically ill patients. Gautret *et al* extended their previous results confirming the fast reduction of viral load in 80 hospitalised Covid-19 patients treated with HCQ, achieved in 93% of them after 8 days and in 100% after 12 days.[5] Moreover, 81% of treated patients were discharged after an average of 4.6 days and all patients improved, except for one still in intensive care unit and another 86-year-old patient who was not admitted in intensive care and died in infectious disease ward.[5] A randomised Chinese trial (not yet peer reviewed), on 62 patients with Covid-19, confirmed the effectiveness of HCQ added to standard of care, compared with standard of care alone, in improving both signs and symptoms of pneumonia (significantly faster resolution of cough and fever) and CT findings (improved in 80.6% of patients receiving HCQ compared with 54.6% of those receiving standard therapy); moreover, none of the patients in the active arm were admitted in the intensive care unit.[7]

Of course, the open-label nature of some studies and the small sample size, together with the heterogeneity of data, do not allow to conclude whether HCQ could effectively change the disease course, especially in more severe patients. International, randomised, ongoing studies will provide more robust evidence on the antiviral effect of different drugs, including HCQ.

Moiseev and colleagues may be right when referring to the attempt to provide antimalarials for off-label use to people at risk of infection. Perhaps, we should also consider that almost half of SARS-CoV-2-infected subjects are asymptomatic.[9] However, in such a global health emergency, with more than 2.600.000 people with confirmed infection and almost 185.000 deaths (as of 23 April 2020), we do not feel to give any judgement on the opportunity of planning studies on the prophylactic effect of HCQ in subjects at highest risk of infection.

As rheumatologists, we are facing every day the consequences of the enthusiasm around HCQ: the optimistic perception of its effectiveness among lay public people has contributed to the shortage of the drug and to the serious risk of widespread self-medication. As the European League Against Rheumatism President Iain McInness has recently underlined, the consequence of this diffuse use of HCQ is already evident, and there is an urgent need to increase its production to protect people with ARDs who depend on it for their well-being.[10]

In Italy the drug supply has been problematic; luckily, the pharma companies ensured the availability of HCQ both for patients with Covid-19 and chronic illnesses. The issue of fair allocation of resources is a matter of debate and the necessity of not overlooking the needs of non-Covid-19 patients is becoming evident.

In conclusion, we appreciate the great effort of the scientific community in the search for prevention and cure for Covid-19 through well-designed studies; on the other hand, we firmly believe that the pandemic-generated focus on antimalarials should not penalise patients with rheumatological diseases, in whom such drugs have widely demonstrated their benefits.

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Table 1 Clinical trials on hydroxychloroquine in patients with Covid-19

Study	Enrolled patients, n	Controls (Y/N, n)	HCQ regimen	Primary endpoint	Main results
Gautret *et al*[8]	36	Y (16)	HCQ 200 mg three times daily for 10 days±azithromycin (500 mg on day 1, 250 mg on days 2–5)	Viral load	HCQ induces viral clearance in a significantly higher percentage of patients after 6 days of treatment (70% HCQ alone, 100% HCQ+azithromycin vs 12.5% controls).
Gautret *et al*[6]	80	N	HCQ 200 mg three times daily for 10 days+azithromycin (500 mg on day 1, 250 mg on days 2–5)	Viral load, oxygen requirement or ICU admittance, hospital stay length	83% negative at day 7, and 93% at day 8. 12/80 (15%) required oxygen and 3/80 (3.75%) ICU. Mean length of stay of 4.6 days.
Chen *et al*[5]	30	Y (15)	HCQ 200 mg two times per day for 7 days	Viral load	No difference in viral load at day 7.
Chen *et al*[7]	62	Y (31)	HCQ 200 mg two times per day for 5 days	Time to clinical recovery	Median time to body temperature and cough recovery were significantly shorter in HCQ arm. Significantly greater percentage of HCQ-treated patients with CT improvement of pneumonia.
Molina *et al*[4]	11	N	HCQ 200 mg three times daily for 10 days+azithromycin (500 mg on day 1, 250 mg on days 2–5)	Viral load	8/10 patients still positive at day 5. (1/11 discontinued for QT prolongation).

HCQ, hydroxychloroquine; ICU, intensive care unit.

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Patients with lupus are not protected from COVID-19

The comment provided by Joob and Wiwanitkit contains serious factual errors that need to be urgently corrected to prevent harm to patients.[1] Their claim that 'there is no case of SLE with covid-19' is false. It is puzzling how they can make such a claim without providing supporting evidence. An initial analysis of patients included in the COVID-19 Global Rheumatology Alliance registry 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.[2] The frequency of patients with lupus who have been diagnosed with COVID-19 was overrepresented at ~50% of that reported for rheumatoid arthritis, a disease that is ~4 to 8 times more prevalent than lupus in the adult population in the USA.[3] Although selection and reporting bias and differences in comorbidities might contribute to this disproportionately high frequency of COVID-19 in patients with lupus, there is reason to be cautious. Evidence supported by mechanistic data indicates that patients with lupus are inherently more susceptible to viral infections.[4] Indeed, we recently suggested that patients with lupus might be particularly more susceptible to severe acute respiratory syndrome (SARS)-CoV-2 infection and to a more complicated course of COVID-19.[5]

The claim that 'anti-HIV drug is proven for efficacy against the novel coronavirus' is also false. The authors here cite another of their own 'Letter to the Editor' also making unsupported claims that 'HIV-infected patients receiving standard anti-HIV drug might not have increased risk for COVID-19'.[6]

To support their claim that 'Hydroxychloroquine is also reported for efficacy against covid-19', the authors cite a review from 2017 before SARS-CoV-2 or COVID-19 was even reported.[7] There are multiple ongoing studies and clinical trials under way to examine possible effects of hydroxychloroquine in COVID-19, but the clinical data we have available at this point in time are not convincing.[8]

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No evidence so far on the protective effect of hydroxychloroquine to prevent COVID-19: comment by Joob and Wiwanitkit

We read with interest the comment by Joob and Wiwanitkit[1] on the letter published by Monti *et al* in the *Annals of the Rheumatic Diseases* (ARD).[2] In it, the authors state that there are no reported cases of patients with systemic lupus erythematosus (SLE) with COVID-19 and suggest that this may be due to a protective effect of hydroxychloroquine, a mainstay treatment taken by most patients with SLE. A similar suggestion had already been made earlier this month in the ARD by colleagues from Italy,[3] the first hardly-hit western country, and was reinforced by yet another recently published letter.[4]

As is now widely known, this old antimalarial drug, which has been part of the daily therapeutic armamentarium of rheumatologists for decades, has reached the global spotlight after demonstration of antiviral efficacy in vitro[5] and some suggestions of clinical efficacy in studies with methodological limitations and fast peer-review processes.[6] The scientific discussion on the potential validity of these findings—which were to be confirmed—was seized by some politicians who quickly transformed it into a matter of belief and conviction. Moreover, an additional problem was created in several countries, where a general run to antimalarials led to nationwide drug shortage and prevented patients with rheumatic diseases from accessing these critical drugs to control their disease.

The yearning for an effective treatment for COVID-19 should not deter the scientific community from critically evaluating available evidence. Rather, it should make it raise the bar even higher to avoid that possible spurious findings are used in the wrong way.

In this regard, we would like to dispute both the statement and the suggestion by Joob and Wiwanitkit in their comment.[1] Indeed, the authors comment on the letter by Monti *et al*, who studied a cohort of 320 patients with rheumatoid arthritis (RA) or spondyloarthritis, but did not have a single patient with SLE.[2] Still, out of the eight patients who developed a clinical picture compatible with COVID-19, three were already on hydroxychloroquine, making it confusing to suggest a protective effect of this drug. Recently, the COVID-19 Global Rheumatology Alliance launched a worldwide register for patients with rheumatic diseases with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.[7] In the initial report that was just published, 19 out of 110 patients had SLE, although no treatment/outcome details were provided specifically for these patients.[8]

Additionally, we report two cases of patients with SLE under long-term treatment with hydroxychloroquine, who developed COVID-19 (table 1). Both were young patients, with controlled disease activity prior to the infection. Both had confirmed close contacts with subjects later diagnosed with COVID-19, developed mild disease and fully recovered. While these two cases do not provide any definite answer to the question of whether antimalarials can prevent COVID-19 or severe disease, they show that, indeed, patients with SLE can develop disease, even if on stable hydroxychloroquine therapy. The mild disease course should not be attributed to the concomitant antimalarial. Rather, it is likely related to other factors known to be associated with better outcomes, such as female sex and younger age.

Table 1 Clinical features of two patients with SLE who developed COVID-19

	Patient 1	Patient 2
Age	30	38
Sex	Female	Female
Disease duration	6.8 years	2.1 years
SLE clinical manifestations	Oral ulcers, photosensitivity, inflammatory arthralgia	Malar rash, photosensitivity, alopecia, fatigue, inflammatory arthralgia, Raynaud's phenomenon
SLE-related laboratorial features	ANA (1/320), anti-Sm, anti-dsDNA, LAC	ANA (1/320), anti-Sm, anti-RNP, anti-Ro, anti-dsDNA, leucopenia, neutropenia, hypergammaglobulinaemia
Comorbidities	Chronic urticaria	Plaque morphea (childhood onset), hypothyroidism
Smoking status	Non-smoker (ever)	Non-smoker (ever)
csDMARDs, dose (duration)	HCQ, 400 mg/day (7 years)	HCQ, 400 mg/day (2.8 years) MTX, 15 mg/week (3.5 years)
Glucocorticoids, dose (duration)	No	PDN, 5 mg/day (2.1 years)
NSAIDs	No	No
ACEi/ARB	No	No
SLEDAI (prior to COVID-19)	0	0
Epidemiological link	Close contact with confirmed case (colleague)	Short close contact (30 min) with two subjects arriving from Madrid (Spain)
Time from contact to symptom onset	6 days	6 days
COVID-19 symptoms	Headache, myalgia, rhinorrhoea, mild unproductive cough	Anosmia, dysgeusia
Time from symptom onset to first positive RT-PCR test	8 days	5 days
Hospitalisation	No	No
Antiviral treatment	No	No
Symptom duration	16 days	7 days
Time from symptom onset to two negative RT-PCR tests	26 days	28 days
csDMARD discontinued	No	MTX, stopped until recovery
SLE symptoms/flare post-COVID-19	Inflammatory arthralgia, oral ulcers	No
Complete recovery	Yes	Yes

ACEi, angiotensin-converting enzyme inhibitor; ANA, antinuclear antibody; ARB, angiotensin-II receptor blocker; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; HCQ, hydroxychloroquine; LAC, lupus anticoagulant; MTX, methotrexate; NSAIDs, non-steroidal anti-inflammatory drugs; PDN, prednisolone; RNP, ribonucleoprotein; RT-PCR, reverse transcription polymerase chain reaction; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index.

In these agitated, confusing times, caution is warranted in interpreting the vast amount of information emerging on COVID-19. Until robust evidence is available, we should stick to what we know by now: antimalarials are crucial drugs for patients with SLE, RA and other rheumatic diseases, who also seem to be susceptible to infection by SARS-CoV-2. Whether they are effective drugs for the prevention or treatment of COVID-19 is yet an open avenue. One we should not rush into without decisive, firm steps.

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SLE patients are not immune to covid-19: importance of sending the right message across

In vitro inhibition of the novel coronavirus, SARS-CoV-2, by hydroxychloroquine (HCQ) has triggered further exploration of the clinical efficacy of this drug in covid-19. Rheumatologists all over the world are analysing registries of patients with systemic lupus erythematosus (SLE) and other rheumatic diseases to look at the prevalence of covid-19 in patients who have been on HCQ. On coming across the title, 'SLE, hydroxychloroquine and no SLE patients with covid-19: a comment', we read it with interest. The authors say, "There are several thousands of patients with covid-19 worldwide. Nevertheless, there is no case of SLE with covid-19". There is no mention of any reference or source to the claim. They go on to extrapolate, "Hence, hydroxychloroquine use might be an explanation for no report on SLE patient with covid-19".[1]

Initial data from the COVID-19 Global Rheumatology Alliance registry reveal that, of the 110 patients with rheumatic diseases who contracted the infection, 19 had SLE.[2] Few studies examining the efficacy of HCQ in covid-19 have recently come into public domain through journals or preprint servers. The rapid spread of the pandemic has not allowed these early studies to have a design suited to answering the research question satisfactorily, thus dampening the level of evidence. Concerns about the scientific contents of a published manuscript examining the role of HCQ in covid-19 have led to an 'additional independent peer review'.[3]

In this age of social media, any information (truthful or not) spreads like wildfire. On one hand, a social media message saying "no case of SLE with covid-19" may create a false sense of security among patients with SLE, possibly diluting their efforts towards other measures like hand hygiene, isolation and distancing. On the other hand, a message saying "hydroxychloroquine use might be an explanation for no report on SLE patient with covid-19" may lead to unjustified use of the drug by lay people as well as doctors. The rapid spread of false information has led the WHO to acknowledge that, we are also fighting an infodemic—an excessive amount of information about a problem, making the solution even more difficult to find. Unsubstantiated public claims of clinical efficacy of HCQ in covid-19 (as though it were a magic bullet) have led to its shortage for patients with rheumatic diseases.[4] The drug has found its way into prophylaxis and treatment protocols for covid-19.[5] Some governments had at a point in time acquired all the stocks of HCQ for patients with covid-19, making it unavailable to patients with rheumatic diseases.[6]

Such times (even more so) call for the scientific community to abide by the principles of scientific publishing and carefully review what information goes out through our trusted medium.

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Response to: 'Patients with lupus are not protected from COVID-19: a comment' by Sawalha, 'No evidence so far on the protective effect of hydroxycloroquin to prevent COVID-19: response to the Comment by Joob and Wiwanitkit' by Romão *et al* and 'SLE patients are not immune to COVID-19: importance of sending the right message across' by Goyal

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We thank the readers who share interesting ideas on our publication entitled 'SLE, hydroxychloroquine and no SLE patients with COVID-19: a comment'.[1] First, we really appreciate the reply by Monti and Montecucco.[2] We agree that large registry data are required to clarify the incidence of COVID-19 in patients with systemic lupus erythematosus (SLE).[1] Seeking for a new drug against the emerging COVID-19 is a challenge. In this pandemic situation, urgent search for drug and vaccine is necessary. Not only hydroxychloroquine but also other drugs such as antiretroviral drugs are widely used without complete clinical trials. We feel glad that our article can stimulate data sharing on the important issue of the inter-relationship between COVID-19, SLE and hydroxychloroquine. Additional correspondences by Sawalha,[3] Romão *et al*[4] and Goyal[5] are examples of new data. At the time our correspondence with Monti and Montecucco's[1] paper was prepared and submitted, there were no publications on the existence of COVID-19 in patients with SLE. All the SLE cases in the correspondences are new cases reported after we first proposed a hypothesis. The proposed idea was a possible explanation of the data at that time. In science, new findings might or might not support the hypothesis. The correspondences to our article refer to registry data that were published after our article (we submitted our correspondence/comment on 31 March 2020, and our article was published on 15 April 2020 and the registry data published on 16 April 2020). Therefore, our first hypothesis was not based on the unpublished registry data at that time. With the increasing number of COVID-19 cases globally, more than one million patients, there may now be new data on SLE patients with COVID-19. We hereby acknowledge contributions that COVID-19 does occur in patients with SLE. At present, the efficacy or lack of efficacy of hydroxychloroquine remains a speculation in the absence of trials, and although the efficacy of hydroxychloroquine still requires further scientific proof the drug has been widely used in the current COVID-19 outbreak situation.[6] Further systematic evaluation on the benefits of this drug is required. Hydroxychloroquine is not recommended for use in the prevention of COVID-19 in healthy people. Our article did not recommend the drug for COVID-19 prevention. There may now be reports of SLE patients with COVID-19, but an important consideration is whether or not these patients received hydroxychloroquine. A recent report on the first few SLE patients with COVID-19 showed that hydroxychloroquine at standard dose could not help prevent severe COVID-19.[7] If hydroxychloroquine has a pharmacological effect against COVID-19, dosage of this drug that can effectively counteract the infection remains unknown.

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Are patients with systemic lupus erythematosus at increased risk for COVID-19?

The global health emergency generated by the SARS-CoV-2 outbreak has complicated the management of patients with comorbidities, which together with old age seem to be the strongest predictor of mortality from COVID-19.[1] We read with great interest the letter published by Mathian and colleagues about the clinical course of COVID-19 in patients with systemic lupus erythematosus (SLE) treated with hydroxychloroquine.[2] Their preliminary data seem to suggest a particularly high incidence of severe and even fatal cases of infection, confirming that, despite ongoing treatment with antimalarial drugs, patients with SLE have a high risk of unfavourable course during the current pandemic.

The critical point that remains to be clarified at present is the real incidence of COVID-19 in patients with SLE, regardless of the current treatment. Being operative in the maximum epicentre of the outbreak in Italy (Milan, Lombardy), we have had to face in these weeks the emergency related to the management of such a fragile population and we have tried to obtain from our cohort of patients information useful to solve the outstanding issues. In particular, between 25 February and 10 April we circulated a survey that explored the frequency of nasopharyngeal swabs positive for COVID-19, the onset of suspicious symptoms due to viral infection (fever >37.5°C, cough, dyspnoea) and the impact of the pandemic on the behaviour and treatment of our patients. The survey was administered face to face to all patients who attended an outpatient visit or by telephone to those who missed a scheduled visit during the period under examination. The study population encompassed more than 900 patients, including 62 (91% females, mean age 44.1 years) with SLE and a mean disease duration of 12.6 years. About half of the patients (51.6%) were treated with biological drugs (26 belimumab and 6 rituximab), 30 (48.3%) were receiving hydroxychloroquine while another 20 were taking another conventional synthetic disease-modifying drug (7 methotrexate, 11 mycophenolate, 2 azathioprine). Forty-six (74.6%) also took corticosteroids (28 at a dose greater than 5 mg/day). No cases of nasopharyngeal swab positivity were observed, while eight patients (including five on hydroxychloroquine) reported symptoms consistent with a viral infection, rapidly resolving without specific treatment. Only three patients reported contact with confirmed cases of COVID-19 and none of them developed suspicious symptoms. None of the patients changed their current rheumatological therapy and 93.5% defined their disease as stable during the entire period under review. Overall, therefore, the impact of COVID-19 in our patients with SLE was very low, in line with the low burden we observed in the rest of our cohort with inflammatory arthritis.[3] The adoption of strict rules for the prevention of contagion, such as the use of face masks, homeworking and social distancing, was reported by almost all patients (95%). This approach, likely induced by the rheumatic disease itself, has probably played a decisive role in reducing the incidence of COVID-19 among our patients.[4] Noteworthy, the majority of patients included had a long-term disease and were therefore already used to adopt measures to minimise the infectious risk before the COVID-19 outbreak.

In conclusion, our preliminary data, although still limited in number, do not seem to suggest an increased risk of SARS-CoV-2 infection for patients with SLE. Therefore, while considering the severe course of COVID-19 reported in SLE, our data support rheumatologists in encouraging patients to maintain the ongoing treatment to avoid dangerous flare-ups of the disease and to strictly enforce the rules for prevention of infection.

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Response to: 'Are patients with systemic lupus erythematosus at increased risk for COVID-19?' by Favalli *et al*

We thank Favalli *et al* for their interest in our study reporting the course of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease 2019 (COVID-19) in a case series of 17 patients with systemic lupus erythematosus (SLE) under long-term treatment with hydroxychloroquine (HCQ).[1 2] As mentioned in our study, we did not aim at reporting the incidence rate and the severity of COVID-19 in SLE, because our cohort most likely over-represents the most symptomatic and severe cases, as a result of a bias in the selection procedure of the patients used by the physicians.

Favalli *et al*, by studying the nasopharyngeal carriage of SARS-CoV-2, confirm the low prevalence of COVID-19 in their cohort of patients with SLE, similar to that reported in the general population even in epicentres of the outbreak. By May 11, when France eased the COVID-19 lockdown, Salje *et al* projected that only 5.7% (range3.5 – 10.3) of the general French population had been infected and that this proportion was likely to be 12.3% (range 7.9 – 21.3) in Ile-de France, which includes Paris, and 11.8% (range 7.4 – 20 .5) in Grand Est, the two most affected regions of the country.[3] Furthermore, in the general population, as probably in patients with SLE, most infected patients display only mild symptoms, if any, without the need for hospital care, whereas even in the case of hospitalisation, death occurs in less than 6% of cases during the course of the disease.[4] Therefore, it is not surprising that Favalli *et al*, in a series of 62 patients with SLE, did not observe cases of nasopharyngeal swab positivity for SARS-CoV-2 and that only eight (13%) patients reported symptoms consistent with viral infection. Only larger studies describing the incidence and severity of COVID-19 in patients with SLE, based on the detection of SARS-CoV-2, as well as specific antiviral antibodies, will help decipher the prevalence and the risk factors of severe COVID-19 in this fragile population suffering from comorbidities such as cardiovascular or chronic kidney disease. However, even if their cohort size is rather limited, the authors' observation that patients with SLE respecting strict contagion prevention rules do not show an increased risk of developing COVID-19 is very encouraging.

Data collected through the COVID-19 Global Rheumatology Alliance registry recently confirmed that patients with lupus on baseline therapy with HCQ are not universally protected from COViD-19.[5] Therefore, we commend Favalli *et al* to emphasise the primordial role of physical distancing and the adoption of strict rules for the prevention of contagion, because of the uncertain protection of patients with SLE from severe SARS-CoV-2 infection by HCQ treatment.

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Correction: *Knee osteoarthritis risk in non-industrial societies undergoing an energy balance transition: evidence from the indigenous Tarahumara of Mexico*

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The following sections have been updated: Contributors, Patient and public involvement statement and Ethics approval.

Contributors: IJW, DTF, SW and DEL designed research; IJW, ALB and DEL conducted fieldwork and acquired data among the Tarahumara in Mexico; DTF, MC and PA acquired data from Framingham residents in the United States; IJW, DTF, JD, MC, PA, GNE and JJS analysed data; IJW and SW performed statistical analyses; and IJW, DTF, SW and DEL wrote the paper.

Patient and public involvement statement: During fieldwork in Mexico, traditional local Tarahumara leaders approved the study and inspected the data collection process. Directors of the clinics in Mexico where research took place also approved the study.

Ethics approval: Permission to conduct research among the Tarahumara was granted by the Comisión Nacional para el Desarrollo de los Pueblos Indígenas in Mexico. The study was also approved by the Institutional Review Boards of Boston University Medical Center, Harvard University and Massachusetts General Hospital.

Ann Rheum Dis 2021;**80**:e27. doi:10.1136/annrheumdis-2019-215886corr1

Correction: *High prevalence of spondyloarthritis-like MRI lesions in postpartum women: a prospective analysis in relation to maternal, child and birth characteristics*

Renson T, Depicker A, De Craemer A-S, et al. High prevalence of spondyloarthritis-like MRI lesions in postpartum women: a prospective analysis in relation to maternal, child and birth characteristics. Ann of Rheum Dis 2020;79:929-34.

There is a mislabeling of MRI sequences within the figures. The figures have been amended:

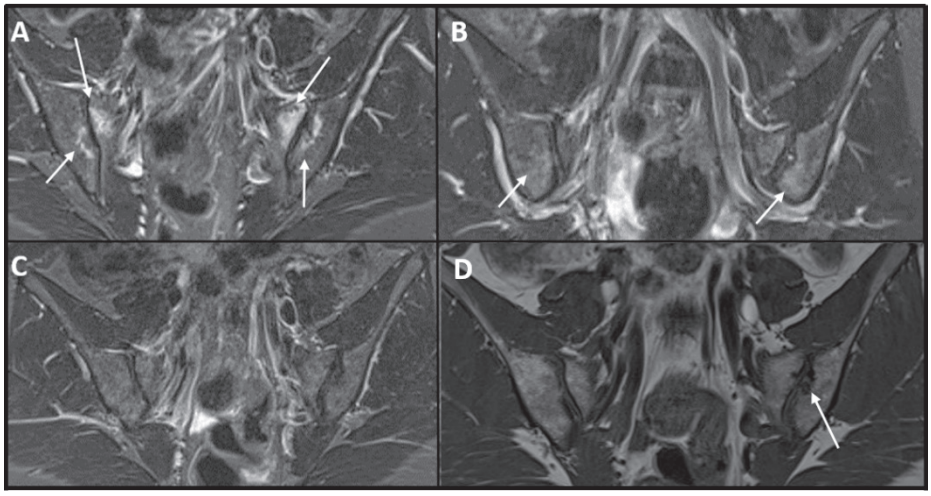

Figure 2 Sacroiliac joint MRI examinations of a 31 year-old postpartum woman. A: extensive sacroiliac bone marrow oedema on stir images at baseline; B: decrease of the bone marrow oedema after 6 months; C: vanishing of the bone marrow oedema after 12 months; D: T1 sequences of the month 12 MRI showing sacroiliac erosions.

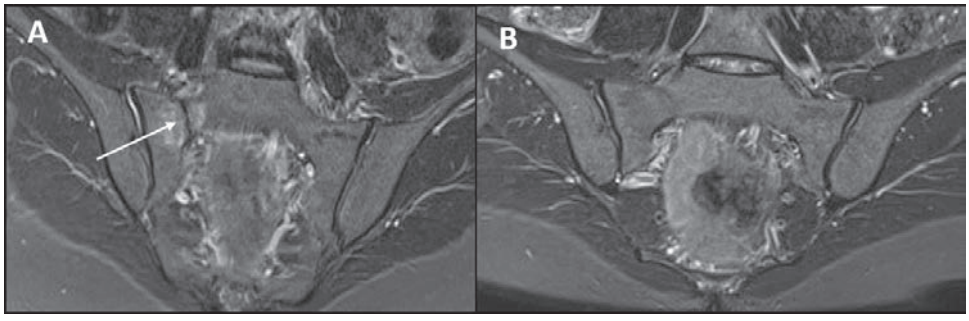

Figure 3 A postpartum sacral fracture on sacroiliac joint MRI of a 28 year-old woman. A: stir sequences of the baseline MRI show a clear fracture of the sacral bone; B: stir sequences show a healed sacral fracture after 6 months.

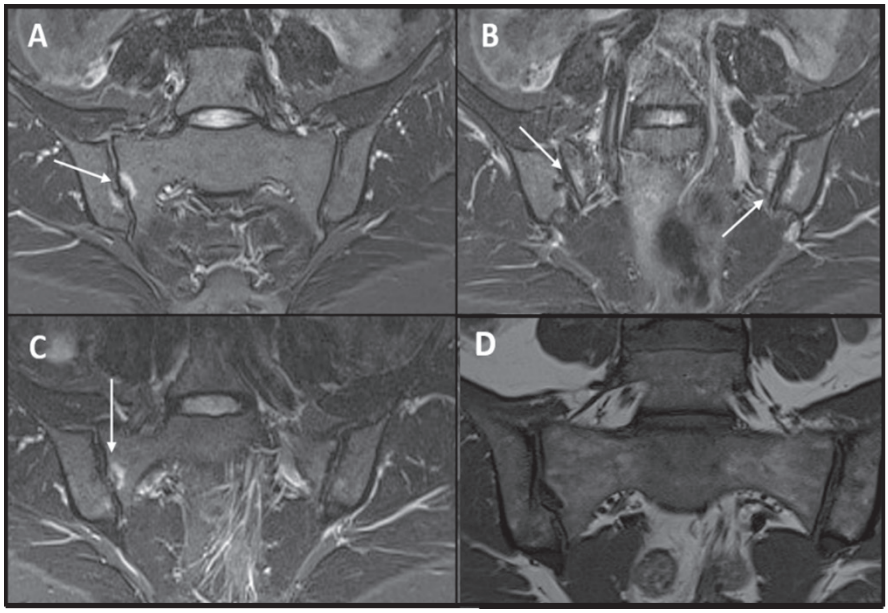

Figure S3 Postpartum sacroiliac MRI images of a 32 year-old woman. A and B: intense sacroiliac bone marrow edema on stir sequences immediately after giving birth; C: decrease of the sacroiliac bone marrow edema on the month 6 MRI stir sequences; D: no structural lesions on the month 12 MRI T1 images. doi:10.1136/annrheumdis-2020-217095.

Ann Rheum Dis 2021;**80**:e28. doi:10.1136/annrheumdis-2020-217095corr1