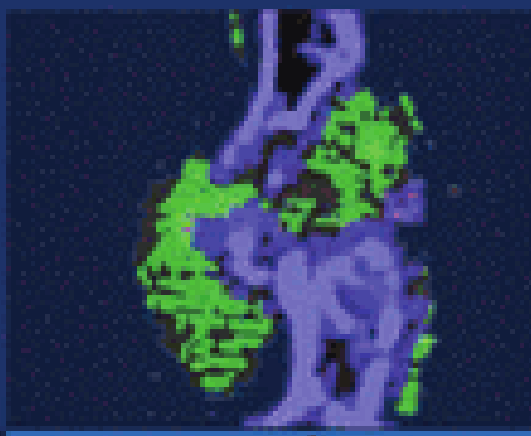
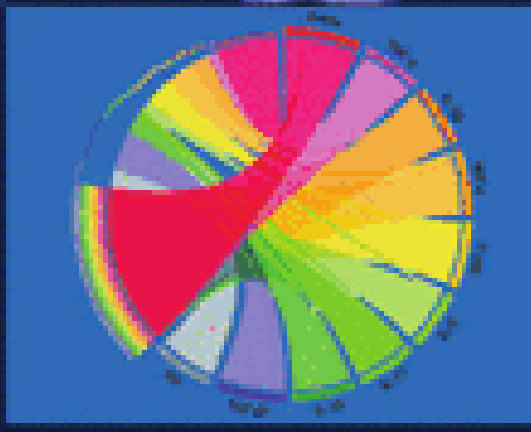
May 2022 Volume 81 Issue 5

Annals of the Rheumatic Diseases

The EULAR Journal





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ARD is published monthly; subscribers receive all supplements
ISSN 0003-4967 (print); 1468-2060 (online)

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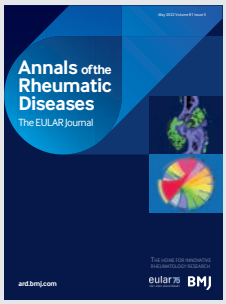

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Twitter: **@ARD_BMJ**
ISSN: 0003-4967 (print)
ISSN: 1468-2060 (online)

ARD is published by BMJ Publishing Group Ltd typeset by Exeter Premedia Services Private Ltd, Chennai, India and printed in the UK on acid-free paper.

Annals of the Rheumatic Diseases, ISSN 0003-4967 (USPS 2152) is published monthly by BMJ Publishing Group Ltd, BMA House, Tavistock Square, WC1H 9JR London. Airfreight and mailing in the USA by agent named World Container Inc, 150-15, 183rd Street, Jamaica, NY 11413, USA. Periodicals postage paid at Brooklyn, NY 11256. US Postmaster: Send address changes to Annals of the Rheumatic Diseases, World Container Inc, 150-15, 183rd Street, Jamaica, NY 11413, USA. Subscription records are maintained at BMA House, Tavistock Square, WC1H 9JR London. Air Business Ltd is acting as our mailing agent.

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The 2021 European Alliance of Associations for Rheumatology/American College of Rheumatology points to consider for diagnosis and management of autoinflammatory type I interferonopathies: CANDLE/PRAAS, SAVI and AGS

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Handling editor Désirée van der Heijde

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221814).

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This article is published simultaneously in *Arthritis & Rheumatology*.

Received 7 November 2021
Accepted 11 January 2022
Published Online First 25 March 2022

To cite: Cetin Gedik K, Lamot L, Romano M, *et al*. *Ann Rheum Dis* 2022;**81**:601–613.

ABSTRACT

Objective Autoinflammatory type I interferonopathies, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature/proteasome-associated autoinflammatory syndrome (CANDLE/PRAAS), stimulator of interferon genes (STING)-associated vasculopathy with onset in infancy (SAVI) and Aicardi-Goutières syndrome (AGS) are rare and clinically complex immunodysregulatory diseases. With emerging knowledge of genetic causes and targeted treatments, a Task Force was charged with the development of 'points to consider' to improve diagnosis, treatment and long-term monitoring of patients with these rare diseases.

Methods Members of a Task Force consisting of rheumatologists, neurologists, an immunologist, geneticists, patient advocates and an allied healthcare professional formulated research questions for a systematic literature review. Then, based on literature, Delphi questionnaires and consensus methodology, 'points to consider' to guide patient management were developed.

Results The Task Force devised consensus and evidence-based guidance of 4 overarching principles and 17 points to consider regarding the diagnosis, treatment and long-term monitoring of patients with the autoinflammatory interferonopathies, CANDLE/PRAAS, SAVI and AGS.

Conclusion These points to consider represent state-of-the-art knowledge to guide diagnostic evaluation, treatment and management of patients with CANDLE/PRAAS, SAVI and AGS and aim to standardise and improve care, quality of life and disease outcomes.

INTRODUCTION

Autoinflammatory type I interferonopathies are genetically defined (monogenic or digenic) immunodysregulatory disorders characterised by the presence of a type I interferon (IFN) signature in peripheral blood and variable systemic inflammation.[1–3] In this expanding group of ultra-rare diseases, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature/proteasome-associated autoinflammatory syndrome (CANDLE/PRAAS), stimulator of interferon genes (STING)-associated vasculopathy with onset in infancy (SAVI) and Aicardi-Goutières syndrome (AGS) are the most common.

Patients with type I interferonopathies present early in life often within the first week of life; prenatal onset has been reported in patients with AGS; however, late-onset cases presenting at ages 14, 18 and 5.6 years with CANDLE/PRAAS, SAVI and AGS, respectively, have been reported.[4–11] Despite CANDLE/PRAAS, SAVI and AGS having distinct clinical phenotypes of varying disease severity, the individual clinical manifestations of these diseases can overlap, and all are associated with high morbidity and mortality if untreated.[4 12] Recent advances in the genetic description of these disorders permit better characterisation of disease-specific clinical manifestations, and provide evidence supporting the pathogenic role of type I IFN signalling.[1 2 12 13] These developments prompted the Task Force lead by the steering committee (two convenors (PAB, RG-M), a neurologist (AV), two methodologists (BMF, ED) and three paediatric rheumatologists/EULAR fellows (KCG, LL, MR) and a rheumatologist (ST)) to review the existing data and develop consensus statements, with the aim of formulating state-of-the-art guidance on the diagnosis, treatment and long-term monitoring of patients with these rare diseases.

Thus, the objective of this project was to develop points to consider for the diagnosis, treatment and long-term monitoring of patients with CANDLE/PRAAS, SAVI and AGS.

The Task Force targets their guidance to paediatricians, internists and subspecialists involved in the care of patients with autoinflammatory type I interferonopathies and to patients and caregivers. These points to consider were developed not only to provide a resource for physicians to facilitate management but also for policy makers governing who have a role in authorising patients' access to various diagnostic tools and treatment options; all with the ultimate goal to harmonise the level of care and to improve quality of life and disease outcomes in this patient population.

METHODS

The European Alliance of Associations for Rheumatology (EULAR)[14] and the American College of Rheumatology (ACR) standardised operating procedures (SOPs) were followed during the project period (see online supplementary methods). With approval from the EULAR and ACR Executive Committees, an international Task Force consisting of worldwide recognised experts from North America, South America, Europe and Australia convened to develop points to consider for the diagnosis, treatment and long-term monitoring of three type I interferonopathies: CANDLE/PRAAS, SAVI and AGS. The Task Force members were selected based on expertise in treatment and care of these patients.

A face-to-face meeting in August 2019 defined the goal of the project and the target population. Then, the Task Force developed research questions related to diagnosis, treatment and long-term monitoring of these diseases using the Population, Intervention, Comparison, Outcome (PICO) format. Search terms were derived from PICO questions and a systematic literature review (SLR) was performed by three research fellows (KCG, MR, LL), with support from a librarian and an epidemiologist (DH and DP), and a senior methodologist (ED) to identify relevant literature published before September 2020.

Two rounds of pre-consensus meeting questionnaires, using the Delphi technique,[15] included questions pertaining to diagnosis, treatment and long-term monitoring were sent to all Task Force members to indicate their agreement with each question or statement with yes/no using the Delphi technique; the Delphi questionnaire was sent to 28 Task Force members, of whom 22 were voting members. The Task Force members were asked to indicate their agreement with each statement, and a free text option was provided to capture every member's comment for each statement. Draft statements and items in questions with 80% or higher agreement were retained for voting at the consensus meetings. Statements and items in questions that did not reach a greater than 80% consensus were reviewed and reworded and sent out in a second round of the Delphi questionnaire. The original and the revised/modified draft statements with the previously achieved level of agreement and the participants' comments were included in the second survey. A free text option to capture comments and additional items was again included. Draft statements with 80% or higher agreement were retained for voting at the consensus meetings, and statements, which did not achieve 80% agreement, were marked for further discussion and refinement at the two consensus meetings. Responses were anonymous.

Based on the SLR findings and two pre-consensus meeting Delphi questionnaires, draft statements were refined by the steering group and were sent to the voting members prior to the consensus meetings. These draft statements were reviewed, discussed, revised and voted on in two consensus meetings that were held online in October 2020 due to the COVID-19 pandemic, one for CANDLE/PRAAS and SAVI, and one for AGS.

Two conveners (RGM, PAB), three methodologists (BMF, ED, DA), three fellows, an allied health professional and three disease experts attended both consensus meetings and, otherwise, participation was based on disease-specific expertise. The voting panel included 19 experts, 1 allied health professional and 1 patient representative for each disease. The joint statements addressing all three interferonopathies were voted on by the entire voting panel; CANDLE/SAVI-specific statements were voted on by 10 experts, 1 allied health professional, 1 SAVI and 1 CANDLE/PRAAS patient presentative, and AGS specific statements were voted on by 14 experts, 1 allied health professional and 1 AGS patient representative. During the meetings, statements that achieved at least 80% agreement were accepted; statements with <80% were discussed a final time in a Nominal Groups round robin discussion (https://www.cdc.gov/healthyyouth/evaluation/pdf/brief7.pdf) and were only accepted if the revised statement reached an 80% agreement.

The Oxford Levels of Evidence (LoE) were applied to each point to consider.[16] The strength of each statement ranged from A (directly based on level I evidence) to D (directly based on level IV evidence or extrapolated recommendations from level I, II or III evidence).[16] Finally, the finalised statements were circulated in a post-consensus meeting Delphi questionnaire to determine level of agreement (LoA). Members of the Task Force were asked to provide their final LoA for each point to consider using a scale of 0 (completely disagree) to 10 (completely agree), which is reported in the tables below.

RESULTS

Systematic literature review

A summary of the literature search strategy and results are provided as supplementary material (online supplementary methods). Based on SLR and consensus conferences, 4 overarching principles and 17 disease-specific points to consider pertaining to the genetically defined interferonopathies (table 1) with their respective LoE, grade of recommendation (GoR) and LoA were generated.[17]

Overarching principles guiding the management of patients with CANDLE/PRAAS, SAVI and AGS

The systemic inflammatory multiorgan involvement in patients with CANDLE/PRAAS, SAVI or AGS can ultimately result in progressive organ injury and early mortality.[4] Damage accrues over time, often manifesting later in life, thus highlighting the importance of early diagnosis and treatment.[1 12]

Autoinflammatory syndromes may present with phenotypic overlap early in life, which poses diagnostic challenges.[12] In addition, mutations in individuals genes may be associated with considerable phenotypic heterogeneity and variable disease severity.[18 19] Genetic confirmation is thus essential for making a precise diagnosis which then facilitates targeted therapy and initiation of genetic counselling with the goal of achieving better clinical outcomes. Patients, their parents and siblings should have access to formal genetic counselling. Genetic counselling can initiate the risk assessment process depending on the type of inheritance for specific disease-causing mutation and help patients understand their test results, including the medical implications for themselves, their reproductive health concerns and impact on their relatives. Patients with clinical symptoms of CANDLE/PRAAS, SAVI or AGS who do not harbour any of the disease-causing mutations described here should be referred

Table 1 Points to consider for the diagnosis, treatment and long-term monitoring of patients with type I interferonopathies, CANDLE/PRAAS, SAVI and AGS

		LoE/GoR	LoA (0–10) Mean±SD
Overarching principles		**C/S/AGS**	
A	Patients with autoinflammatory interferonopathies CANDLE/PRAAS, SAVI or AGS present with chronic systemic and organ-specific inflammation; when untreated, chronic inflammation results in progressive organ damage, early morbidity and increased mortality.	4C/4C/4C	9.8±0.7
B	A confirmed genetic diagnosis is required to make the diagnosis of CANDLE/PRAAS, SAVI and AGS, which facilitates initiation of targeted treatments, genetic counselling, screening for complications and informs prognosis.	5D/5D/4C	9.5±1.0
C	The goal of treatment of type I interferonopathies is to reduce systemic and organ inflammation to prevent or limit the development of and/or the progression of organ injury and damage, and to improve quality of life.	2B/2B/2B	9.8±0.5
D	In CANDLE/PRAAS, SAVI or AGS, long-term monitoring of disease activity, organ-specific injury/damage and of treatment-related complications is required and involves a multidisciplinary team.	5D/5D/4C	9.9±0.3
Individual points to consider			
I.Points to consider for diagnostic evaluation			
1	Patients presenting with unexplained systemic inflammation (including elevations of CRP, ESR and/or an IFN signature) and clinical features* that include rashes, lipodystrophy, musculoskeletal, neurologic, pulmonary and metabolic findings should receive a prompt diagnostic workup for CANDLE/PRAAS, SAVI and AGS comprising: ► Genetic evaluation ► Clinical evaluation focusing on the extent of inflammatory organ involvement ► Screening for disease-related comorbidities	4C/4C/4C	9.8±0.7
2	Patients with clinical symptoms of CANDLE/PRAAS, SAVI or AGS who do not carry any of the disease-causing mutations described here should be referred to specialty/research centres that can guide further workup and treatment.	5D/5D/5D	9.8±0.5
Genetic evaluation			
3	Mutations in the following disease-causing genes should be included in the genetic analyses: ► CANDLE/PRAAS: *PSMB8, PSMA3, PSMB4, PSMB9, PSMB10, POMP* and *PSMG2*. ► SAVI: *STING1* (previously *TMEM173*). ► AGS: *TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR1, IFIH1, LSM11*† and *RNU7-1*†.	4C/4C/4C	9.8±0.6
4	Genetic mimics of CANDLE/PRAAS, SAVI and AGS are recognised and should be included in the diagnostic workup (a non-exhaustive list is below for reference): ► For CANDLE-like conditions: Splice variants in *IKBKG,* frameshift mutations in *SAMD9L*, and recessive mutations in *RNASEH2 (A, B, C*). ► For SAVI-like conditions: *TREX1*, *ADA2* and *COPA*. ► For AGS-like conditions: *RNASET2*.	4C/4C/4C	9.4±0.9
Clinical evaluation (see also tables 3 and 4***)***			
5	In patients with suspected CANDLE/PRAAS, SAVI or AGS, assessment for disease and treatment related comorbidities should include screening for: ► *Skin manifestations*: Nodular rashes, violaceous annular rashes, panniculitis, lipodystrophy or vasculopathic skin lesions. ► *Neurological manifestations*: Intracerebral calcifications, leukoencephalopathy, progressive microcephaly or cerebral atrophy. ► *Pulmonary manifestations*: Interstitial lung disease/pulmonary hypertension. ► *Hepatic manifestations*: Hepatic steatosis, hepatitis, hepatosplenomegaly. ► *Metabolic manifestations*: Hypertension, hyperlipidaemia, glucose intolerance (=metabolic syndrome). ► *Musculoskeletal manifestations*: Arthritis, contractures and myositis. ► *Growth and development*: Growth retardation, osteoporosis, bone development delay, pubertal delay. ► *Haematological manifestations*: Cytopenias (eg, more specifically lymphopenia, thrombocytopenia). ► *Ophthalmologic manifestations*: Episcleritis, keratitis, retinopathy, glaucoma. ► *Cardiac manifestations*: Cardiomyopathy.	4C/4C/4C	9.7±0.6
6	Neuroimaging should be performed in individuals with suspected neurologic symptoms. ► MRI best identifies white and grey matter changes. ► CT is generally more sensitive for detecting cerebral calcification and can be considered when calcium-sensitive modalities on MRI are not available or do not detect calcifications.	4C/4C/4C	9.8±0.4
7	In patients with presumed CANDLE/PRAAS, SAVI or AGS, tissue sampling as appropriate (eg, CSF if neurologic involvement is suspected, or lesional skin biopsies) may support the diagnosis.	4C/4C/4C	9.4±1.1
8	All patients should undergo a basic immunodeficiency workup that includes a history of infections, lymphocyte subsets and immunoglobulin levels, as a minimum.	4C/4C/4C	9.3±1.5
II. Points to consider for treatment			
9	Treatment of patients with CANDLE/PRAAS, SAVI and AGS should be aimed at achieving disease control or low disease activity to prevent progression of organ damage. For patients with SAVI and CANDLE/PRAAS, disease control should be maintained with the lowest possible dose of glucocorticoid.	2B/2B/2B 4C/4C/NA	9.4±1.2
10	Janus kinase inhibitors (JAKIs) are of benefit for improving symptoms‡ in CANDLE/PRAAS, SAVI and AGS.	2B/2B/2B	9.3±0.9
11	In patients with CANDLE/PRAAS, SAVI or AGS on JAKI, screening for treatment-related comorbidities is important. We currently recommend monitoring for BK viral loads in urine and blood to prevent viral organ injury such as nephropathy.	4C/4C/5D	9.3±1.6
12	Glucocorticoids are of benefit for improving symptoms‡ in CANDLE/PRAAS or SAVI. Chronic glucocorticoids do not improve the neurological features of AGS, although acute courses of glucocorticoids may be useful for the treatment of non-CNS inflammatory conditions.	4C/4C/5D	9.0±1.3
III. Points to consider for long-term monitoring and management			

Continued

Table 1 Continued

		LoE/GoR	LoA (0–10) Mean±SD
	Disease related comorbidities and disease progression		
13	A multidisciplinary management team is required for optimal care of patients with CANDLE/PRAAS, SAVI and AGS, that is customised based on patient's disease manifestations.	5D/5D/5D	9.9±0.3
14	Disease activity and burden of disease should be monitored regularly depending on disease activity and severity (see table 4) ► Symptom control can be monitored by assessing disease-specific symptoms‡ using validated patient-reported outcome and quality of life assessments, and by recording missing school or workdays.	5D/5D/5D 5D/5D/5D	9.3±1.8
15	Growth and development of children should be monitored at each visit.	5D/5D/5D	9.8±0.4
	Risk of COVID-19		
16	At the time of writing, there is no evidence to suggest that risks to patients with CANDLE/PRAAS, SAVI or AGS of COVID-19 are any different from the healthy population. Therefore, treatment for interferonopathy should not be stopped unless a specific contraindication to ongoing treatment arises.	5D/5D/5D	9.5±0.8
	Vaccinations		
17	Generally, for CANDLE/PRAAS and SAVI, all routine vaccines (live and killed) are indicated when not receiving immunosuppressive treatments or glucocorticoids, although this should be considered on a case-by-case basis.	5D/5D/5D	9.4±0.9

LoE and GoR are reported separately for each disease.
GoR: A: based on consistent level 1 studies; B: based on consistent level 2 or 3 studies or extrapolations from level 1 studies; C: based on level 4 studies or extrapolations from level 2 or 3 studies; D: based on level 5 studies or on troublingly inconsistent or inconclusive studies of any level. LoE: 1a: systematic review of randomised controlled trials (RCTs); 1b: individual RCT; 2a: systematic review of cohort studies; 2b: individual cohort study (including low-quality RCT); 3a: systematic review of case-control studies; 3b: individual case-control study; 4: case-series (and poor-quality cohort and case-control studies); 5: expert opinion without explicit critical appraisal, or based on physiology, bench research or 'first principles'.
*Disease-characteristic clinical features are listed in table 3.
†These two genes were published after the consensus meeting occurred.
‡Clinical symptoms are listed in tables 3 and 4.
C/S/AGS: CANDLE/PRAAS/SAVI/AGS; AGS, Aicardi-Goutières syndrome; CANDLE/PRAAS, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature/proteasome-associated autoinflammatory syndrome; CNS, central nervous system; CRP, C-reactive protein; CSF, cerebrospinal fluid; ESR, erythrocyte sedimentation rate; GoR, grade of recommendation; IFN, interferon; JAKI, Janus kinase inhibitors; LoA, level of agreement; LoE, level of evidence; NA, not applicable; SAVI, STING-associated vasculopathy with onset in infancy.

to specialty/research centres that can guide further workup and treatment. There is no cure for type I interferonopathies. Current treatment options therefore aim to prevent development or progression of end organ damage by controlling systemic and organ inflammation,[20 21] to improve quality of life and to improve disease outcomes.[1] Given the paucity of long-term outcome data on newly available treatments, monitoring of disease activity, and development of organ-specific and treatment-related complications is essential.[1 22 23] A multidisciplinary team is required to provide optimal care in the context of multiorgan system involvement.[24 25]

Points to consider 1–8: diagnostic evaluation focuses on raising an early suspicion and on facilitating genetic testing, appropriate clinical and laboratory workup and early treatment

Diagnostic evaluation

The presence of a chronically elevated peripheral blood IFN signature is a common finding in patients with the type I interferonopathies CANDLE/PRAAS, SAVI and AGS. In contrast, traditional inflammatory markers such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are typically elevated in CANDLE/PRAAS and SAVI but rarely in patients with AGS.[2 7 12 18 26–30] A peripheral blood IFN signature may be measured using different methodologies, including a 28-gene IFN scoring system using NanoString technology or by quantitative reverse transcriptase (RT) PCR methods of gene subsets should be measured repeatedly to establish chronic elevation.[13] Scores may be negative in the diagnostic phase in patients with milder disease; or in response to glucocorticoid treatment. In addition, patients with AGS with *RNASEH2B* mutations may have a negative IFN signature even with active disease.[31] A practical barrier is the limited number of centres with the ability to check an IFN signature. Thus, a chronically elevated peripheral blood IFN signature is not required for diagnosis but can be very useful in raising the suspicion of an interferonopathy. For most IFN signatures, sensitivity and specificity data are not available. However, in a retrospective study, the IFN signature at a set cut-off score was helpful in differentiated patients with an interferonopathy from healthy controls and from patients with a cryopyrin-associated periodic syndrome (an interleukin-1-mediated autoinflammatory disease). The IFN signature demonstrated an area under the receiver operator characteristic (ROC) curve of 0.98, with sensitivity and specificity exceeding 0.8.[12] Currently, the IFN signature should be interpreted in the context of normal values of the laboratory that conducts the test, since no internationally standardised methodologies or reference ranges are currently available.

Genetic evaluation

As there can be significant overlap of clinical features across several autoinflammatory disorders, a confirmed genetic diagnosis is critical to facilitating a precision medicine approach and targeted therapy. Next-generation sequencing (eg, targeted gene panel, whole exome or whole genome sequencing) to screen for pathogenic variants rather than single gene Sanger sequencing is recommended. Sanger sequencing of individual genes may still be cost effective in patients with known familial disease; and may be the only available option if next-generation sequencing is not yet available to the patient. However, this increasingly outdated 'gene by gene' approach ultimately may result in diagnostic delay and may not be cost-effective.[32] In addition to the known disease-causing genes[1 2 5 7 12 18 31 33–39] (table 1), screening should be considered for diseases that can mimic one of these disorders; their genetic causes[8 12 40–45] are listed in table 2. Allelic, monogenic or digenic, double heterozygous mutations in

Table 2 List of genetically defined disease and genes that should be considered in the differential diagnosis of CANDLE/PRAAS, SAVI and AGS

Genetically defined diseases*	Genes
CANDLE/PRAAS mimics/overlaps	
Differential diagnoses:	
► NEMO Deleted exon 5 Autoinflammatory Syndrome (NEMO-NDAS)	*IKBKG* (exon 5 deletion/splice variant)
► SAMD9L-associated autoinflammatory disease (SAAD)	*SAMD9L* (frame shift mutations)
► Other	*RNASEH2B*
SAVI mimics/overlaps	
Differential diagnoses:	
► Deficiency of the enzyme adenosine deaminase 2 (DADA2)	*ADA2*
► Familial chilblain lupus (CHBL)	*TREX1, SAMHD1*
► COPA syndrome	*COPA*
AGS mimics/overlaps	
Differential diagnoses:	
► Other	*RNASET2*
Other disorders with partially overlapping phenotypes	
Differential diagnoses:	
► Spondyloenchondrodysplasia (SPENCD)	*ACP5*
► Singleton Merten syndromes	*IFIH1, DDX58*
► Retinal vasculopathy with cerebral leukodystrophy (RVCL)	*TREX1*
► Trichohepatoenteric syndrome (THES)	*TTC37, SKIV2L*
► Lipopolysaccharide responsive and beige-like anchor protein (LRBA) deficiency	*LRBA,*
► Monogenic early onset lupus	eg, *C1Q (A, B, C),* several other

*Based on current evidence, all type I interferonopathies, including but not limited to the genetically defined diseases listed in the table should be considered in the differential diagnosis of CANDLE/PRAAS, SAVI or AGS because of overlapping clinical and laboratory features.
AGS, Aicardi-Goutières syndrome; CANDLE/PRAAS, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature/proteasome-associated autoinflammatory syndrome; SAVI, STING-associated vasculopathy with onset in infancy.

genes encoding proteasome or immunoproteasome subunits are the cause for CANDLE/PRAAS, with biallelic pathogenic *PSMB8* variants being the most common cause. Digenic disease causing mutations including *PSMB8*, *PSMA3*, *PSMB4* and *PSMB9*,[1 2 26] compound heterozygous mutations including *PSMB4*, *PSMB8* and *PSMG2*[2 12] and autosomal dominant loss-of-function mutations in *POMP*[2] also cause CANDLE/PRAAS but are rarer. However, novel disease-causing genes are being added as causes for CANDLE/PRAAS. All proteasome genes should be specifically assessed in a patient with a suggestive clinical phenotype. Both parents may need to be tested to confirm digenic inheritance. The inheritance of SAVI is mostly autosomal dominant, and most patients harbour a de novo heterozygous missense mutations in the *STING1* gene that confers a gain-of-function by increasing TANK-binding kinase 1-mediated IRF3 phosphorylation and *IFNB1* transcription.[7 46] Liu *et al* also reported somatic mosaic mutations in one patient (OMIM-615934). So far only additive *STING1* gain-of-function mutations in p.R284W require homozygosity to confer disease.[47] Furthermore, mostly loss-of-function mutations in genes encoding proteins that regulate nucleic acid metabolism or signalling cause AGS.[34] These include biallelic null mutations in *TREX1* and *SAMHD1;* biallelic null mutations in the disease-causing genes, *RNASEH2A*, *RNASEH2B*, *RNASEH2C* or *ADAR1* have not been reported. Disease-causing *IFIH1* variants are all heterozygous gain-of-function mutations that increase type I IFN signalling.[34] Recently, biallelic mutations in *LSM11* and *RNU7-1*, which encode components of the replication-dependent histone pre-mRNA–processing complex extend defects in nucleic acid metabolism to histone mRNAs.[48] It is important to note that large deletions, such as deletions in AGS-related genes including *SAMHD1*, may be missed on exome sequencing and need to be reviewed using other testing modalities.[31 49 50] If following routine genetic workup, a molecular diagnosis is not established in a patient with suggestive phenotypic features, referral to a research centre of excellence for further evaluation should be considered.

Clinical evaluation

In patients with undifferentiated autoinflammatory diseases or otherwise unexplained systemic inflammation, certain clinical features are suggestive of CANDLE/PRAAS, SAVI or AGS (tables 1 and 3).

The following clinical features are relevant to the workup of patients with suspected interferonopathies:

Cutaneous manifestations

Inflammatory skin lesions are present in all three diseases; however, the nature of the rash differs. Nodular rashes or violaceous annular rashes should prompt a diagnostic workup for CANDLE/PRAAS. Another specific cutaneous finding for CANDLE/PRAAS is panniculitis (particularly neutrophilic panniculitis) and panniculitis-induced lipodystrophy, which are hallmarks of the disease.[1 2 9 12 18 36 37 51]

The presence of vasculopathic skin lesions such as pernio ('chilblain lesions') or acral ischaemia presenting as Raynaud's phenomenon, and/or 'purple toes' is suggestive of SAVI[7 44 47] and AGS,[33 52–55] the development of gangrene with prolonged ischaemic attacks is a feature of SAVI[1 7 44] (table 3). Skin involvement is the most common symptom in patients with SAVI at presentation[1 7 56–59] but some patients can present with severe lung disease and only minimal skin involvement.[8 46 60 61]

In addition to chilblain-like lesions and acrocyanosis, other skin manifestations such as periungual erythema, or necrotic lesions of the toes, fingers and outer helix, can be seen in patients with AGS.[33 52–55] Moreover, some patients with AGS can have panniculitis as well.[34] Finally, some patients with AGS have recurrent oral ulcers.[50 62]

Lesional skin biopsies in areas that can safely be biopsied can be beneficial in revealing the neutrophilic dermatosis, small vessel vasculitis (from necrotic area), fasciitis[57] and granulomatous nodular dermatitis,[59] thus supporting the diagnosis of SAVI while in AGS specifically, a lesional biopsy can demonstrate deposition of immunoglobulin and complement in the walls of small vessels.[63]

Neurological manifestations

Although CANDLE/PRAAS-affected patients present with headaches and may develop aseptic meningitis,[24] neurological findings are most common and severe in AGS and include subacute or acute neurologic decline, unexplained developmental delay, progressive microcephaly, dystonia, spasticity, encephalopathy, irritability and focal motor findings. A lumbar puncture typically shows sterile cerebrospinal fluid (CSF) pleocytosis.[11 64 65]

Neuroimaging should be performed in individuals with a suspected diagnosis of an interferonopathy in the presence of neurologic symptoms. The initial workup may include MRI of the brain which identifies best white and grey matter changes.[41] CT head should be considered when calcium-sensitive modalities on MRI are not available or not able to detect calcifications, since it is more sensitive for the detection of cerebral calcification.[66] Risks and benefits of sedating a child for MRI brain should be considered.[67] It is useful to have a baseline brain MRI to assess the severity and to monitor disease-associated complications; however, this is not a diagnostic prerequisite, especially for SAVI and CANDLE/PRAAS. Neuroimaging may be particularly helpful in patients with suspected AGS due to the dominant neurological phenotype which should be differentiated from mimickers of interferonopathies.

Basal ganglia or other intracerebral calcifications are overlapping neuroimaging findings for all three diseases[68]; they are

Table 3 Clinical features suggestive of CANDLE/PRAAS, SAVI and AGS

Systemic inflammation	
CANDLE/PRAAS, SAVI, AGS	*Clinical features*: Recurrent fever, hepatosplenomegaly *Laboratory features:* Elevated CRP, ESR and IFN signature
Skin manifestations	
CANDLE/PRAAS	Neutrophilic panniculitis, nodular rashes, violaceous annular rashes, lipodystrophy
SAVI	Vasculopathy (ie, chilblain lesions, acral ischaemia ranging from Raynaud's phenomenon to gangrene), loss of digits
AGS	Chilblain lesions, acral lesions (including Raynaud's phenomenon), panniculitis
Neurological manifestations	
CANDLE/PRAAS	*Clinical features*: Headache, cognitive impairment *Lumbar puncture*: Sterile pleocytosis *Neuroimaging:* Basal ganglia calcifications
SAVI	*Neuroimaging:* Basal ganglia calcifications (rare)
AGS	*Clinical features*: Subacute or acute onset of neurologic symptoms including developmental delay, irritability, neurological impairment or regression, dystonia and spasticity, focal motor findings, progressive microcephaly, seizures *Lumbar puncture*: Sterile pleocytosis, elevated CSF neopterin and tetrahydrobiopterin, elevated interferon alpha *Neuroimaging*: Leukoencephalopathy, cerebral calcifications, early and rapid cerebral atrophy with or without calcification, Moyamoya disease*
Pulmonary manifestations	
CANDLE/PRAAS	Pulmonary hypertension without fibrosis
SAVI	Interstitial lung disease with or without secondary pulmonary hypertension
AGS	Pulmonary hypertension
Hepatic manifestations	
CANDLE/PRAAS	Elevated transaminases, hepatic steatosis
AGS	Elevated transaminases, autoimmune hepatitis
Metabolic and endocrine manifestations	
CANDLE/PRAAS	Hypertension, hyperlipidaemia, glucose intolerance (=metabolic syndrome)
AGS	Hypothyroidism, diabetes insipidus, diabetes
Musculoskeletal manifestations	
CANDLE/PRAAS, SAVI, AGS	Myositis
CANDLE/PRAAS, SAVI, AGS	Arthritis, joint contractures
Growth and development	
CANDLE/PRAAS, SAVI, AGS	Growth retardation, osteoporosis, bone development delay, pubertal delay
Haematological manifestations	
CANDLE/PRAAS, SAVI, AGS	Anaemia, leucopenia, lymphopenia and/or thrombocytopenia
Ophthalmologic manifestations	
CANDLE/PRAAS	Episcleritis and keratitis
SAVI, AGS	Retinopathy, glaucoma
Cardiac manifestations	
AGS	Cardiomyopathy, valve calcifications

*Vasculopathy characterised by progressive narrowing of the terminal intracranial portion of the internal carotid artery and circle of Willis.
AGS, Aicardi-Goutières syndrome; CANDLE/PRAAS, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature/proteasome-associated autoinflammatory syndrome; CRP, C-reactive protein; CSF, cerebrospinal fluid; ESR, erythrocyte sedimentation rate; IFN, interferon; SAVI, STING-associated vasculopathy with onset in Infancy.

more common, more severe and typically start earlier in life in patients with AGS compared with CANDLE/PRAAS, while calcifications are rare in SAVI.[8 41 68 69] In addition, the presence of leukoencephalopathy is suggestive of AGS and typically starts early in life in AGS patients with severe disease; it is unusual in CANDLE/PRAAS or SAVI.[11 70 71] Other supportive neuroimaging characteristics for AGS are early and rapid cerebral atrophy with or without calcifications, cerebral white and grey matter changes and Moyamoya disease.[12 41 69 70 72–74] Intracerebral large vessel vasculitis or Moyamoya can be seen and is associated with *SAMHD1* mutations.[49 74–77]

Additional workup for neurodegenerative diseases in patients with suspected AGS may also be considered. Lumbar punctures are not required to make the diagnosis of AGS but may support the diagnosis[72] and characterise the immunological features of the central nervous system (CNS) inflammation, including the presence of lymphocytosis and raised levels of interferon-alpha (IFN-α), CXCL10 and CCL2 in the CSF.[31 54 69] The CSF studies are most beneficial if a molecular diagnosis of AGS is not confirmed by genetic testing and provide support for additional molecular testing.[72]

Pulmonary manifestations

The presence of early onset interstitial lung disease (ILD) raises suspicion for SAVI, in particular in the context of unexplained systemic inflammation.[1 7 46 56 61] Many patients with SAVI are reported to have lung involvement, mostly manifested as ILD, ranging from mild ILD with no respiratory symptoms to lung fibrosis. Also, alveolar haemorrhage is reported as the presenting feature in a few cases with SAVI.[47 60] Although ILD is a major concern for patients with SAVI, it is rarely present in patients with CANDLE/PRAAS[1 18 51] and not reported in AGS. Low radiation chest CT and pulmonary function tests (PFTs) are recommended modalities to screen for ILD.[8] Lung biopsies may distinguish infectious from inflammatory disease but are not required to make the diagnosis of SAVI.[7 46 60 61]

Another significant pulmonary manifestation is pulmonary hypertension, which is a potentially life threatening and possibly underdiagnosed complication of CANDLE/PRAAS and AGS.[1 12 78] While CANDLE/PRAAS and AGS are known to affect the vascular system, the full impact of systemic vasculopathy is currently undercharacterised. All patients with suspected CANDLE/PRAAS and AGS should undergo regular evaluation for pulmonary hypertension; echocardiography is recommended as a screening and monitoring tool.

Hepatic manifestations

Forty to eighty per cent of patients with CANDLE/PRAAS develop metabolic syndrome and hepatic steatosis, often in the first decade of life.[1] In addition, patients may develop hepatosplenomegaly which could be due to extensive metabolic disturbance in fat processing.[2 5 9 36 37 39 51] In an open-label trial in CANDLE/PRAAS, it is reported that baricitinib did not significantly improve hepatic steatosis in two patients with hepatic steatosis prior to baricitinib treatment nor prevent it in three patients with hyperlipidaemia at baseline pointing to the role of proteasome dysfunction in the aetiology of hepatic steatosis.[1]

In AGS, hepatosplenomegaly and/or transaminitis can be an initial presentation in the neonatal period when it resembles congenital viral infection.[31 33 72 79] Patients can develop autoimmune hepatitis, the presence of liver-specific antibodies has been described.[34 62 80]

Transaminases should be evaluated at presentation and may be monitored as a marker for hepatic disease activity in patients with type I interferonopathies, although it should be noted they can also be elevated in CANDLE/PRAAS and AGS due to myositis.[12]

Information about the clinical features of hepatic involvement in patients with SAVI is limited. However, case reports of patients with SAVI presenting with hepatic disease, such as necrotising granulomatous hepatitis, cholestatic hepatitis and cholangitis and multiple biliary cysts are presented.[58 81]

Metabolic manifestation

Metabolic abnormalities are significant concerns in patients with CANDLE/PRAAS and patients can develop metabolic syndrome defined by Ford *et al* (presence of at least three of the following five criteria: hypertriglyceridaemia ≥110 mg/dL, low high-density lipoprotein cholesterol ≤40 mg/dL, abdominal obesity with waist circumference ≥90th percentile (sex specific), hyperglycaemia ≥110 mg/dL, systolic or diastolic blood pressure ≥90th percentile (age, height, sex specific)).[82] In addition, these patients can have increased abdominal girth secondary to intra-abdominal fat deposition.[1 51] The workup in CANDLE/PRAAS should include screening for metabolic abnormalities.

Patients with AGS may have hypothyroidism, often requiring replacement therapy, and insulin-dependent diabetes mellitus is reported.[34 49 53 54 77 83–85] Other endocrine manifestations include central diabetes insipidus, growth hormone deficiency and adrenal insufficiency.[34 83]

Musculoskeletal manifestations

Myositis is a common feature of patients with CANDLE/PRAAS. It is usually patchy in distribution and can be demonstrated by muscle MRI.[1 39 51] In addition, most patients with CANDLE/PRAAS will develop variable degrees of joint contractures in the hands and feet; these can be severely disabling.[1 2 9 37 51] Myopathy is described in individual case reports in AGS.[86] In AGS-affected patients, joint involvement can include a lupus-like arthritis, or progressive arthropathy with joint contractures.[50 87 88] Articular involvement in SAVI is seen in one-third of the patients.[8] Rheumatoid factor (RF) positivity was reported in majority of cases (57%)[8] while anti-cyclic citrullinated peptide (anti-CCP) was not common in patients with SAVI but systematic testing has not been performed. Interestingly, the course of the arthritis in SAVI can be destructive, especially in childhood, when associated with RF and anti-CCP antibodies.[7 43]

Growth and development

Many children with chronic inflammation, including patients with type I interferonopathies, have lengths/heights and bone mineral density (BMD) that are below that of age-matched controls. Height and BMD are further decreased in the context of treatment with glucocorticoids. Weight percentiles can increase sharply with high doses of glucocorticoids, and this should be taken into consideration when evaluating weight.[1]

In addition to abnormalities in stature, patients with AGS can have significant developmental delay; after a subacute onset most individuals develop profound neurological regression and present with severe impairment in psychomotor development.[22 23 34] Patients with AGS and CANDLE/PRAAS may also present with mild developmental delay[5 22 51]; these delays are not reported in patients with SAVI.[8]

Haematological manifestations

Cytopenias can occur in all three diseases due to temporary bone marrow suppression or homing changes and may correlate with disease activity.[1 12] Cytopenias including autoimmune cytopenias occur more frequently in patients with CANDLE/PRAAS and AGS but are also seen in patients with SAVI.[1 8 18 33 50 52 54 60 79 83 89] Thrombocytopenia in patients with AGS can be present during the neonatal period mimicking congenital infection, but also later during the course of the disease associated with other haematological abnormalities such as anaemia and leucopenia.[19 79] Complete blood count with differential should be evaluated at presentation and may be monitored as a marker for disease activity in patients with type I interferonopathies.

Ophthalmological manifestations

Patients with type I interferonopathies can develop different types of ophthalmological manifestations. While patients with CANDLE/PRAAS can present with keratitis and/or episcleritis,[2 18 51] patients with SAVI and AGS can develop glaucoma.[8 54 76] Glaucoma has been reported in 6.3% of patients with AGS (up to 20.8% of patients with *SAMHD1* mutations), with most cases presenting in the first 6 months of life, in patients who were not receiving glucocorticoids.[34 76] Retinopathy has been described in AGS and SAVI but it remains unclear whether this occurs in the context of secondary mutations.[90]

Cardiac manifestations

Patients with AGS, especially those with mutations in *TREX1*, are prone to develop infantile-onset hypertrophic cardiomyopathy.[31 34] There is an important risk of cardiac valve calcification in disease related to mutations in *IFIH1* and *ADAR*.[91]

Other considerations

Immunodeficiency workup

Patients with known type I interferonopathies may have some degree of immunodeficiency, either due to chronic disease and cytopenias or due to treatment with immunosuppressants.[92] Early manifestations may overlap with non-type I interferonopathy immunodeficiencies. Therefore, a basic immunologic workup should be considered even in the context of a confirmed diagnosis. The workup should include a history of infections and assessment of lymphocyte subsets and immunoglobulin levels, as a minimum.[1 12 93]

Infections in patients with CANDLE/PRAAS can be associated with the development of macrophage activation syndrome (MAS). Opportunistic infections in patients with other CANDLE/PRAAS mutations or SAVI and AGS are rare, although pneumocystis infection has been reported in a patient with SAVI who was not on any immunosuppressive treatment.[89] Furthermore, defects in maturation of CD8+ cells are identified in patients with CANDLE/PRAAS,[2 94] and in some patients with SAVI.[8 57 89] Severe infections are reported in two patients with *POMP* mutations,[94] which may be modified by additional genetic variants.

Points to consider 9–12: treatment focus on optimising inflammatory disease control

The goal of treatment is the control of the systemic and organ-specific disease manifestations and to manage complications of existing organ damage that are consequences of untreated disease.

Pharmacological treatment with Janus kinase inhibitors (JAKIs), particularly baricitinib, is widely used to treat patients with type I interferonopathies.[1 95–98] The JAKIs are reported

to be beneficial in controlling inflammatory symptoms and in preventing progression of end organ damage. Specifically, treatment with baricitinib resulted in a significantly lower daily diary score as well as significant reduction in glucocorticoid use in patients with type I interferonopathies in different open-label trials.[1 95] In the study by Sanchez *et al*, none of the patients had achieved remission before initiating baricitinib treatment, and 50% of patients with CANDLE/PRAAS achieved lasting remission with no clinical symptoms, normalisation of inflammatory markers on baricitinib, all discontinued glucocorticoids. In addition, patients with CANDLE/PRAAS had improvement in myositis and cytopenias (haemoglobin, lymphocyte and platelets). Moreover, significant clinical improvement, including fewer vasculitis flares, prevention of skin involvement/progression of spontaneous amputations/the development of gangrene, and stabilisation of ILD by preserving pulmonary function, was achieved in patients with SAVI.[1] However, to date, no patient with SAVI treated with JAKI achieved complete remission. Furthermore, JAKIs reduce IFN-α-mediated STAT-1 phosphorylation in a dose-dependent manner in patients with interferonopathy,[26 56] thus demonstrating an in vivo effect of the JAKI on type I IFN signalling. The JAKIs, ruxolitinib and tofacitinib, are also reported as potential treatment options.[44 56 59 98] Population pharmacokinetics and pharmacodynamic analyses in children treated with baricitinib showed a substantially shorter half-life in paediatric than in adult populations requiring more frequent dosing, and led to a proposed weight-based and estimated glomerular filtration rate-based dosing regimen to guide dose adjustments in the growing child.[26] Doses of JAKI used to treat these conditions that were published are summarised in online supplemental table 4. A beneficial effect of JAKI on inflammatory disease manifestations is also observed in patients with AGS, including in an open-label trial. The treatment led to a decrease in interferon signalling genes expression scores and improvement of AGS-related symptoms, including neurologic disability, crying, sleep disturbances, irritability, seizures, fever and skin inflammation of the trunk, arms and legs.[95–97] In all instances, pre-existing organ damage is irreparable (ie, the neurological manifestations) stressing the need for early treatment. In patients with AGS, treatment with HIV-1 reverse-transcriptase inhibitors reduced IFN scores, however, clinical benefit was not demonstrated[99] and thus it is unclear if these drugs can be recommended.

Viral reactivation including BK viral reactivation has been reported in type I interferonopathy patients treated with JAKI.[1 59] BK polyomavirus reactivation caused by therapeutic immunosuppression a commonly reported complication in renal transplant patients that can result in nephropathy and renal allograft loss. There is no proven treatment for BK nephropathy and management is limited to early detection and to controlling BK viral load by reducing the dose of immunosuppressive medications.[100 101] Monitoring for BK viral load in blood and urine and renal function prior to initiation of JAKI, at baseline, and then routinely at each visit is recommended.

Other viral reactivations, such as herpes, are reported in CANDLE/PRAAS and SAVI[1]; however, there are insufficient data to routinely recommend anti-viral drug prophylaxis for patients with CANDLE/PRAAS and SAVI treated with JAKI. Similarly, in AGS, viral prophylaxis for patients on JAKI is not currently recommended.

Finally, the data from an open-label trial indicated that patients with AGS who are receiving baricitinib should be monitored closely for thrombocytosis, leucopenia and infection, especially those with underlying thrombotic risk factors or those who are receiving systemic glucocorticoids or immunosuppressive regimens,[95] while no such events were reported in two other reports.[96 97]

Glucocorticoids are generally considered useful in CANDLE/PRAAS and SAVI patients with systemic inflammation, although their use is limited by toxicity.[1] When used for a prolonged time, glucocorticoids cause serious side effects including growth arrest, truncal obesity, hypertension, glucose intolerance and osteopenia.[102] Therefore, the lowest possible dose of glucocorticoids should be targeted for disease control.

There is generally no role for chronic glucocorticoids in AGS, as glucocorticoids do not improve the long-term neurological features nor outcome of AGS. However, short courses of glucocorticoids to treat acute CNS and non-CNS inflammatory manifestations, such as cytopenias and hepatitis, may be beneficial.

Points to consider 13–17: long-term monitoring and management focus on assessing inflammatory organ manifestations, minimising treatment-related toxicities, and encouraging general health measures, including vaccines, and fostering of self-management skills and medical decision-making

A multidisciplinary team approach to regular clinical follow-up is recommended and may include access to medical subspecialists, including a rheumatologist, geneticist, neurologist, ophthalmologist, pulmonologist, cardiologist, hepatologist, gastroenterologist, haematologist, immunologist, dermatologist, endocrinologist, nephrologist, and access to supportive services including a physiatrist, wound care specialist, psychologist, bone health specialist, physical therapist, dental/oral surgeon, dietitian, psychiatrist, rehabilitation care, orthopaedic care and social support services. With current treatment strategies the ultimate treatment goal in inflammatory diseases, namely inflammatory remission, can only be achieved in a subset of patients. Remission is mainly described in patients with CANDLE/PRAAS.[1] The current treatment goal is therefore to reduce systemic and organ inflammation and to prevent or limit the development or progression of organ injury/damage. This requires treatment adjustments and close monitoring of disease progression. Table 4 provides general and disease-specific guidance for the monitoring of disease activity and assessment of organ damage. The monitoring should include (1) assessment of the level of systemic inflammation, and of growth and sexual development, (2) the assessment of general and disease-specific clinical signs and symptoms including the use of validated instruments when available,[1 22 23] (3) monitoring of disease-specific organ manifestations and (4) monitoring of the development of autoimmune features (see online supplemental table 5 for autoantibody associations with organ-specific autoimmune manifestations in CANDLE/PRAAS, SAVI and AGS), cytopenias, treatment-related complications and infections (immunodeficiencies). Preliminary guidance regarding the monitoring of JAKI treatment (tables 3 and 4) is provided but may need to be adjusted as experience with treatment of interferonopathies grows.

All patients should be evaluated at each visit for the presence of disease-specific symptoms and presence of systemic inflammation (table 4).

Chronic inflammation and chronic glucocorticoid treatment negatively affect bone health (eg, osteoporosis), growth (stunting) and development.[1] These parameters should be monitored regularly, as well as cardiac (eg, hypertension) and ophthalmologic complications of chronic glucocorticoid use.

Table 4 Evaluation of inflammatory disease manifestations and organ involvement with proposed interval monitoring

		Follow-up frequency*
A. Monitoring of systemic inflammation and development		
	ESR, CRP, CBC with differential (cytopenias), IFN signature when available	At each visit*
	Urinalysis (proteinuria, renal disease) Renal ultrasound	At each visit* To consider at baseline
	Hepatosplenomegaly and lymphadenopathy	At each visit*
	Height and weight DEXA scan† (BMD) Sexual development	At each visit* As clinically indicated As clinically indicated
B. Monitoring of clinical disease signs and symptoms		
CANDLE/PRAAS		
	Fever, rash, progressive lipodystrophy, headache, musculoskeletal symptoms (joint pain, contractures, weakness), shortness of breath, weight changes, developmental assessment, fatigue	At each visit*
SAVI		
	Fever, rash, peripheral acral vasculitis and dystrophic changes, respiratory symptoms (shortness of breath, tachypnoea, digital clubbing), fatigue	At each visit*
AGS		
	Developmental assessment, changes in neurologic tone affecting joint integrity, skin findings, musculoskeletal findings, clinical evidence of cytopenias, endocrine disturbance, ocular abnormalities or cardiomyopathy	At each visit*
C. Monitoring of organ manifestations		
CANDLE/PRAAS		
Skin disease	Skin exam, assessment of lipodystrophy Lesional skin biopsy (neutrophilic panniculitis)	Every 3–6 months until stable then every 6–12 months Baseline only
Musculoskeletal disease	Arthritis, contractures, weakness CK, aldolase, LDH for myositis	Every 6–12 months
Endocrine, metabolic disease†	Metabolic syndrome Lipid profile (dyslipidaemia), fasting glucose, Haemoglobin A1C, serum insulin (insulin resistance) BP measurement (systemic hypertension)	Every 12–36 months depending on symptoms. At each visit* At each visit*
Hepatic disease†	ALT, AST, GGT, liver elastography or screening for hepatic steatosis with the best available method	Every 6–12 months
Pulmonary arterial hypertension†	Echocardiography Cardiology and/or pulmonology referral if signs of PAH	Every 6–12 months, if PAH then as clinically indicated
CNS disease†	Lumbar puncture (if headaches), Brain MRI	Every 12–36 months depending on symptoms
Eye disease†	Scleritis, episcleritis, keratitis	Yearly or based on clinical need
Dental disease	Tooth abnormalities (tooth agenesis, hypodontia), delayed tooth eruption	Yearly or based on clinical need
SAVI		
Skin disease	Wound care (including wound culture as necessary)	As needed
Pulmonary disease†	Low radiation chest CT	At baseline and then as needed
	PFTs	Every 3–6 months As needed
AGS		
Neurological damage/progression†	Brain MRI (cerebral white and grey matter changes) MRI/MRA in patients with *SAMHD1*-associated AGS (intracerebral vasculitis) Electroencephalogram (epilepsy) Muscle MRI or ultrasound (myositis)	At baseline and then as needed At baseline and then as needed Yearly As needed
Hepatic disease†	ALT, AST, GGT, bilirubin total and direct, albumin, and INR (autoimmune hepatitis)	Every 6–12 months
Endocrinopathies	TSH (hypothyroidism) GH testing and glucose tolerance test	Yearly As needed based on symptoms
Renal disease	Urinalysis	Every 6–12 months
Eye disease†	Ophthalmologic evaluation (glaucoma)	Yearly
Cardiorespiratory	Echocardiogram (cardiomyopathy and PAH)	Every 1–2 years
Scoliosis, hip dislocation†	Hip X-rays and spine screening in non-ambulatory patients (hip dislocation)	Every 6–12 months
D. Monitoring of autoimmunity, cytopenias, immuno-deficiency and JAK inhibitor-related complications		
Autoimmunity and cytopenias and immunodeficiency	Screening for autoimmunity (autoantibodies as indicated), CBC with differential (screening for anaemia, thrombocytopenia, leukopenias) History of infections, lymphocyte subsets, immunoglobulin levels.	Every 6–12 months and when indicated
	Consider immunology or haematology referral	At baseline and then every 3–6 months
Infections	Clinical history, viral reactivation (on JAK inhibitors), opportunistic infections	At each visit
JAK inhibitor monitoring	CBC with differential, LFTs, urinalysis, renal function, creatinine clearance, BK viral loads in urine and blood, urine beta 2 microglobulin	At each visit

*The visit frequency is set according to clinical need and the patient's disease activity. If there is no active disease, then patients should be followed every 3 months to assess disease activity and monitor drug toxicity.
†Requires subspecialty evaluation.
AGS, Aicardi-Goutières syndrome; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMD, bone mineral density; BP, blood pressure; CANDLE/PRAAS, chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature/proteasome-associated autoinflammatory syndrome; CBC, complete blood count; CK, creatine kinase; CRP, C-reactive protein; DEXA, dual energy X-ray absorptiometry; ESR, erythrocyte sedimentation rate; GGT, gamma-glutamyl transferase; GH, growth hormone; IFN, interferon; ILD, interstitial lung disease; INR, international normalised ratio; JAK, Janus kinase; LDH, lactate dehydrogenase; LFTs, liver function tests; MRA, magnetic resonance angiography; PAH, pulmonary arterial hypertension; PFTs, pulmonary function tests; SAVI, STING-associated vasculopathy with onset in infancy; TSH, thyroid-stimulating hormone.

Patients with CANDLE/PRAAS should also be monitored for headaches, skin and musculoskeletal disease, development of metabolic syndrome (hypertension, hyperglycaemic and hepatic steatosis) and for development of primary pulmonary hypertension. Pulmonary hypertension can be insidious in onset. Although ILD is rare, it should be screened for at baseline and monitored as indicated by PFTs and low radiation chest CT. Ophthalmologic and dental assessment may be required in

patients with eye inflammation and hypodontia and tooth eruption problems.[1 2 5 9 18 36 37 39 51]

Patients with SAVI may require wound care (including wound culture as necessary), and close assessment of ILD and the development of secondary pulmonary hypertension. Patients should be screened for systemic hypertension, otolaryngology, ophthalmology and dental disease at baseline and be followed as indicated. Patients should be instructed in self-care, including keeping peripheries warm, and in emergency management of acute ischaemic digits (eg, with, but not limited to, intravenous fluids, pentoxyphylline or intravenous vasodilators), prompt use of antibiotics if infection is suspected, and meticulous wound care.[1 8 103]

Patients with AGS are monitored for progression of neurological disease including gross and fine motor function and cognitive function using validated scales when available.[22 23] Patients with *SAMHD1* mutations require yearly MRI and MR angiography studies to screen for intracerebral artery disease (eg, Moyamoya).[49 74 77] Patients should be monitored for the development of systemic hypertension, pulmonary hypertension and cardiomyopathy.[78] Other complications include autoimmune hepatitis[25 83] and autoimmune endocrinopathies, most frequently hypothyroidism.[34] Other manifestations that can develop insidiously include glaucoma and epilepsy, and should be monitored as clinically indicated.[76 104] Neurological tone abnormalities in non-ambulatory patients can lead to joint dislocation and scoliosis and should be monitored. Families should be instructed in prevention of skin complications, physical therapy, management of disturbed sleep–wake patterns and irritability commonly seen in AGS. Families can also participate in home stretching programmes, and appropriate positioning of children with tone abnormalities.

The heightened type I interferon-mediated autoimmune response contributes to the development of autoantibodies and autoimmune diseases[105] (see online supplemental table 5). Antinuclear antibodies (ANA) are seen in up to 62.5% of patients with SAVI,[8] in up to 42% of patients with CANDLE/PRAAS[1 2 5 9 18 39 51 93] and 23% of patients with AGS.[62] Moreover, antiphospholipid antibodies are present in patients with CANDLE/PRAAS, SAVI and AGS.[1 7 62] Antineutrophil cytoplasmic antibodies (ANCA) are, intermittently, elevated in up to 71% of patients with SAVI and 18% of patients with AGS[8 62]; and RF positivity is reported in patients with SAVI (see above). Urinalysis for kidney dysfunction and screening for autoimmunity based on the disease symptoms are recommended as kidney disease is reported mostly in patients with AGS[50 62 79] and SAVI.[8 106 107] Antibodies associated with specific autoimmune diseases including autoimmune arthritis, pauci-immune glomerulonephritis, autoimmune cytopenias, thyroiditis and/or hepatitis have been described in CANDLE/PRAAS, SAVI or AGS with variable frequencies (online supplemental table 5). As it remains difficult to diagnose these diseases based on clinical symptoms, regular screening for autoantibodies as outlined in table 4 is currently recommended. Renal pathology prior to treatment with JAKI should be assessed by a baseline renal ultrasound and urine protein/creatinine ratio (or albumin/creatinine ratio).

All patients and families should have access to formal genetic counselling and may require social and other support. Supportive care, including adaptive equipment (eg, orthoses, walkers, wheelchairs, seating equipment), may be required.

Treatment during infections including COVID-19

Disease flares and progression can occur if immunosuppressive treatment is held[108] and disease can flare in the context of an infection. Thus, any patient who develops an acute infection (or other complications) may require adjustment of immunosuppressive treatment (and/or institution of other supportive treatment), which should be conducted only under expert supervision. In line with these suggestions, recently published ACR guidance recommends continuing or initiating immunosuppressants when indicated in patients with paediatric rheumatic diseases in the context of exposure to SARS-CoV-2 or if experiencing asymptomatic SARS-CoV-2 infection. Immunosuppressants may be temporarily delayed or withheld if a patient has symptomatic COVID-19.[109]

Vaccination

Whether vaccination may trigger disease flares in interferonopathies is an important and currently unanswered question. There are no data suggesting that patients with CANDLE/PRAAS and SAVI develop disease flares to routine childhood vaccinations and the Task Force therefore recommended compliance with local regulations when patients are not treated with immunosuppressive treatments or glucocorticoids. No such consensus was achieved for AGS: the safety of vaccines in this population is not fully evaluated, and anecdotal reports of vaccine-induced neurological regression were concerns debated by the Task Force. No specific recommendation on vaccination for AGS was therefore possible. In line with the general EULAR guidance, the Task Force recommends avoiding live vaccines in patients with CANDLE/PRAAS, SAVI and AGS while on treatment with JAKI or other immunosuppressive medications.[110] Treatment discontinuation can result in withdrawal flares. In general, we suggest following recommendations for other autoimmune and inflammatory rheumatic diseases,[110 111] we however currently do not advise treatment adjustments for treatments recommended for the type I interferonopathies including JAKI.

RNA-based SARS-CoV-2 vaccines are not live vaccines, suggesting that they may be safe for immunosuppressed patients. Whether vaccines against COVID-19 have the potential to provoke a disease flare is unknown, theoretical concerns about disease flare in type I interferonopathies caused by RNA vaccines exist. There are currently no data to back specific recommendations.

CONCLUSION

The aim of these points to consider is to address the unmet need to provide guidance for healthcare professionals involved in the care of patients with the recently characterised type I interferonopathies, CANDLE/PRAAS, SAVI and AGS. A lack of high-level evidence is a limitation to these points to consider and reflect the challenges of studying novel, ultra-rare diseases. To address these challenges, the Task Force generated guidance statements based on results from a thorough SLR and on specialists'/experts' opinions where evidence was lacking or was insufficient. The Task Force included various specialists with broad expertise in relevant clinical areas and representing different regions, disease interests and practice environments.

Important areas of future research are outlined in box 1. The cost and availability of genetic testing, interferon signature assays and JAKI treatment are substantial barriers that currently prevent optimised care for patients with interferonopathies. Furthermore, patients with the autoinflammatory interferonopathies CANDLE/PRAAS, SAVI and AGS live in many different

Box 1 Research agenda

- To define autoinflammatory disease outcomes, including:
  - Develop validated remission criteria for each disease including patient reported outcome measures.
  - Develop minimal disease activity criteria.
  - Validate sensitive biomarkers of progression of organ disease (including central nervous system).
- To further assess efficacy of Janus kinase inhibitors (JAKI) and other type I IFN targeted therapies.
- To assess long-term safety with treatment of JAKI.
  - Assess long-term effect of chronic BK viral reactivation.
  - Recommend monitoring guidance including frequency of BK viral loads measurements and management of BK viraemia.
- To assess requirement of viral prophylaxis on JAKI.
- To identify novel therapeutic targets and better treatments.
- To validate an interferon signature to diagnose and monitor patients (eg, number of interferon response genes to include, sensitivity and specificity of score).
- To evaluate the effect of vaccination in triggering or exacerbating disease activity in patients with type I interferonopathies while on or off treatments with immunosuppressive medications and/or glucocorticoids.
- To identify new genetic causes for interferonopathies.

countries and are managed in different healthcare systems. These points to consider address the multiple challenges of managing patients with these ultrarare diseases, by providing guidance on improving clinical recognition, support for decision-making on genetic testing as well as treatment and long-term management. These points to consider were developed to increase awareness of these diseases, and to standardise the level of care by characterising the diagnostic and therapeutic tools that can improve care.

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Correction notice This article has been corrected since it published Online First. The title has been corrected.

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Acknowledgements The Task Force gratefully thanks the librarian Darren Hamilton (London Health Sciences Center, London, Ontario, Canada) for his contribution to the systematic literature search, Brian Feldman, Hayyah Clairman and Natasha Naraidoo for their support of conducting the Delphi questionnaires using the Redcap platform and the European Alliance of Associations for Rheumatology and the American College of Rheumatology for financial and logistical support. This project is part of a series of 'points to consider' consensus efforts to standardise the diagnosis, treatment, and long-term monitoring of patients with the 3 major groups of known autoinflammatory diseases including (1) the IL-1-mediated diseases CAPS, TRAPS, MKD and DIRA, (2) the autoinflammatory interferonopathies CANDLE/PRAAS, SAVI and AGS and (3) the on early diagnosis, treatment, and long-term monitoring of inflammatory conditions with the potential progression to HLH/MAS. This research was supported in part by the intramural research program of the NIH institutes, NIAID (KCG, RGM), NHGRI and NIAMS and by the Deutsche Forschungsgemeinschaft, grant CRC237 369799452/B21 to MAL-K. We would like to acknowledge and are grateful for the generous and invaluable financial and organisational support from the Autoinflammatory Alliance and the systemic JIA foundation. The Autoinflammatory Alliance substantially contributed to an international meeting and workgroup organisation in August 2019 that developed the outline of the points to consider project. The funds for this preceding international meeting were largely due to patient fundraisers, online fundraising and the work of countless volunteers who made this project possible.

Contributors All authors contributed to the formulation of the points to consider. In details, the steering committee of the Task Force (RG-M, PB, AV, BF, ED) defined the research questions for the SLR. A systematic literature review was conducted by KCG, MR, LL with support from a librarian and epidemiologist (DH and DP) under supervision of a senior methodologist (ED). KCG, LL, MR extracted the data. RGM, PB and AV synthesised the results form SLR and Delphi questionnaires and generated draft statements. The manuscript was drafted by KCG, LL and MR and revised by RG-M, PB, AV, ED and BF. DA oversaw the proceedings and provided advice of this points to consider project as EULAR methodologist. All other authors participated in the Task Force meetings, in two pre-meeting Delphi questionnaires, suggested and agreed upon the research questions, read the final statements prior to the manuscript, discussed results and made contributions to the text. All authors approved the final version of the manuscript.

Funding This work was funded by European Alliance of Rheumatology Associations/American College of Rheumatology.

Competing interests EMF received NIH Grant "Clinical Outcomes in Aicardi Goutières Syndrome" (Grant number 5U01NS106845-02) and participated in an advisory board of Biogen. EPH: Spouse employed by Eli Lilly and received stock options in 2019 and 2020. SO1: NIH Grant "Clinical Outcomes in Aicardi Goutières Syndrome" (Grant number 5U01NS106845-02) and participated in advisory board of Biogen. CP received consulting and lecture fees from Novartis. BMF is associate editor of Arthritis and Rheumatology and member of the ACR guidance document

committee. AV received grant support from Eli Lilly. PAB received grants from Roche and consulting and lecture fees and travel support from Roche, SOBI and Novartis. RG-M received study support under government CRADAs from Eli Lilly, IFM and SOBI. The consensus meetings were sponsored as EULAR/ACR projects.

Patient consent for publication Not applicable.

Ethics approval This study does not involve human participants.

Provenance and peer review Not commissioned; externally peer reviewed.

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Call for action in ANCA-associated vasculitis and lupus nephritis: promises and challenges of SGLT-2 inhibitors

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Handling editor Josef S Smolen

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Received 8 September 2021
Accepted 27 October 2021
Published Online First 29 November 2021

To cite: Säemann M, Kronbichler A. *Ann Rheum Dis* 2022;**81**:614–617.

ABSTRACT

Sodium–glucose cotransporter- 2 inhibitors (SGLT- 2i) have recently been demonstrated to exert profound cardio- and nephroprotection in large cardiovascular outcome trials. They reduce progression of chronic kidney disease (CKD) including albuminuria and improve outcomes in heart failure patients with and without type 2 diabetes on top of angiotensin-blocking agents. These benefits translate into improved mortality in cardiorenal risk patients. While the detailed molecular mechanisms underlying these surprising clinical outcomes are not fully understood, their antidiabetic properties are not causative. Rather reduction of glomerular hyperfiltration and tubuloprotection are involved as root cause mechanisms of their clinical effects. Finally, their side effect profile is advantageous especially in non-diabetic patients also reducing the risk of acute kidney injury. Among the independent risk factors for excess mortality, CKD is still one of the strongest predictors of a poor prognosis in patients with both ANCA- associated vasculitis (AAV) and lupus nephritis (LN). Since patients with autoimmune disease were excluded from all recent large renal outcome trials with SGLT-2i and given their strong nephroprotective potential, we herein advocate to study this unique class of disease-modifying therapies when it comes to kidney and cardiovascular health in patients with AAV and LN.

The story of sodium–glucose cotransporter-2 inhibitors (SGLT-2i) is fascinating since they were initially studied as antidiabetic drugs due to their ability to increase glycosuria by blocking sodium and glucose reabsorption in proximal tubules of the kidney. While their antidiabetic efficiency has turned out to be at best modest, initial trials have unexpectedly found potent effects on cardiovascular (CV) morbidity and heart failure (HF) in patients with type 2 diabetes. Specifically, four large CV outcome trials (CVOTs) demonstrated encouraging kidney-specific outcomes, although being primarily designed to assess CV protection in high CV-risk patient populations.[1] Beyond these benefits in CVOTs, the CREDENCE trial included patients with diabetic kidney disease, estimated glomerular filtration rate (eGFR) of 30–90 mL/min/1.73 m^2 and albuminuria. Canagliflozin reduced the risk of the primary composite endpoint (doubling of serum creatinine, terminal kidney disease and renal or CV death) by 30%. Importantly, the risk of end-stage kidney disease (ESKD) was reduced by 32%, as were risks of major adverse CV events and hospitalisation for HF. This dedicated chronic kidney disease (CKD) study complements the DAPA-HF and EMPEROR-REDUCED trials that included patients with HF with reduced ejection fraction with and without diabetes, demonstrating efficacy and safety in cardiorenal patients.[2 3] These data demonstrated that the benefits of SGLT-2i are independent from their antidiabetic effects. The overall robust cardioprotective and nephroprotective efficiency of SGLT-2i has led to the rapid adoption of SGLT-2i as strong therapy recommendation by many international guidelines for patients with diabetes and CKD as well as patients with HF with and without diabetes.[4–6]

A giant step towards our understanding of CKD was the DAPA-CKD trial that included 4304 patients also without diabetes with CKD between eGFR 25 mL/min/1.73 m^2 and 75 mL/min/1.73 m^2 and albuminuria.[7] Patients with vasculitis and lupus nephritis (LN) were excluded due to potential necessities of acute immunosuppression. Patients were randomised to dapagliflozin or placebo in addition to maximum tolerated doses of renin–angiotensin system (RAS) inhibitors. In participants without diabetes, the most common cause of CKD was either glomerulonephritis (GN) or ischaemic/hypertensive nephropathy. DAPA-CKD had to be prematurely stopped, since dapagliflozin reduced the primary endpoint (composite of sustained >50% eGFR decline, ESKD and renal or CV death) by 39% resulting in a number needed to treat of 19. Importantly, no interaction was seen regarding the primary endpoint and diabetes status, and effect size was consistently large for other endpoints like decline in eGFR and overall mortality. Potential SGLT-2i side effects, including diabetic ketoacidosis or hypoglycaemia, were at placebo level while acute kidney injury was reduced. Further analysis of patients with advanced CKD down to a GFR of 25 mL/min/1.73m^2 demonstrated that SGLT-2i were still significantly nephroprotective, while no increased safety signals were observed.[8 9] Of note, the DAPA-CKD trial included many patients with GN, especially with immunoglobulin A (IgA) nephropathy (IgAN). Patients with IgAN displayed a 71% risk reduction for the primary endpoint, including a reduction of albuminuria by 26%,[10] suggesting that specific patients with GN may benefit more from treating CKD than from immunosuppression.[11]

An intense discussion surrounds the causative mechanisms of the cardioprotective and nephroprotective effects of SGLT-2i (figure 1).[12] An initial drop of eGFR after SGLT-2i administration (2–5 mL/min) is detected during the first weeks of treatment reflecting a reduction of intraglomerular pressure. SGLT-2i are considered to alter glomerular haemodynamics via affecting the tubuloglomerular feedback. Under physiological conditions, sensing of tubular electrolytes especially sodium and chloride exerts signals, including

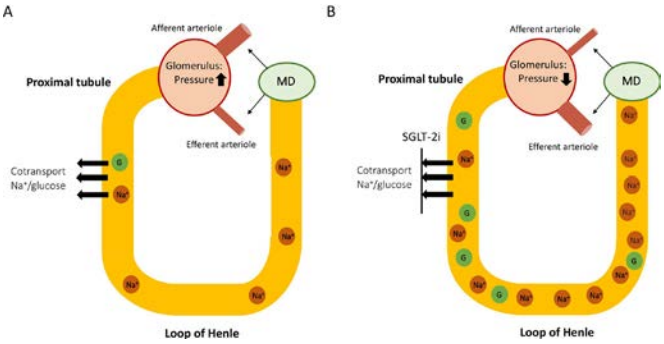

Figure 1 Schematic illustration of the effects of SGLT-2i at the single nephron level. By blocking SGLT-2, reabsorption of both luminal glucose and sodium in the proximal tubuli is blocked. This further leads to enhanced sodium delivery in the distal tubuli that triggers via the MD, the secretion of several mediators, including adenosine. This leads to potential vasoconstriction of glomerular afferent arterioles or vasodilation of the efferent arterioles of the glomerulus impacting glomerular haemodynamics. Thereby, SGLT-2i reduce glomerular hyperfiltration occurring during CKD as part of their nephroprotective potential. Furthermore, by reducing the workload of the proximal tubuli especially under conditions of reduced nephron number, SGLT-2i may further contribute to alleviate kidney damage. CKD, chronic kidney damage; MD, macula densa; SGLT-2i, sodium–glucose cotransporter-2 inhibitors.

adenosine to the afferent and efferent arterioles to influence renal haemodynamics. While tubuloglomerular feedback is distorted especially during glomerular hyperfiltration as it occurs in diabetes and CKD leading to increased glomerular perfusion, SGLT-2i rebalance tubuloglomerular feedback, thereby reducing glomerular hyperfiltration (figure 1). Furthermore, SGLT-2 is centrally positioned within proximal tubules, where most of the demanding work of reabsorption and secretion occurs via energy-dependent transport processes. Hence, SGLT-2i might mitigate metabolic stress of remnant nephrons processing the glomerular filtrate, thereby preserving nephron integrity and reducing CKD progression.[12] Also, other kidney-relevant influences of SGLT-2i have been observed, including natriuresis, osmotic diuresis (decreased interstitial fluid overload), anti-inflammatory and antifibrotic effects and increased erythropoietin production. The clinical relevance of these effects is, however, unclear. Effects pertinent to cardioprotection could be driven to a substantial degree by those linked to nephroprotection, while distinct haemodynamics affecting, for example, cardiac preload and afterload are under investigation.[13]

In general, patients with ANCA-associated vasculitis (AAV) experience after their first year of diagnosis an increased long-term mortality risk compared with the age-matched and sex-matched general population, while CV disease remains the most important cause of death besides malignancy and infection.[14–16] Apart from the inflammatory nature of the disease itself, including endothelial dysfunction and arterial stiffening, also long-term effects of immunosuppressive treatment, especially if poorly controlled, significantly contribute to the heightened CV risk in patients with AAV.[17 18] Among the independent risk factors for excess mortality, CKD remains one of the strongest predictors of a poor prognosis. Hence, patients with AAV and kidney involvement clearly have a significantly increased risk of CV morbidity and mortality as part of the inherent association of CKD with increased CV risk.[19] Furthermore, several unique pathophysiological features affecting the cardiorenal axis occur more frequently in patients with AAV such as diastolic dysfunction and pulmonary hypertension along with reduced systolic function that are all clearly positively impacted by SGLT-2i.[20 21] Patients with AAV and kidney involvement would be perfectly suited to benefit from the nephroprotective properties of SGLT-2i, once the initial phase of induction immunosuppression is completed and kidney function along with the overall clinical situation of the patient has stabilised. Currently, trials are in the set-up phase to test dapagliflozin in AAV, such as DAPA-vasculitis.

CV morbidity and mortality are also substantially increased in patients with systemic lupus erythematosus (SLE) and CV disease is the most common cause of mortality in SLE, followed by infection and severity of disease activity.[22–24] As with AAV, both systemic inflammation as well as the cumulative dose of immunosuppressants influence the long-term CV risk. Hence, while significant advances have been made in the treatment of SLE and especially LN, the heightened mortality in patients remains a major concern in management, especially for patients with LN.[25 26] Among CV and renal risk factors such as arterial hypertension, which is occurring in most affected patients,[27] even patients with SLE with mild disease experience a significantly increased CV mortality risk.[28] All patients with LN have by definition CKD, since they display albuminuria to varying degrees. While albuminuria is a classical sign of kidney damage, a substantial portion of patients will also have structural and functional impairment of their kidney function as hallmark of CKD, that is, glomerular hyperfiltration and albuminuria. In the past, RAS blockade has already conferred nephroprotective potential in patients with LN; however, a substantial residual renal risk remains in all forms of CKD.[29 30] Since CKD is per se one of the strongest CV risk factors, any manoeuvres to prevent CKD progression, including reduction of albuminuria and prevention of eGFR decline, will likely have profound influences on patient outcomes. Furthermore, distinct complications of SLE may also seem to be amenable to the therapeutic potential with SGLT-2i such as the increased occurrence of pulmonary hypertension, metabolic syndrome and increased blood pressure.[24 31]

Principal nephroprotective strategies in AAV and LN relied hitherto on (1) aggressive blood pressure control, (2) reduction of albuminuria, especially with RAS inhibitors, (3) optimising lifestyle, including cessation of smoking, treatment of metabolic syndrome/diabetes as well as increased exercise and (4) avoiding nephrotoxic drugs. At advanced CKD stages, addressing of CV risk factors is also important, including dyslipidaemia, anaemia and secondary hyperparathyroidism.[19] Management of AAV and LN goes beyond immunosuppressive and anti-inflammatory treatment, but also involves a coordinated approach towards nephroprotection and CV risk reduction as these patients are cardio-reno-metabolic risk patients especially when kidney involvement has already occurred. SGLT-2i exert unequivocal cardioprotective and nephroprotective effects by reducing albuminuria and impacting eGFR decline by affecting unique CKD pathophysiology such as glomerular hyperfiltration and tubular workload along with significantly reducing the incidence of acute kidney injury. These profound clinical effects suggest that SGLT-2 inhibition is an ideal therapeutic avenue for patients with AAV and SLE especially when signs of heart or kidney damage have already manifested. Importantly, an advantageous safety profile has been established in cardiorenal patients, possibly due to diminished glycosuria in CKD. Finally, metabolism of SGLT-2i is via simple hepatic glucuronidation and no interference occurs with P450 enzymes or P-glycoprotein pathways via which most immunosuppressive agents are metabolised.

The DAPA-CKD trial significantly changed our view of CKD therapy, since CKD with all its diverse aetiologies ranging from

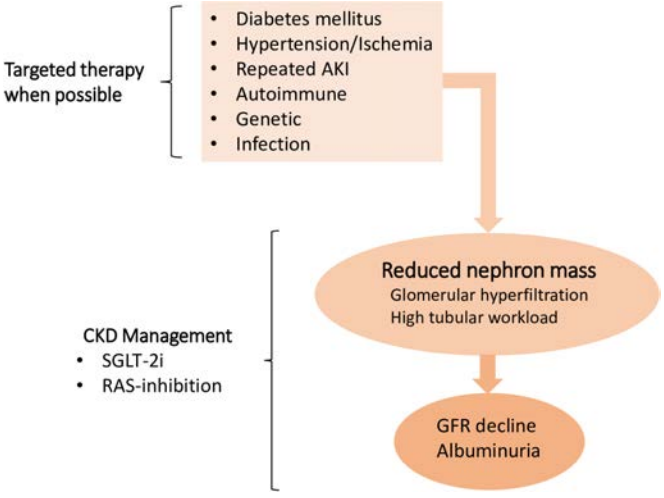

Figure 2 Modern concept of CKD management. While nephron loss is the structural and functional hallmark of CKD resulting in glomerular hyperfiltration and excessive tubular workload of the remaining nephrons, the primary aetiologies leading to a reduced nephron mass are highly divergent and only some of them are potential targets of specific therapy. Patients with CKD (reduced GFR and albuminuria) should have potent nephroprotective therapies such as SGLT-2i and inhibitors of the RAS affecting both hyperfiltration and tubular capacity as part of their CKD management, thereby reducing CKD progression and CV risk. CKD,chronic kidney disease; GFR, glomerular filtration rate; RAS, renin–angiotensin system; SGLT-2i, sodium–glucose cotransporter-2 inhibitors.

diabetes and hypertension to several forms of GN should be primarily seen as a unique form of organ dysfunction that can be successfully treated (figure 2). For example, in slowly progressing CKD like in IgAN, the benefit-to-harm ratio of immunosuppression could be too small compared with the profound nephroprotection exerted by SGLT-2i.[11] Therefore, future CKD trials even with specific therapies will have to implement SGLT-2i as standard therapy due to their strong effect size on CKD progression.

The clinical promises of SGLT-2i obtained from large clinical trials and real-world evidence can only be realised if SGLT-2i are rapidly implemented in clinical practice. Establishing novel treatments is naturally a slow process and even adoption of RAS inhibitors in patients with CKD is even at present far from satisfying. While prospective controlled trials with hard outcomes are urgently required to establish firm evidence in patients with AAV and LN, we envision the adoption of SGLT-2i as part of an integral CKD management strategy in these patients addressing their CKD apart from their original disease. Future trials will have to study the ideal time of initiation of SGLT-2i therapy: hence, should SGLT-2i be administered when first signs of kidney damage are detected such as small amounts of albuminuria or when nephron loss has already occurred or should they even be part of a regular cardioprotective and nephroprotective standard therapy such as RAS inhibitors in afflicted patients?

Given both excellent safety profile and with considerable cardioprotective and nephroprotective potential, SGLT-2 inhibition as simple and cheap therapeutic strategy could ultimately turn out to exert not only organ protection but also increase health and life span of afflicted individuals by reducing their overall CV risk.

Contributors MS drafted the first version of the manuscript. AK revised the manuscript and approved the final version.

Competing interests MS has received honoraria for consulting and lectures from Astra Zeneca, Bayer, Boehringer-Ingelheim, Novartis, Otsuka and Vifor Pharma. AK has received research grants from Otsuka and Vifor Pharma, honoraria for lectures from TerumoBCT and Vifor Pharma and consulting fees from UriSalt and Catalyst Biosciences.

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 applicable.

Provenance and peer review Commissioned; externally peer reviewed.

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Marking the 50th anniversary of a seminal paper in rheumatology: did Baruj Benacerraf and Hugh McDevitt get it right?

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Received 7 January 2022
Accepted 10 February 2022
Published Online First 2 March 2022

To cite: Rosenbaum JT, Gill T, Martin TM, *et al*. *Ann Rheum Dis* 2022;**81**:618–621.

Great scientific advances provide novel insight while simultaneously creating a platform to ask additional questions.

In January 1972, in *Science*, Baruj Benacerraf and Hugh McDevitt summarised their remarkable discovery of major histocompatibility complex (MHC)-related immune response genes[1] (figure 1). To label their work as a paradigm shift in rheumatology is wholly appropriate.

In their essay, the authors wrote, 'there is considerable reason to believe that this type of genetic control of specific immune responses may play an important role in susceptibility to a variety of diseases in both animals and man.'[1] They specifically speculated about immune response genes and 'the frequency of autoantibodies in a number of clinical diseases, such as rheumatoid arthritis, systemic lupus erythematosus, thyroiditis, and other autoimmune diseases'.[1] The hypothesis that human leukocyte antigen (HLA) molecules (the human version of MHC) influence susceptibility to immune-mediated disease was validated about 15 months later by two independent studies, both showing that the HLA antigen now known as HLA-B27 profoundly affected susceptibility to ankylosing spondylitis.[2] Benacerraf and McDevitt were writing in an era that preceded the conceptualisation of immune-mediated diseases as a spectrum ranging from truly autoimmune to autoinflammatory. Their essay went beyond autoimmunity by also recognising that MHC molecules could be exerting control over the immune response to a foreign antigen. For example, they cited work showing that mouse susceptibility to a leukaemia virus was under the control of the MHC.

The Nobel Prize in Physiology or Medicine in 1980 was awarded to Jean Dausset, George D Snell and Baruj Benacerraf. Dausset was the first to identify MHC molecules on cells in peripheral blood.[3] Snell recognised the mouse MHC as early as 1951 for its role in transplant rejection.[4] He did pioneering mouse breeding studies that proved essential for the mapping of the genes within the MHC. Working with guinea pigs, Benacerraf (figure 2) and colleagues, who included Bill Paul and Ira Green, showed that immune responses were under genetic control.[5] But it was McDevitt (figure 3) and colleagues like Michael Sela, who collaborated with McDevitt when he was studying at Mill Hill in London[6] or Len Herzenberg, with whom he collaborated at Stanford,[7] who were able to identify the MHC as the location for genes controlling the immune response. Many felt that McDevitt's omission by the Nobel committee was a serious oversight (Leslie Brent, The Guardian, 2 October 2011, 'Letter: Baruj Benacerraf obituary').

McDevitt and Benacerraf travelled different paths to arrive at their seminal insights. Hugh McDevitt grew up in Wyoming, Ohio just outside of Cincinnati (Stanford Historical Society, oral history project, interview with Kim Smuga-Otto, 2015). His father was a general surgeon who was chagrined that Hugh's four older siblings elected not to go into medicine. So, beginning in third grade, his father began to take Hugh on morning rounds. On Sunday, this was a special treat because it enabled him to miss church. While his father would chat in a patient's room, the charming scientist-to-be was left in the nurse's station where he was happily plied with chocolates. Dad tried to inspire Hugh with medical stories including one about Paul Ehrlich who had won the Nobel in 1908. Ehrlich's 'side chain theory' that cells have receptors to detect signals from other cells might be considered the forerunner of McDevitt's work on the MHC and antigen presentation. And destiny was fulfilled in 1987 when McDevitt won Germany's foremost award for medical research, the Ehrlich-Darmstaedter Prize.

Baruj Benacerraf was born in Caracas, the son of Sephardic Jewish parents who had immigrated to Venezuela from North Africa. The family, which owned highly successful import, banking and textile businesses, moved to Paris when Baruj was 5, but returned to Venezuela in 1939 in time to avoid the Nazi reign over France. One year later, Benacerraf moved to New York City for his undergraduate studies at Columbia University. In his Nobel acceptance speech, he recounts that he was accepted to only one medical school, the Medical College of Virginia. He attributed this to his foreign birth and Jewish heritage, both obstacles to gaining medical school admission in that era. While McDevitt's father tried to persuade his son to be a physician, Benacerraf 's father tried to dissuade his son from that pursuit. The family business was the father's preference. Benacerraf passed away in 2011, having enjoyed 68 years of marriage. He and his wife were said to be nearly inseparable. In the morning, before Baruj could turn his attention to his passion in the lab, his wife would bring him the letters and paperwork which he needed to complete so that his corporate interests could continue to thrive.

Just as Watson and Crick in 1953 inspired generations of molecular biologists with their insightful comment: 'it has not escaped our notice that the

Histocompatibility-Linked Immune Response Genes

A new class of genes that controls the formation of specific immune responses has been identified.

Baruj Benacerraf and Hugh O. McDevitt

Figure 1 The title of a seminal paper by Benacerraf and McDevitt as it appeared in *Science*, 21 January 1972.

specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material',[8] so the concept of HLA and disease has inspired thousands of investigations.

Fifty years after their seminal paper, however, there remains a dearth of evidence that the human MHC controls the immune response to a self-antigen. Maybe it is time to reconsider the mechanism by which HLA molecules predispose to autoimmune disease?

One paradigm that would seem ideal to clarify the mechanism by which HLA molecules influence disease pathogenesis would be adverse reactions to medications. Such a reaction has a discrete trigger and a discrete time course. Indeed, HLA molecules are closely linked to an array of adverse drug reactions.[9] Probably the best studied is the connection between HLA*B57:01 and abacavir-induced hypersensitivity reaction in the treatment of HIV infection; the phenomenon has not been shown to occur in the absence of HLA*B57:01.[10] One hypothesis, which might apply to some drug reactions and not others, is that the medication haptenates a protein and it is the immune response to the hapten that induces disease. In the case of abacavir, the best explanation to date is that the medication affects the binding groove such that the repertoire of immunogenic molecules is shifted[9] (figure 4A).

Gluten-sensitive enteropathy is an example of a disease in which an HLA allele impacts the immune response to a foreign protein. HLA-DQ2.5 predisposes to sprue while HLA-DQ2.2 does not. The gluten-derived peptides presented by DQ2.5 are strikingly different from the peptides presented by DQ2.2[11] and the magnitude of the T cell response to HLA-presented peptides correlates with the likelihood of disease. In spondyloarthritis, an HLA-B27 subtype that predisposes to disease can differ from a non-predisposing subtype by a single amino acid in the peptide-binding groove.[12] As in sprue, the small structural change alters the peptide repertoire.[12] However,

Figure 2 Photograph of Hugh McDevitt. Source: Grete Sonderstrup, Hugh's wife.

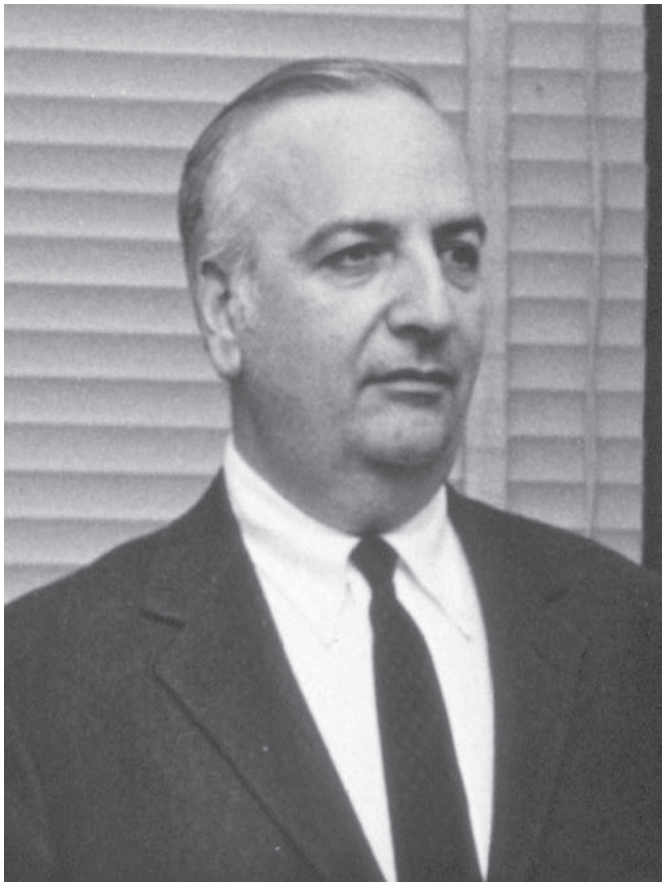

Figure 3 Photograph of Baruj Benacerraf, source: Wikimedia.

unlike coeliac sprue, no corresponding self-antigen or foreign antigen has been convincingly identified in spondyloarthritis.

An immune response to self-antigens can be pathogenic. These autoantigens include cyclic citrullinated peptides in rheumatoid arthritis (RA), double-stranded DNA in systemic lupus, the thyroxine receptor in Graves' disease, the acetylcholine receptor in myasthenia gravis, and proteinase-3 or myeloperoxidase in granulomatosis with polyangiitis. Although each of these diseases has an HLA association, to our knowledge no study has linked the predisposing HLA allele to an immune response to the autoantigen (figure 4B). As we have had 50 years of opportunity to establish this connection, we suggest that it is an appropriate time to test additional hypotheses. Some possibilities include linkage to other genes encoded within the MHC, or in the case of spondyloarthritis, the ability of HLA-B27 to dimerise on the cell surface and activate natural killer (NK) cells, or the propensity of HLA-B27 to misfold and thus activate the unfolded protein response[13] (figure 4C,D). We propose that a viable possibility is that many HLA alleles predispose to disease by virtue of their effect on the immune response to the microbiome (figure 4E).

The microbiome refers to the collection of micro-organisms which cohabit a species. In humans, this ecosystem includes bacteria, viruses, archaea, fungi and sometimes other parasites which reside in the gastrointestinal tract predominantly. The microbiome has a profound effect on the immune system and its function. If a mouse is delivered sterilely through a caesarean section and reared germ-free such that bacteria never colonise the gut, the immune system is relatively impoverished with underdeveloped lymph nodes and spleen and a limited ability to make an immune response to a foreign antigen.[14] Some have argued that the gut is the largest organ in the immune system. Geva-Zatorsky and colleagues[15] identified 53 different enteric bacterial species which affected the mouse immune

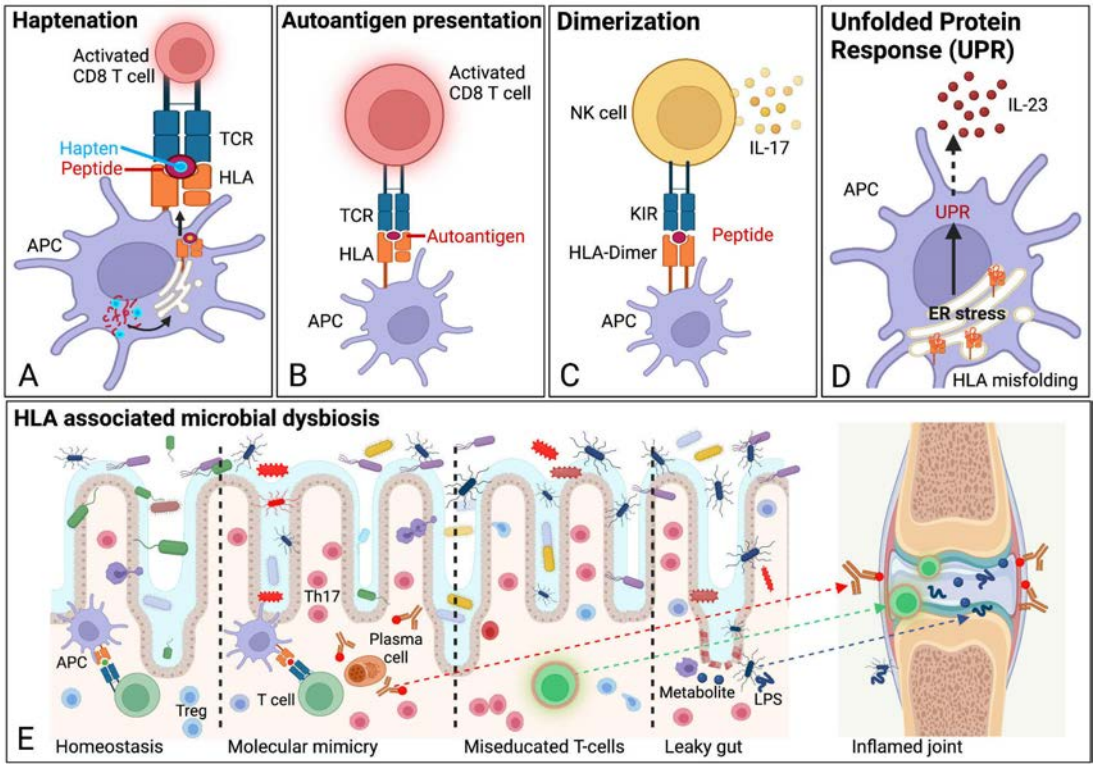

Figure 4 Examples of mechanisms to account for HLA-disease associations. This figure has been drawn using BioRender software. The figure illustrates several of the mechanisms by which HLA might predispose to immune-mediated disease. (A) Some medications could potentially haptenate a peptide and render it immunogenic; (B) the HLA allele could present an antigen to a T cell to trigger an immune response. The illustration shows a class I HLA molecule presenting to a CD8 T cell but the presentation could also arise from an HLA class II molecule presenting to a CD4 T cell. The illustration shows an autoantigen as could occur in theory, but the HLA molecule could alternatively present a foreign antigen as is believed to occur with peptide derived from gluten in coeliac sprue; (C) the HLA allele could dimerise on the cell surface to result in activation of NK cells as is thought to occur with HLA-B27; (D) instability of the HLA molecule within the cell could trigger the unfolded protein response as has been proposed for HLA-B27; (E) the antigen presented by HLA could be derived from the microbiome. Such an immune response should perturb the normal homeostasis of the microbiome and result in a dysbiosis, which in turn could cause disease through molecular mimicry; miseducated T cells that migrate to a target organ; a leaky gut that allows bacterial products like cell wall or metabolites like inosine to escape the bowel; and/or a change in a vital immune cell population such as the number of T cells synthesising interleukin-17 or the number of regulatory T cells. Certainly, combinations of these mechanisms could also be at play. APC, antigen presenting cell; ER, endoplasmic reticulum; IL, interleukin; KIR, killer-cell immunoglobulin-like receptor; LPS, lipopolysaccharide; NK, natural killer; TCR, T-cell receptor; Th17, Helper cell that synthesizes interleukin-17; Treg, regulatory T-cell.

system if introduced as unique bacteria into germ-free mice. Like prior studies, this report[15] implicated bacteria in a range of immunological effects including impacting the number of T cells that synthesise interleukin-17 and the number of T cells that have a regulatory or suppressive effect.

The microbiome is an ecosystem such that perturbation of a single species will create a ripple effect that alters the population of multiple species.[16] The unparalleled polymorphism of the HLA system has been suggested to protect species' survival during a pandemic.[17] The diversity of bacteria within the intestine would seem to guarantee that a specific HLA allele would impact the ecosystem within the bowel. The effect of the MHC on the intestinal microbiome has been shown in rodent models[18 19] and in humans as well.[20–23] A change in the intestinal microbiome that results in disease is labelled a dysbiosis. A dysbiosis has been described for virtually every immune-mediated rheumatic disease including RA,[24] systemic lupus erythematosus,[25] ankylosing spondylitis,[26] psoriatic arthritis[27] and scleroderma.[28] Some argue that the change in the microbiome could be secondary to intestinal pathology rather than secondary to the MHC. We have demonstrated, however, that healthy individuals without known bowel pathology but with alleles such as HLA-B27,[20] HLA-A29[21] or HLA-DRB1[20] have an altered microbiome. Factors such as diet and geography presumably affect the intestinal microbiome to a greater extent than the MHC. We contend that even a subtle alteration in the microbiome could be an important factor that contributes to disease.

A change in the microbiome could, of course, be an epiphenomenon rather than causally related to disease. However, in rodent models such as those that resemble spondyloarthritis, germ- free animals are almost completely protected from gut inflammation.[29] Furthermore, faecal microbiome transplantation has therapeutic benefit as has been shown, for example, in ulcerative colitis.[30] In a faecal transplant study on patients with psoriatic arthritis,[31] patients who received a mock transplant fared better than patients receiving a faecal transplant. While these results are clinically disappointing, they confirm the potential of the microbiome to influence inflammatory disease activity.

We have previously proposed that the gut microbiome could be causally related to ankylosing spondylitis.[32] Once a dysbiosis occurs, multiple mechanisms could contribute to disease,[33] for example, the dysbiosis could create a leaky gut which allows bacteria or bacterial products to escape the intestine. This permeability could allow bacterial cell wall to deposit at a site such as synovium as has been shown for RA[34] or reactive arthritis.[35] Gut bacteria, as noted above, affect major aspects of the immune system such as regulatory T cell populations. A bacterially derived peptide could mimic a self-epitope to stimulate an autoimmune response. This is the presumed

pathogenesis for rheumatic fever[36] or Guillain-Barre syndrome.[37] Finally, lymphocytes might be educated in the gut and then circulate to other organs where their 'education' might induce them to cause disease.

At the American College of Rheumatology (ACR) annual meeting in 2021, Chriswell and colleagues presented experimental data relevant to RA and in strong support of the above hypothesis (Chriswell, M. *et al*., Plasmablast-autoantibodies from individuals at risk for RA that target RA-relevant antigens are polyreactive with arthritogenic bacteria, ACR annual meeting, 2021, abstract 0461). This group studied individuals at risk of RA. They described antibodies that reacted to a bacterial isolate of *Ruminococcaceae subdoligranulum* and cross-reacted to autoantigens. This bacterium stimulated lymphocytes and this stimulation could be blocked by an antibody to HLA-DR4. Furthermore, intestinal colonisation of germ-free mice with *R. subdoligranulum* proved arthritogenic and induced autoantibodies.

Benacerraf and McDevitt truly did 'get it right'. Presciently they predicted that genes of the MHC would influence susceptibility to immune-mediated disease. However, in 50 years of inspiration from their insights, the world of rheumatology is still looking for evidence that HLA molecules directly stimulate an immune response to an autoantigen. We suggest a potentially indirect mechanism worthy of further exploration: that the effects of HLA molecules on the intestinal microbiome may predispose to autoimmune disease.

Contributors JTR conceived the idea for this report and wrote the first draft. TG created the figure. All authors contributed to the critical reading and revision of the report.

Funding This work was supported by NIH Grant NEI RO1 EY029266, NCATS KL2R002370 and P30 EY010572; the Oregon Health & Science University Department of Medicine Wheels Up Award; the Medical Research Foundation of Oregon; and donations from the Stan and Madelle Rosenfeld Family Trust, the Grandmaison Fund for Autoimmunity Research, the William and Mary Bauman Foundation, and Research to Prevent Blindness. TG is the recipient of the Jane Bruckel Early Career Investigator Award from the Spondylitis Association of America.

Competing interests JTR consults for Abbvie, Novartis, Gilead, UCB, Corvus, Revolo, Neoleukin, Santen, Affibody and Horizon. He receives grant support from Horizon, the Malassezia Foundation and Pfizer. He receives royalties from UpToDate. He serves on a data monitoring board for Celgene-BMS. MF consults for Revolo.

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.

Provenance and peer review Not commissioned; externally peer reviewed.

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CLINICAL SCIENCE

Baricitinib further enhances disease-modifying effects by uncoupling the link between disease activity and joint structural progression in patients with rheumatoid arthritis

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Handling editor David S Pisetsky

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221323).

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Received 10 August 2021
Accepted 1 February 2022
Published Online First 22 February 2022

To cite: Lopez-Romero P, de la Torre I, Haladyj E, *et al*. *Ann Rheum Dis* 2022;**81**:622–631.

ABSTRACT

Objectives To evaluate if baricitinib, a Janus kinase inhibitor, further enhances disease-modifying effects by uncoupling the link between disease activity and structural damage progression in patients with rheumatoid arthritis (RA) using two phase III randomised, double-blinded trials.

Methods In RA-BEAM, patients with established RA and inadequate response to methotrexate (MTX-IR) received placebo (PBO), baricitinib 4 mg or adalimumab 40 mg on background MTX. In RA-BEGIN, conventional synthetic disease-modifying antirheumatic drug (csDMARD)-naïve patients received MTX, baricitinib 4 mg or baricitinib 4 mg plus MTX. Using linear regression analyses, joint damage progression (assessed by change from baseline in van der Heijde modification of the Total Sharp Score) was compared between treatment groups for patients achieving certain disease activity states by the Clinical Disease Activity Index. Time-averaged postbaseline responses were used to week 24 (RA-BEAM) and week 52 (RA-BEGIN).

Results For MTX-IR patients, structural damage progression was reduced regardless of disease activity states in baricitinib-treated patients (p=0.6), whereas in PBO patients there was a clear dependence on disease activity states, being significantly lower in those who achieved remission/low disease activity (REM/LDA) compared with moderate/high disease activity (MDA/HDA) (p=0.02). Furthermore, the baricitinib MDA/HDA group had less damage progression than the PBO MDA/HDA group (p<0.001). For csDMARD-naïve patients, progression was lower in REM/LDA versus MDA/HDA within the MTX group (p<0.001). However, for baricitinib+MTX (p=0.5) or baricitinib monotherapy (p=0.07), progression was similar regardless of disease activity. In MDA/HDA groups, progression was lower with baricitinib+MTX (p<0.001) and numerically lower with baricitinib monotherapy (p=0.07) versus MTX. C reactive protein (≤5 mg/L and >5 mg/L) sensitivity analyses supported the primary findings.

Conclusions Baricitinib reduces structural damage progression versus PBO with background MTX and/or MTX, even in patients with MDA/HDA, showing a disease-modifying effect across all disease activity states.

Key messages

What is already known about this subject?

- In patients with rheumatoid arthritis, tumour necrosis factor inhibitors, interleukin 6 inhibitors and rituximab have been shown to uncouple the link between disease activity and radiographic progression such that patients are protected from structural damage progression even if remission/low disease activity (REM/LDA) is not achieved.

What does this study add?

- In two distinct populations of patients with rheumatoid arthritis (patients naïve to conventional synthetic disease-modifying antirheumatic drugs or with inadequate response to methotrexate (MTX)), either baricitinib alone and/or in combination with MTX enhanced disease-modifying properties by uncoupling the link between disease activity and structural damage progression, with the uncoupling being more evident for baricitinib in combination with MTX.
- In the baricitinib groups, joint damage was controlled regardless of disease activity level, unlike in the control groups.
- Patients with residual moderate or high disease activity who received baricitinib with background MTX or in combination with MTX had less structural damage progression than the control groups (MTX or placebo with background MTX).
- Validation analysis showed a similar uncoupling of inflammation and structural damage progression when patients were stratified by high-sensitivity C reactive protein.

INTRODUCTION

The high propensity to destroy cartilage and bone constitutes a major hallmark of rheumatoid arthritis (RA). While it appears that over the last two decades progression rates of joint damage have declined,[1] patients with established disease entering clinical trials still have high baseline radiographic scores, implying aggressively damaging RA.[2] Indeed, joint damage shows a significant positive association with both swollen joint counts and acute phase reactant levels.[3–8] Joint swelling or synovitis characterises the local and acute phase reactants reflect the systemic inflammatory response, which usually go

Key messages

How might this impact on clinical practice or future developments?

- The uncoupling of disease activity and structural damage progression by baricitinib, a Janus kinase inhibitor, provides evidence which was not previously available for this mechanism of action.
- Preservation of structural progression regardless of disease activity might ensure medium-term to long-term prevention of disability in patients who cannot achieve REM/LDA or require more time to reach this target.
- This may inform treatment decisions in patient groups who have not, or have not yet, achieved sufficient clinical improvement.

hand in hand, but joint swelling is more strongly associated with progression of joint damage than the acute phase response.[4 9]

Effective treatment with conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) reduces the rate of structural progression and ultimately halts joint structural damage accrual once stringent remission is achieved and sustained afterwards.[5 10–13] However, while joint damage progression is reduced on csDMARDs, structural deterioration still continues to occur in correlation with residual disease activity.

Several years ago, it was observed that the strong relationship between the extent of structural changes and inflammatory disease activity was blunted with infliximab use, a tumour necrosis factor α (TNF) inhibitor.[14] In contrast to patients continuing methotrexate (MTX) treatment, who showed a linear progression of structural damage in relation to their disease activity state, patients who received infliximab plus MTX would not progress significantly, even if their disease activity remained moderate or high.[15] Similar results were observed for other TNF inhibitors in later studies.[16 17]

The reason for the disruption of the tight link between disease activity and joint damage may be ultimately explained by a threshold effect: activation of osteoclasts, which induce bone erosions, requires higher TNF concentrations than induction of the inflammatory response. Therefore, when this cytokine is blocked to a level that still allows an unmitigated perpetuation of synovitis, this reduction may still suffice to not allow continued activation of osteoclasts, a major mediator of joint destruction.[18]

Interestingly, this interference with structural progression despite high disease activity is not confined to TNF inhibitors, but was also found for interleukin 6 (IL-6) inhibition and rituximab, usually combined with MTX.[19 20] This suggests that biological (b)DMARDs reduce the inflammatory load of cytokines like TNF and IL-6 to a much larger extent than csDMARDs alone and thus downregulate the amplifying activity of these messenger molecules on osteoclastogenesis.[21]

Janus kinase (JAK) inhibitors (Jakinibs) belong to the most recent class of targeted synthetic (ts)DMARDs, which have shown at least similar efficacy as bDMARDs.[22] JAKs are needed for signal transduction of various cytokine receptors, including those for IL-6 and interferons,[23] but not TNF or most B cell receptors. Baricitinib, a Jakinib widely approved for treating RA, is a JAK1 and JAK2 inhibitor which has shown efficacy across RA subsets[24–26]; this efficacy includes structural damage inhibition. However, knowledge on structural damage reduction beyond the decrease in disease activity is limited. Indeed, when patients with poor prognostic factors were assessed for joint damage progression, another Jakinib, tofacitinib, failed to show a significant reduction compared with bDMARD.[27]

This question is the focus of research addressed in the present study.

METHODS

Study design

In the 52-week RA-BEAM trial, adult patients with active RA (≥6/68 tender joints, ≥6/66 swollen joints and a high-sensitivity serum C reactive protein (hsCRP) ≥6 mg/L) and no previous bDMARD therapy were included.[25] Patients had either ≥3 joint erosions or ≥1 joint erosions plus seropositivity for rheumatoid factor or anticitrullinated peptide antibodies (ACPA). Patients had an inadequate response to MTX (MTX-IR) and received background MTX throughout the study. Randomisation was 3:3:2 to placebo (PBO) (n=488), baricitinib 4 mg (n=487) and adalimumab 40 mg (n=330).

In RA-BEGIN, adult patients with active RA who had received no/limited treatment with csDMARDs (up to 3 weekly doses of MTX allowed) and no treatment with bDMARDs were included.[24] Patients had ≥6/68 tender joints and ≥6/66 swollen joints and serum hsCRP level ≥3.6 mg/L and were seropositive for rheumatoid factor or ACPA. Randomisation was 4:3:4 to MTX (n=210), baricitinib 4 mg (n=159) and baricitinib 4 mg plus MTX (n=215) for 52 weeks.

Outcome measures

Joint damage progression was assessed using the van der Heijde modification of the Total Sharp Score (mTSS).[28 29] Two independent readers, who were unaware of the chronological order, patient identity and treatment group, scored the radiographs and the mean score between the readers was used.

Disease activity was assessed by the Clinical Disease Activity Index (CDAI), stratified by the states of remission/low disease activity (REM/LDA; CDAI ≤10) and moderate/high disease activity (MDA/HDA; CDAI >10). Systemic inflammation was assessed by hsCRP stratified at ≤5 mg/L and >5 mg/L.

Analyses populations

In RA-BEAM, analyses were performed for endpoints at week 24 to allow comparison with the PBO group; at week 24 all patients randomised to PBO were switched to baricitinib 4 mg and therefore analyses at week 52 were not done. Analyses in RA-BEGIN were done for endpoints at week 52 because in this study patients who were randomised to MTX were followed up until week 52, unless they were rescued or discontinued from the study, and thus baricitinib could be appropriately compared with this control population in the longer term.

Only completers of the relevant study endpoint were included, excluding patients who switched treatment, were rescued or lost to follow-up before the time point defined for analysis. In RA-BEAM, 329, 427 and 273 patients in the PBO, baricitinib and adalimumab groups, respectively, were defined as completers. Patients with missing structural data and/or missing data on the covariates used in the models were also excluded from the analysis. Thus, 318, 407 and 262 patients, respectively, were included in the CDAI analysis in RA-BEAM. In RA-BEGIN, 142, 129 and 170 patients in the MTX, baricitinib monotherapy and baricitinib+MTX groups, respectively, were defined as completers. After excluding patients with missing data, 134, 125 and 166 patients, respectively, in these groups were included in the CDAI analysis.

Statistical analysis

Analyses of change from baseline in mTSS in RA-BEAM and RA-BEGIN have been done stratifying by treatment and by postbaseline CDAI response at week 24 and week 52, respectively. Adjusted means for the mTSS change from baseline were estimated using linear regression models adjusted by baseline CDAI. Adjusted means for change from baseline in mTSS responses are displayed as effects plots. The adjusted means were estimated from the multivariable models, with continuous covariates fixed at their mean values and categorical covariates fixed at their proportional distribution in the data.

As sensitivity analyses, similar analyses of change from baseline in mTSS were done stratifying by hsCRP. Adjusted means for the mTSS change from baseline, stratified by treatment and by postbaseline hsCRP response at week 24 in RA-BEAM and week 52 in RA-BEGIN, were estimated using linear regression models adjusted by baseline hsCRP.

Change from baseline in CDAI at week 24 in RA-BEAM and week 52 in RA-BEGIN in patients with postbaseline CDAI >10 was estimated using linear regression analyses (observed values). Similar analyses were undertaken for hsCRP in patients with elevated postbaseline hsCRP.

For both CDAI and hsCRP postbaseline responses, individual time point responses were averaged until the end of the period of analysis (week 24 or week 52) to define the response at the specific time point of analysis. This analytical process tends to diminish the potential influence of extreme values, especially for hsCRP, and also reduces the number of patients with missing data at individual endpoint visits. The definition of disease activity and hsCRP response using a time-averaged CDAI and hsCRP has been used previously.[16]

RESULTS

Baseline demographics and patient characteristics

Baseline demographics and patient characteristics have been published previously.[24 25] Summaries of these data for the analyses populations are presented in table 1 (CDAI response analyses) and table 2 (hsCRP sensitivity analyses). The characteristics of the different patient populations at baseline were mostly similar. However, reflective of the different patient populations in the two trials, patients in RA-BEGIN (early RA naïve to csDMARDs) had a shorter disease duration and had lower mTSS scores than patients in RA-BEAM (established RA and MTX-IR).

Treatment response in relation to disease activity

In RA-BEAM, 19.2% (n=61), 37.4% (n=98) and 42.0% (n=171) of patients in the analysed population who received PBO, adalimumab and baricitinib, respectively, achieved REM/LDA. Heatmap plots show individual CDAI longitudinal responses at all postbaseline visits for all patients included in the analyses (figure 1), differentiating REM/LDA and MDA/HDA CDAI responses at week 24.

In RA-BEGIN, at week 52, the proportion of patients in REM/LDA was 39.6% (n=53), 57.6% (n=72) and 62.7% (n=104), respectively, in the MTX, baricitinib monotherapy and baricitinib+MTX groups. Figure 2 displays the heatmap plots showing individual responses to treatment over time for patients stratified by CDAI responses at week 52.

Structural damage progression in relation to disease activity in RA-BEAM (MTX-IR patients with established RA)

Among those achieving REM/LDA, patients who received PBO, adalimumab and baricitinib had adjusted means for mTSS change from baseline of 0.31, 0.15 and 0.24, respectively, compared with 0.84, 0.28 and 0.34, respectively, among patients in these groups with MDA/HDA (figure 3A). Thus, in the PBO group, patients achieving REM/LDA had less structural damage progression than patients with MDA/HDA (adjusted mean difference (95% CI): −0.53 (−0.98 to –0.09), p=0.02). In contrast, there was no significant difference in structural damage progression depending on disease activity states within patients receiving baricitinib (−0.09 (−0.41 to 0.22), p=0.6) or adalimumab (−0.13 (−0.53 to 0.26), p=0.5). Given that those with MDA/HDA did not have greater progression than those with REM/LDA, this reveals an uncoupling of disease activity and structural damage progression with baricitinib treatment.

Among patients with MDA/HDA, the adjusted mean difference (95% CI) for adalimumab compared with PBO was −0.56 (−0.87 to –0.25, p<0.001), in line with previous findings on the dissociative capacity of TNF inhibition on the link between disease activity and joint damage progression. The adjusted mean difference for baricitinib versus PBO was −0.50 (−0.78 to –0.23, p<0.001), indicating that, among those with MDA/HDA, there was significantly less structural damage progression in patients receiving baricitinib. This further suggests an uncoupling of disease activity and structural damage progression with baricitinib, which did not occur with PBO. There was no difference in structural damage progression between patients in the baricitinib and adalimumab groups (0.06 (−0.26 to 0.37), p=0.7).

Structural damage progression in relation to disease activity in RA-BEGIN (csDMARD-naïve patients with early RA)

Among patients who achieved REM/LDA, the adjusted means for mTSS change from baseline to week 52 were 0.43, 0.39 and 0.28 in the MTX, baricitinib monotherapy and baricitinib+MTX groups, respectively (figure 3B). Patients in the same treatment groups with MDA/HDA had progressed by 1.69, 1.05 and 0.50, respectively. Thus, patients receiving MTX in REM/LDA had significantly less structural damage progression compared with those with MDA/HDA (−1.26 (−1.95 to –0.57), p<0.001). In contrast, there were no significant differences in progression among patients who received baricitinib+MTX (−0.22 (−0.85 to 0.41), p=0.5) or those who received baricitinib in monotherapy (−0.65 (−1.36 to 0.06), p=0.07), although this difference was numerically larger for baricitinib monotherapy.

The magnitude of differences between the MTX (1.69), baricitinib (1.05) and baricitinib+MTX (0.50) groups among patients with MDA/HDA again reveals an effect of both baricitinib monotherapy and combination therapy on structural damage progression inhibition relative to MTX treatment. Compared with MTX, the adjusted mean difference for baricitinib monotherapy was −0.64 (−1.33 to 0.05, p=0.07) and was −1.19 (−1.85 to –0.53, p<0.001) for baricitinib+MTX.

These results indicate an uncoupling of disease activity and structural damage progression with combination therapy and a similar trend with baricitinib monotherapy, which did not occur with MTX monotherapy.

CDAI changes in patients with residual disease activity

In RA-BEAM, among all patients with a postbaseline averaged CDAI classed as HDA (n=229), more were in the PBO group than in the baricitinib and adalimumab groups (49.3%, 29.7% and 21%, respectively). The CDAI change from baseline to week 24 among patients with MDA/HDA was significantly greater in the baricitinib (−22.78) and adalimumab (−22.30) groups, compared with PBO (−17.26, both p<0.001; online

Table 1 Demographic and baseline characteristics of patients in RA-BEAM (MTX-IR patients with established RA) and RA-BEGIN (csDMARD-naïve patients with early RA) stratified by averaged CDAI

	RA-BEAM						RA-BEGIN					
	CDAI ≤10			CDAI >10			CDAI ≤10			CDAI >10		
	PBO (n=61)	Bari 4 mg (n=171)	ADA (n=98)	PBO (n=257)	Bari 4 mg (n=236)	ADA (n=164)	MTX (n=53)	Bari 4 mg (n=72)	Bari 4 mg ± MTX (n=104)	MTX (n=81)	Bari 4 mg (n=53)	Bari 4 mg ± MTX (n=62)
Age, years	54.6±12.4	53.0±11.9	52.1±11.8	52.5±11.5	52.7±12.3	53.2±12.2	50.9±13.7	50.3±13.0	45.9±13.4	49.7±13.2	51.3±13.0	51.0±13.0
Female, n (%)	49 (80.3)	134 (78.4)	73 (74.5)	202 (78.6)	179 (75.8)	125 (76.2)	37 (69.8)	55 (76.4)	73 (70.2)	58 (71.6)	42 (79.2)	49 (79.0)
BMI, kg/m^2	28.0±6.2	26.3±5.6	26.1±5.2	26.8±6.6	27.2±6.2	26.6±5.4	27.1±6.3	27.7±6.6	25.8±5.3	26.5±6.6	25.3±6.3	26.8±6.7
Smoker, yes, n (%)	8 (13.1)	39 (22.8)	18 (18.4)	58 (22.6)	53 (22.5)	38 (23.2)	8 (15.1)	18 (25.0)	20 (19.2)	16 (19.8)	11 (20.8)	15 (24.2)
Duration of RA from diagnosis, years	8.8±7.3	9.0±8.4	7.8±7.4	8.9±7.8	8.3±8.6	8.5±8.2	0.5±1.0	1.5±3.3	1.1±2.2	1.4±4.0	2.0±4.4	1.8±3.7
hsCRP, mg/L	13.7±14.5	18.9±19.4	18.6±16.7	17.9±17.2	25.0±24.6	22.0±20.0	17.2±14.6	19.8±16.3	26.6±28.7	25.2±22.6	24.2±23.5	22.0±28.1
CDAI	28.5±10.6	33.2±10.6	32.4±13.3	38.8±12.4	41.0±11.4	40.3±11.9	38.6±13.7	35.3±13.1	37.9±12.1	39.7±12.6	42.3±11.3	42.9±13.5
HAQ-DI	1.2±0.7	1.4±0.7	1.3±0.7	1.6±0.6	1.7±0.7	1.7±0.6	1.6±0.7	1.5±0.8	1.6±0.7	1.7±0.7	1.7±0.6	1.7±0.7
RF-positive*, n (%)	55 (90.2)	157 (91.8)	90 (91.8)	238 (92.6)	211 (89.4)	150 (91.5)	50 (94.3)	72 (100)	100 (96.2)	79 (97.5)	52 (98.1)	57 (91.9)
ACPA-positive†, n (%)	53 (86.9)	150 (87.7)	89 (90.8)	221 (86.0)	212 (89.8)	145 (88.4)	47 (88.7)	68 (94.4)	98 (94.2)	77 (95.1)	46 (86.8)	53 (85.5)
Pain assessment	47.6±23.2	58.3±21.7	55.2±24.5	60.6±21.1	63.6±21.9	62.5±21.8	62.4±24.4	59.4±24.2	61.2±22.4	65.5±25.1	64.1±19.3	68.0±21.2
mTSS	34.3±41.5	35.9±42.7	34.7±42.0	44.7±50.6	47.1±55.4	50.2±56.4	8.4±21.4	7.4±12.8	8.6±16.2	13.1±17.6	17.0±34.6	17.6±26.4

Data are mean±SD or n (%).
Averaged CDAI responses in RA-BEAM calculated as the mean of postbaseline measurements at weeks 4, 12, 16, 20 and 24 and in RA-BEGIN as the mean of postbaseline measurements at weeks 4, 12, 16, 20, 24, 32, 40 and 52.
*RF-positive >14 units/mL (ULN).
†ACPA-positive >10 units/mL (ULN).
ACPA, anticyclic citrullinated peptide antibody; ADA, adalimumab; Bari, baricitinib; BMI, body mass index; CDAI, Clinical Disease Activity Index; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; HAQ-DI, Health Assessment Questionnaire-Disability Index; hsCRP, high-sensitivity C reactive protein; mITT, modified intent-to-treat; mTSS, modified Total Sharp Score; MTX-IR, inadequate response to methotrexate; n, number of mITT completers; PBO, placebo; RA, rheumatoid arthritis; RF, rheumatoid factor; ULN, upper limit of normal.

Table 2 Demographic and baseline characteristics of patients in RA-BEAM (MTX-IR patients with established RA) and RA-BEGIN (csDMARD-naïve patients with early RA) stratified by averaged hsCRP

	RA-BEAM						RA-BEGIN					
	hsCRP ≤5 mg/L			hsCRP >5 mg/L			hsCRP ≤5 mg/L			hsCRP >5 mg/L		
	PBO (n=63)	Bari 4 mg (n=262)	ADA (n=158)	PBO (n=257)	Bari 4 mg (n=149)	ADA (n=107)	MTX (n=56)	Bari 4 mg (n=73)	Bari 4 mg ± MTX (n=120)	MTX (n=81)	Bari 4 mg (n=54)	Bari 4 mg ± MTX (n=47)
Age, years	52.9±10.6	53.2±12.2	52.3±12.6	52.9±12.0	52.2±12.2	53.2±11.1	49.8±12.5	51.4±12.5	47.7±14.4	50.7±14.0	47.7±11.4	49.5±13.3
Female, n (%)	50 (79.4)	202 (77.1)	121 (76.6)	202 (78.6)	113 (75.8)	78 (72.9)	41 (73.2)	53 (72.6)	89 (74.2)	55 (67.9)	44 (81.5)	34 (72.3)
BMI, kg/m^2	26.2±5.1	25.6±5.2	25.3±4.5	27.3±6.8	29.0±6.5	28.1±5.9	24.9±4.4	26.0±5.9	25.1±5.6	28.1±7.3	27.5±7.2	29.0±5.6
Smoker, yes, n (%)	13 (20.6)	56 (21.4)	28 (17.7)	54 (21.0)	36 (24.2)	30 (28.0)	6 (10.7)	15 (20.5)	30 (25.0)	20 (24.7)	16 (29.6)	6 (12.8)
Duration of RA from diagnosis, years	7.6±7.0	8.4±8.1	6.9±6.6	9.2±7.8	8.9±9.3	10.0±9.3	0.8±1.9	2.0±4.4	1.0±2.1	1.2±3.8	1.2±2.6	2.3±4.1
hsCRP, mg/L	6.3±5.9	17.9±18.6	17.0±16.2	20.1±18.3	30.1±26.8	26.5±21.8	14.8±13.9	19.2±17.9	17.5±18.6	27.0±22.0	24.6±21.4	43.3±39.1
HAQ-DI	1.4±0.7	1.4±0.7	1.5±0.7	1.5±0.7	1.8±0.7	1.7±0.7	1.5±0.7	1.6±0.8	1.5±0.7	1.7±0.7	1.6±0.7	1.9±0.7
RF-positive*, n (%)	57 (90.5)	238 (90.8)	144 (91.1)	238 (92.6)	134 (89.9)	99 (92.5)	56 (100)	73 (100)	113 (94.2)	76 (93.8)	53 (98.1)	45 (95.7)
ACPA-positive†, n (%)	53 (84.1)	233 (88.9)	141 (89.2)	223 (86.8)	133 (89.3)	96 (89.7)	53 (94.6)	67 (91.8)	110 (91.7)	74 (91.4)	49 (90.7)	42 (89.4)
Pain assessment	55.2±22.1	60.0±21.7	57.7±22.1	58.8±22.1	63.6±22.2	62.9±24.3	60.4±25.0	62.0±23.3	61.4±23.9	67.1±24.2	60.9±20.7	69.5±15.3
mTSS	34.4±43.2	43.4±50.1	41.4±47.5	44.7±50.2	40.5±51.6	49.5±58.5	11.4±24.5	10.3±20.1	11.4±20.7	11.0±14.2	12.9±29.9	13.2±21.8

Data are mean±SD or n (%).
Averaged hsCRP in RA-BEAM calculated as the mean of postbaseline measurements at weeks 4, 12, 16, 20 and 24 and in RA-BEGIN as the mean of postbaseline measurements at weeks 4, 12, 16, 20, 24, 32, 40 and 52.
*RF-positive >14 units/mL (ULN).
†ACPA-positive >10 units/mL (ULN).
ACPA, anticyclic citrullinated peptide antibody; ADA, adalimumab; Bari, baricitinib; BMI, body mass index; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; HAQ-DI, Health Assessment Questionnaire-Disability Index; hsCRP, high-sensitivity C reactive protein; mITT, modified intent-to-treat; mTSS, modified Total Sharp Score; MTX-IR, inadequate response to methotrexate; n, number of mITT completers; PBO, placebo; RA, rheumatoid arthritis; RF, rheumatoid factor; ULN, upper limit of normal.

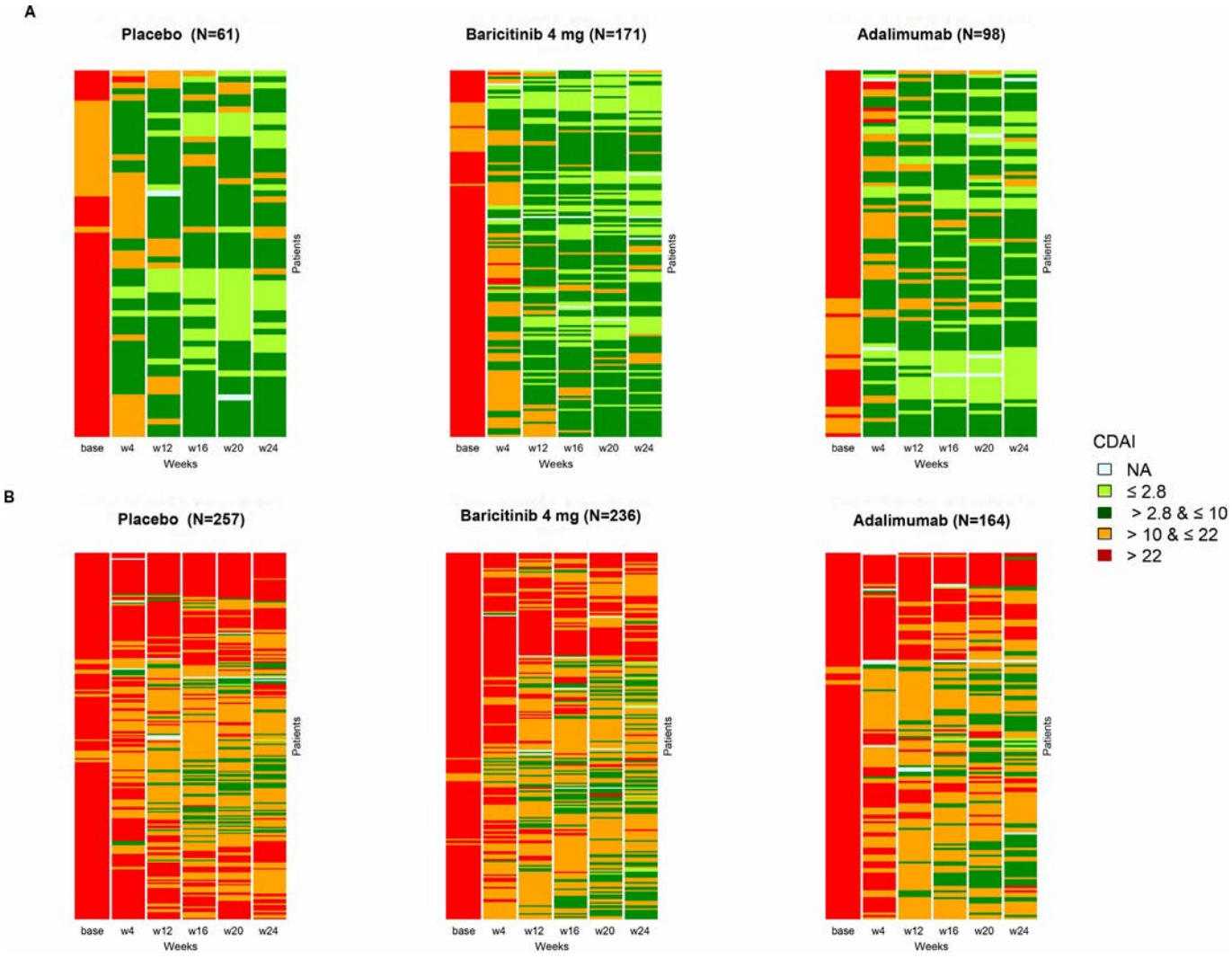

Figure 1 Heatmaps showing individual CDAI responses to treatment in RA-BEAM (MTX-IR patients with established RA) in patients with (A) averaged CDAI ≤10 (remission/low disease activity) and (B) averaged CDAI >10 (moderate/high disease activity). Averaged CDAI responses calculated as the mean of postbaseline measurements at weeks 4, 12, 16, 20 and 24. CDAI, Clinical Disease Activity Index; MTX-IR, inadequate response to methotrexate; NA, not available; RA, rheumatoid arthritis; w, week.

supplemental table 1A). This indicates that even in patients who do not achieve REM/LDA, baricitinib conveys more clinical improvement and radiographic control than PBO, which is evidenced by the dissociation seen in this study (figure 3A).

Similarly, among all patients with HDA (n=40) in RA-BEGIN, more were on de novo MTX (57.5%) than on baricitinib (15.0%) and baricitinib+MTX (27.5%). Interestingly, in contrast to RA-BEAM, among patients classed as having MDA/HDA in RA-BEGIN, the CDAI change from baseline on MTX (−24.8) was not significantly lower than on baricitinib monotherapy (−26.4) or combination therapy (−27.0) (both p>0.05; online supplemental table 1B). Nevertheless, structural progression was higher, suggesting that the capacity of baricitinib to dissociate the tight link between activity and damage goes beyond the mere association with change in disease activity or disease activity states (figure 3B).

Structural damage progression in relation to systemic inflammation

Regarding systemic inflammation, as reflected by hsCRP levels, 80.3% (n=257), 40.4% (n=107) and 36.3% (n=149) of patients receiving PBO, adalimumab and baricitinib, respectively, had a postbaseline averaged hsCRP >5 mg/L up to 24 weeks in RA-BEAM.

The impact of the systemic inflammatory response on structural damage progression within each treatment group differed (figure 4A). Among patients receiving PBO, those with hsCRP >5 mg/L had significantly greater structural damage progression compared with those with hsCRP ≤5 mg/L (0.48 (0.04 to 0.91), p=0.03). However, there were no significant differences among patients receiving baricitinib (0.18 (−0.14 to 0.50), p=0.3) or adalimumab (0.12 (−0.26 to 0.51), p=0.5).

Across treatment groups, among patients with hsCRP >5 mg/L, structural damage progression was lower in those receiving baricitinib compared with PBO (−0.45 (−0.77 to −0.13), p=0.006). Structural damage progression was also lower in patients who received adalimumab versus PBO (−0.55 (−0.90 to −0.20), p<0.01). As in the clinical assessment analyses, there was no essential difference between the baricitinib and adalimumab groups (0.10 (−0.29 to 0.49), p=0.6).

These results indicate that structural damage progression was uncoupled from inflammation in patients receiving baricitinib and show that, even with high systemic inflammation, structural damage progression was inhibited by baricitinib.

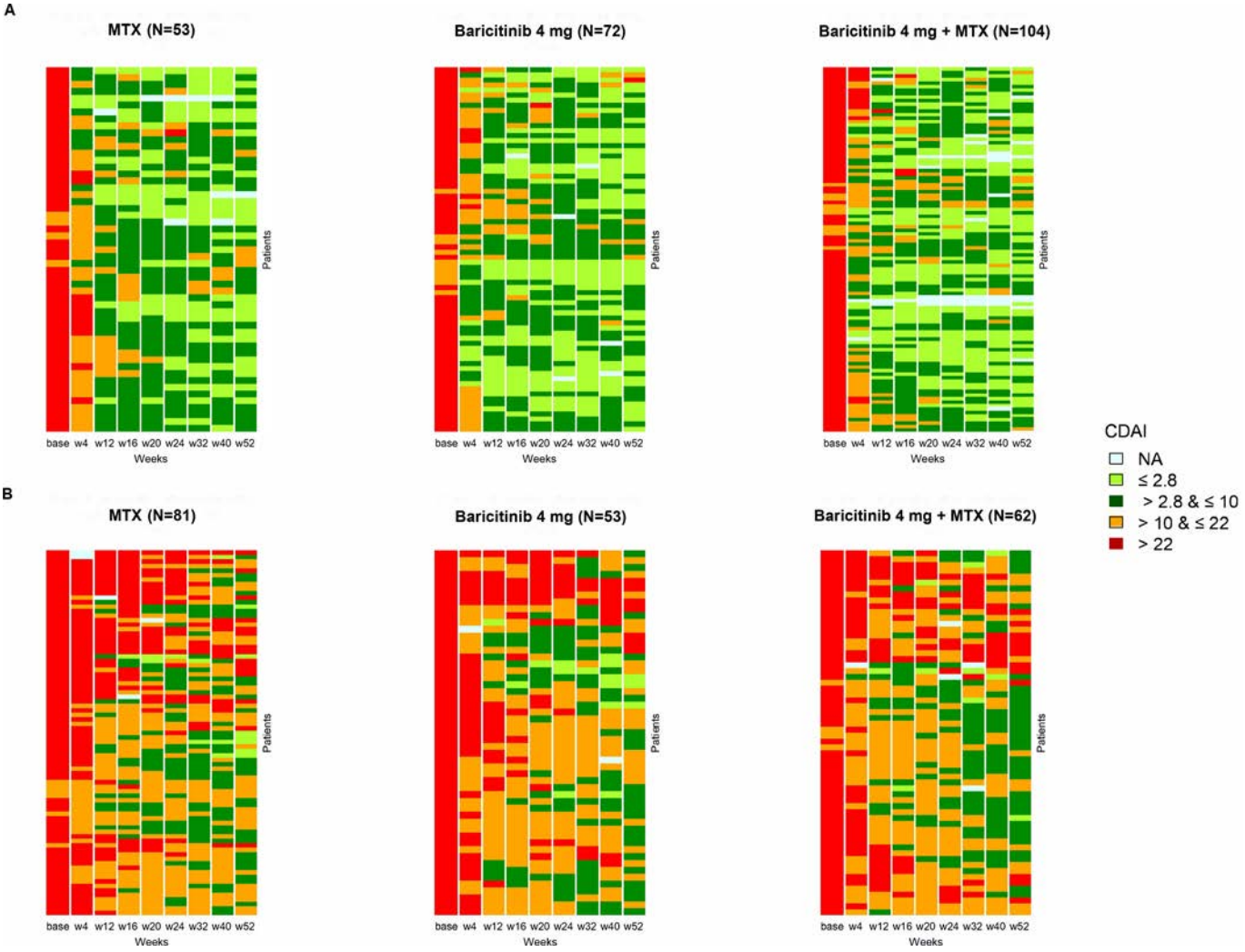

Figure 2 Heatmaps showing individual CDAI responses to treatment in RA-BEGIN (csDMARD-naïve patients with early RA) in patients with (A) averaged CDAI ≤10 (remission/low disease activity) and (B) averaged CDAI >10 (medium/high disease activity). Averaged CDAI responses calculated as the mean of postbaseline measurements at weeks 4, 12, 16, 20, 24, 32, 40 and 52. CDAI, Clinical Disease Activity Index; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; MTX, methotrexate; NA, not available; RA, rheumatoid arthritis; w, week.

In RA-BEGIN, at week 52, 59.1% (n=81), 42.5% (n=54) and 28.1% (n=47) of patients in the MTX, baricitinib and baricitinib+MTX groups, respectively, had hsCRP >5 mg/L. These patients had progressed by 1.51, 1.20 and −0.15, respectively (figure 4B). Structural damage progression was significantly lower in the baricitinib+MTX group compared with the MTX group (−1.66 (−2.36 to –0.95), p<0.001). Damage progression in patients who received baricitinib monotherapy was also numerically lower compared with those who received MTX, but this was not significant (−0.31 (−0.97 to 0.36), p=0.4).

Within treatment groups stratified by acute phase response, structural damage progression was significantly greater in patients with hsCRP >5 mg/L in the MTX (0.73 (0.07 to 1.40), p=0.03) and baricitinib monotherapy (0.89 (0.21 to 1.57), p=0.01) groups, but did not significantly differ in the baricitinib+MTX group (−0.67 (−1.35 to 0.02), p=0.06).

hsCRP changes in patients with residual inflammation

In RA-BEAM, in patients with averaged hsCRP >5 mg/L, the change from baseline in hsCRP was significantly greater in the baricitinib group (−6.61, p<0.001), but not in the adalimumab group (−0.75, p=0.64), compared with PBO (online supplemental table 2A). This might be in line with the direct effect of baricitinib on IL-6 signalling and thus on C reactive protein (CRP). However, the fact that the change of hsCRP on adalimumab was not different from that in PBO again reveals that the dissociation is largely independent of changes of acute phase reactant levels in non-responders, confirmed by the similar inhibitory effect of baricitinib on progression of damage as that of adalimumab (figure 4A). This is further exemplified in RA-BEGIN. The difference in change from baseline in participants with averaged hsCRP >5 mg/L was −1.71 (p=0.35) for baricitinib and −3.94 (p=0.045) for baricitinib+MTX, compared with MTX alone (online supplemental table 2B), although MTX did not inhibit structural damage progression to the same extent as baricitinib, particularly when in combination with MTX (figure 4B).

DISCUSSION

The research presented here originates from observations that in csDMARD-naïve patients with RA, MTX alone might not halt the progression of joint damage if there is ongoing residual disease activity. Early in the disease, as part of the window of opportunity, this is not acceptable, and patients should

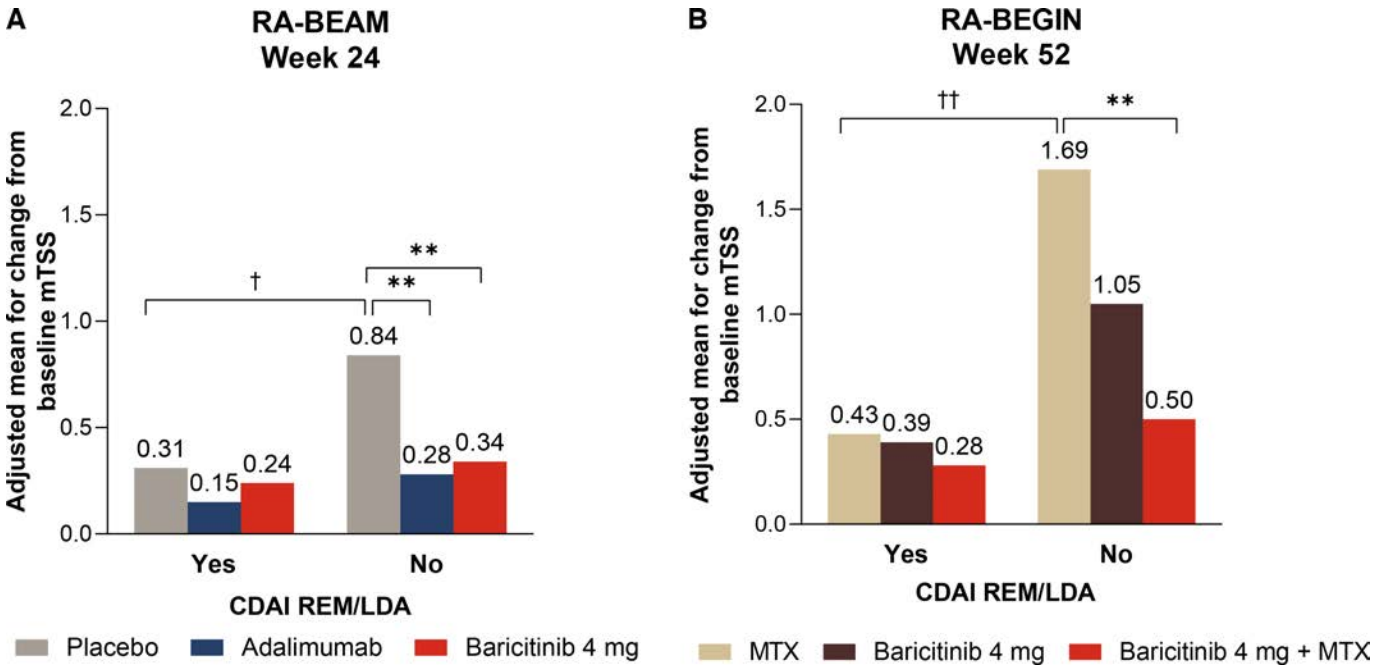

Figure 3 Structural damage progression (adjusted mean for change from baseline mTSS) in relation to averaged CDAI in (A) RA-BEAM (MTX-IR patients with established RA) and (B) RA-BEGIN (csDMARD-naïve patients with early RA). Averaged CDAI responses in RA-BEAM calculated as the mean of postbaseline measurements at weeks 4, 12, 16, 20 and 24 and in RA-BEGIN as the mean of postbaseline measurements at weeks 4, 12, 16, 20, 24, 32, 40 and 52. REM/LDA classified as CDAI ≤10. Between treatment group difference, **p<0.001 versus placebo or MTX; within treatment group difference, †p<0.05 and ††p<0.001. CDAI, Clinical Disease Activity Index; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; LDA, low disease activity; mTSS, modified Total Sharp Score; MTX, methotrexate; MTX-IR, inadequate response to methotrexate; RA, rheumatoid arthritis; REM, remission.

optimise treatments; thus far, tsDMARDs and bDMARDs are approved and recommended options. Later in the disease, in MTX-csDMARD insufficient responders who receive PBO, damage accrual is high, in line with their continued active established disease; in patients who receive either tsDMARDs or bDMARDs (with/without background MTX, but more so with combination therapy), damage could be halted or dramatically reduced even if they continue to have MDA/

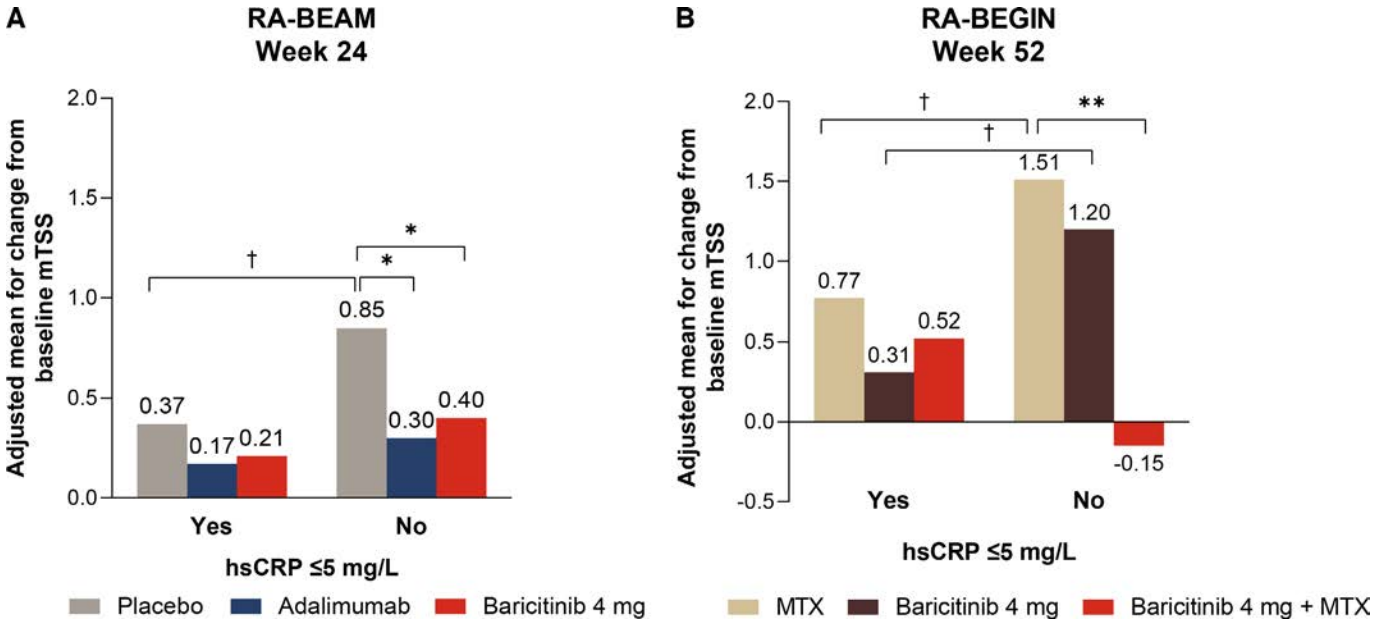

Figure 4 Structural damage progression (adjusted mean for change from baseline mTSS) in relation to averaged hsCRP in (A) RA-BEAM (MTX-IR patients with established RA) and (B) RA-BEGIN (csDMARD-naïve patients with early RA). Averaged hsCRP in RA-BEAM calculated as the mean of postbaseline measurements at weeks 4, 12, 16, 20 and 24 and in RA-BEGIN as the mean of postbaseline measurements at weeks 4, 12, 16, 20, 24, 32, 40 and 52. Between treatment group differences, *p<0.05 and **p<0.001 versus placebo or MTX; within treatment group differences, †p<0.05. csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; hsCRP, high-sensitivity C reactive protein; mTSS, modified Total Sharp Score; MTX, methotrexate; MTX-IR, inadequate response to methotrexate; RA, rheumatoid arthritis.

HDA.[14–17 19 20] These analyses confirmed this hypothesis for baricitinib.

The results presented reveal that baricitinib, with/without de novo MTX or with background MTX, enhances disease-modifying effects by blunting the tight link that is usually seen between disease activity and progression of joint damage in csDMARD-naïve and MTX-IR patients, being more evident when baricitinib is combined with MTX. Thus, baricitinib and baricitinib+MTX exert efficacy in structural terms even in patients who remain in MDA/HDA on this treatment, being significant for baricitinib+MTX. This was also evident when changes from baseline in disease activity were examined in those with residually active disease.

These data are very robust on several grounds. First, they are independent of disease duration and prior treatment. They pertain to patients with early disease who are MTX-naïve, as exemplified in the RA-BEGIN trial analyses, and to patients with established disease who had an insufficient response to MTX, as evaluated in RA-BEAM. Second, the analyses are consistent and confirmatory irrespective of the type of inflammatory marker used. When subgroups for the definition of disease activity are formed according to clinical assessment (CDAI), which is primarily driven by joint counts and thus local inflammation, damage progression is not larger in higher versus lower disease activity states on baricitinib+MTX in contrast to control (MTX in RA-BEGIN and PBO with background MTX in RA-BEAM). These data are confirmed when employing CRP, a systemic inflammatory marker induced by proinflammatory cytokines in the liver, to distinguish patients with higher and lower disease activity.

In RA-BEGIN baricitinib monotherapy was also studied. While there was a trend towards better structural efficacy in patients with higher disease activity states also with baricitinib monotherapy compared with MTX monotherapy, this did not reach statistical significance. It should be considered that in early disease if uptitration of MTX does not control inflammation, structural progression would be higher than with use of a bDMARD or Jakinib, such as baricitinib, as indicated by mTSS progression over time. However, other studies of bDMARDs and tsDMARDs on csDMARD/MTX-naïve patients with early RA have not compared monotherapy, MTX combination therapy and MTX uptitration.

One of the strengths of the present study is the use of time-averaged disease activity, for both CDAI and CRP, so that the effects of extreme values and missing data are mitigated. Interestingly, when following disease activity in individual patients over time, in patients in whom REM/LDA is achieved at endpoint, a drop in activity is already discernible within 4–12 weeks, in line with the treat-to-target recommendations[2] and independent of whether patients have established or early RA. In contrast, those who remain in MDA/HDA never achieve any better status.[30] The visualisation as 'heatmaps' allowed us to show these data for individual patients very clearly. Another strength is the use of the CDAI for clinical disease activity assessment rather than a single measure. Composite scores capture RA better than individual variables.[31 32] Because the CDAI does not include an acute phase reactant,[7] we could validate the clinical findings by using a serological marker.

Our study has some limitations. First, we used different time points for analysis of the early csDMARD-naïve and established MTX-IR RA populations. While the ideal time frame for comparative assessment of radiographic progression is 1 year, as done in the RA-BEGIN analysis, assessments of RA-BEAM data were restricted to the 24-week time point. However, it was more important to have a valid active comparator, and in RA-BEAM all patients in the PBO group received baricitinib after at most 6 months; analyses at 1 year would then have confounded the value of the data. Interestingly though, significant differences between the groups could already be seen at 6 months, which may even increase the importance of the data. Another limitation is our focus on baricitinib and therefore we cannot be sure that our findings can be translated to other Jakinibs. While it is likely this will be the case, there might be some differences based on the different selectivity of the compounds.

In conclusion, the Jakinib baricitinib has shown to have significant inhibitory effects on the progression of structural joint damage even in patients who continue to have MDA/HDA states. This quality has hitherto been described only for bDMARDs and has important clinical value. Adherence to treat-to-target principles calls for a rapid change of treatment with insufficient improvement. However, when a patient improves clinically on baricitinib but not yet to the desired extent,[33] the decision to change treatment could be delayed for a short while in accordance with the patient because joint damage progression and thus irreversible disability need not be feared.

Acknowledgements The authors thank Andrea Hemmingway, PhD (Eli Lilly and Company) for writing and editorial assistance, and Liliana Zaremba-Pechmann, PhD (HaaPACS) for validation of statistical analyses.

Contributors PLR, IdlT, and JSS were involved in conception and design of the work. PLR designed and completed the statistical analyses. All authors contributed to data interpretation, critically reviewed the manuscript, and approved the final version for submission. PLR acts as guarantor for this work.

Funding The RA-BEGIN and RA-BEAM studies were supported by Eli Lilly and Company and Incyte Corporation. Medical writing and editorial support for this manuscript were funded by Eli Lilly and Company.

Competing interests PLR developed this work while an employee of Eli Lilly and Company and is currently an employee of Novartis. IdlT and EH are employees of Eli Lilly and Company. DA reports grants/contacts from AbbVie, Amgen, Eli Lilly and Company, Novartis, Roche, SoBi and Sanofi; and consulting fees from AbbVie, Amgen, Eli Lilly and Company, Merck, Novartis, Pfizer, Roche and Sandoz. JSS reports grants/contracts from AbbVie, AstraZeneca, Eli Lilly and Company and Novartis; consulting fees from AbbVie, Amgen, AstraZeneca, Astro, Bristol Myers Squibb, Celgene, Celltrion, Chugai, Eli Lilly and Company, Gilead, ILTOO, Janssen, Merck Sharp & Dohme, Novartis-Sandoz, Pfizer, Roche, Samsung, Sanofi and UCB; payment/honoraria from AbbVie, Amgen, Bristol Myers Squibb, Celgene, Chugai, Eli Lilly and Company, Janssen, Merck Sharp & Dohme, Novartis-Sandoz, Pfizer, Roche, Samsung and UCB; participation on a data safety monitoring/advisory board for AstraZeneca; a leadership/fiduciary role with European League Against Rheumatism; and other interests as an editorial board member of Annals of the Rheumatic Diseases and textbook editor.

Patient consent for publication Not required.

Ethics approval This study involves human participants. The studies were approved by each centre's institutional review board or ethics committee. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Lilly provides access to all individual participant data collected during the trial, after anonymization, with the exception of pharmacokinetic or genetic data. Data are available to request 6 months after the indication studied has been approved in the US and EU and after primary publication acceptance, whichever is later. No expiration date of data requests is currently set once data are made available. Access is provided after a proposal has been approved by an independent review committee identified for this purpose and after receipt of a signed data sharing agreement. Data and documents, including the study protocol, statistical analysis plan, clinical study report, blank or annotated case report forms, will be provided in a secure data sharing environment. For details on submitting a request, see the instructions provided at www.vivli.org.

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CLINICAL SCIENCE

Anti-RNP antibodies are associated with the interferon gene signature but not decreased complement levels in SLE

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221662).

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Received 12 October 2021
Accepted 19 January 2022
Published Online First 3 February 2022

ABSTRACT

Objectives The goals of these studies were to elucidate the inter-relationships of specific anti-nuclear antibody (ANA), complement, and the interferon gene signature (IGS) in the pathogenesis of systemic lupus erythematosus (SLE).

Methods Data from the Illuminate trials were analysed for antibodies to dsDNA as well as RNA-binding proteins (RBP), levels of C3, C4 and various IGS. Statistical hypothesis testing, linear regression analyses and classification and regression trees analysis were employed to assess relationships between the laboratory features of SLE.

Results Inter-relationships of ANAs, complement and the IGS differed between patients of African Ancestry (AA) and European Ancestry (EA); anti-RNP and multiple autoantibodies were more common in AA patients and, although both related to the presence of the IGS, relationships between autoantibodies and complement differed. Whereas, anti-dsDNA had an inverse relationship to C3 and C4, levels of anti-RNP were not related to these markers. The IGS was only correlated with anti-dsDNA in EA SLE and complement was more correlated to the IGS in AA SLE. Finally, autoantibodies occurred in the presence and absence of the IGS, whereas the IGS was infrequent in anti-dsDNA/anti-RBP-negative SLE patients.

Conclusion There is a complex relationship between autoantibodies and the IGS, with anti-RNP associated in AA and both anti-dsDNA and RNP associated in EA. Moreover, there was a difference in the relationship between anti-dsDNA, but not anti-RBP, with complement levels. The lack of a relationship of anti-RNP with C3 and C4 suggests that anti-RNP immune complexes (ICs) may drive the IGS without complement fixation, whereas anti-dsDNA ICs involve complement consumption.

Key messages

What is already known about this subject?
- Anti-RNP and anti-dsDNA autoantibodies form immune complexes (ICs) that induce interferon (IFN).
- Anti-dsDNA ICs deposit in lupus kidneys and contribute to renal pathology by fixation and activation of complement.

What does this study add?
- Anti-RNP autoantibody ICs have a stronger capacity to induce IFN than anti-dsDNA autoantibodies but are not related to depression of complement.
- Autoantibodies are likely required for the induction of IFN, but the IFN gene signature (IGS) is not required for the production of autoantibodies.

How might this impact on clinical practice or future developments?
- Development of a more precise way to detect the presence of pathogenic ICs and identify the mechanisms underlying the IGS.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease that primarily affects young women and is especially severe in patients of African Ancestry (AA).[1–4] Among the most prominent immunological features of SLE is the production of anti-nuclear antibodies (ANAs); these antibodies target nucleic acids, proteins and protein-nucleic acid complexes.[5] Among ANAs, anti-dsDNA antibodies are unique markers for both classification and disease activity.[6] In SLE, anti-dsDNA antibodies form immune complexes (ICs) that deposit in the kidney to activate complement and provoke inflammation; anti-dsDNA can also stimulate the production of type I interferon (IFN) by cells of the innate immune system.[7 8]

Like anti-dsDNA, ANAs directed to RNA-binding proteins (RBPs) can form ICs inducing IFN.[9–15] As a group, anti-RBPs target RNA–protein complexes, although antibodies bind to the protein and not to the nucleic acid. Although anti-RNP antibodies are frequent in SLE, they are not disease specific and their levels do not obviously change with disease activity.[5 16] Therefore, their role in SLE pathogenesis has been less well studied.

The IFN response is usually assessed by analysis of gene expression of peripheral blood cells, which can show an IFN gene signature (IGS).[17–20] The stimulation of IFN results from the interaction of DNA or RNA with internal nucleic acid sensors, both toll-like receptor (TLR) and non-TLR, following uptake into cells. These internal receptors are elements of an internal host defence system that can recognise foreign DNA or cytoplasmic DNA from cell stress or damage.[21] Recent studies have demonstrated a strong relationship between the IGS and anti-RNP antibodies, suggesting a pathogenic role for these autoantibodies.[15 22 23]

To cite: Hubbard EL, Pisetsky DS, Lipsky PE. *Ann Rheum Dis* 2022;**81**:632–643.

Anti-dsDNA and anti-RBPs, while both targeting nuclear macromolecules, nevertheless differ in their pattern of expression. Thus, anti-dsDNA levels can vary markedly during the course of disease especially during nephritis, and can decrease and even disappear with therapy.[24] During flare, levels of C3 and C4 can decrease, consistent with activation of the complement system by ICs.[25–29] In contrast, anti-RBP levels tend to remain relatively static over time, making the relationship of anti-RBPs and disease flares or complement unclear. With the approval of anifrolumab, a monoclonal antibody to the type I IFN receptor, to treat SLE, it is important to understand the drivers of the IGS and the interplay of biomarkers related to IC formation.[30–32]

In these studies, we investigated the relationship between ANA levels, the IGS and levels of C3 and C4 in patient samples from two clinical trials of tabalumab in SLE, testing the associations of these biomarkers in patients of AA and European Ancestry (EA). As our results indicate, anti-dsDNA levels have an inverse relationship with C3 and C4, whereas anti-RNP levels were not related to depression of complement despite a strong association of anti-RNP with the IGS in both ancestral groups. These findings suggest differences in the properties of ICs formed by anti-dsDNA and anti-RBP antibodies in SLE and the impact of ancestry on serological disease manifestations.

METHODS

Patient involvement

Patients were not directly recruited or involved in this study. Rather, patient's data from previously completed clinical trials were obtained and analysed.[33] SLE patients enrolled in the trials had a clinical diagnosis of SLE defined as having ≥4 of the American College of Rheumatology 1997 criteria, positive ANA, and active disease defined as SELENA-SLEDAI ≥6. Exclusion criteria included having active nephritis, active CNS or peripheral neurologic disease, having previously received rituximab, having received IVIg within 180 days of randomisation, and other criteria relating to previous infections and details relating to treatment regimens.[34]

Clinical information and microarray data from whole blood of patients from two clinical trials of tabalumab, a monoclonal antibody to BAFF, in SLE were downloaded from Gene Expression Omnibus under accession GSE88884.[33–36] Additional clinical metadata was provided by Matthew D. Linnik of Eli Lilly & Co. Anti-dsDNA was determined by IgG INOVA QUANTA Lite SC ELISA (INOVA Diagnostics, San Diego, California, USA). Anti-RNP was determined by QUANTA Lite RNP ELISA (INOVA Diagnostics, San Diego, California, USA). Anti-Sm, anti-SSA, anti-SSB and C3 and C4 levels were also determined by ELISA. Gene expression and autoantibody levels, complement levels and all clinical data were analysed using baseline values, before initiation of study drug. Patients with missing data were excluded from the appropriate analyses.

Gene set variation analysis (GSVA)

The GSVA R package was used as a non-parametric, unsupervised gene set enrichment method.[37] The inputs for the GSVA algorithm were log2 microarray expression values (Affymetrix Human Transcriptome Array V.2.0) and predefined gene sets describing IFN stimulated gene signatures,[15 38–41] TNF[38] and interleukin 1 (IL-1) cytokine signatures.[15] Probes were filtered out if their IQR was equal to zero. GSVA was conducted on the remaining network. Enrichment scores were calculated using a Kolmogorov-Smirnov (KS)-like random walk statistic to estimate variation of predefined gene sets. The enrichment scores take on values between −1 and 1, where 1 represents enrichment of every gene in a given gene set among the samples analysed compared with every other gene not included in the specified gene set, whereas −1 represents a relative lack of enrichment. Each gene in a gene set is given a rank based on expression values and the KS-like random walk statistic is calculated.

Classification and regression trees (CART)

CART analysis was performed in R V.4.0.4 using the rpart, rpart.plot, and ggplot2 packages.[42–44] Regression trees were initially visualised in R and reimaged in GraphPad Prism (V.9.1.0.221). Categorical variables were used as input to the CART algorithm, including autoantibody status of anti-dsDNA, anti-RNP, anti-Sm, anti-SSA and anti-SSB (positive, negative or borderline) and complement C3 and C4 status. C3 levels were considered low if <0.9 g/L and normal if ≥0.9 g/L. C4 levels were considered low if <0.1 g/L and normal if ≥0.1 g/L. A numeric range for C3 and C4 levels considered high was not available. Antibody levels were considered positive if >20 IU/mL, borderline if ≥11 IU/mL and ≤20 IU/mL and negative if <11 IU/mL. GSVA enrichment scores of the core IGS were used as the dependent variable of the estimated regression trees. In every CART analysis, regression trees were pruned once, and then two times but with no observed reduction in cross-validated error, and, therefore the original unpruned trees were decided on as the best estimators of the IGS.

Statistical analyses

Statistical analyses were conducted in GraphPad Prism (V.9.1.0.221) including linear regression. All violin plots, scatterplots and pie charts resulting from these analyses were also rendered in Prism. To compare autoantibody positivity between AA and EA patients, an online calculator (https://wwwsocscistatisticscom/defaultaspx) was used to carry out the χ^2 test with Yates correction.

To determine statistical significance between GSVA enrichment scores of two groups, the Mann-Whitney U test was carried out in GraphPad Prism. To determine statistical significance between enrichment scores of three or more groups, the Kruskal-Wallis and Dunn's multiple comparisons test were performed in GraphPad Prism. Dunn's multiple comparisons test accounts for the number of comparisons made and adjusted p values were reported.

RESULTS

Female SLE patients from GSE88884 (n=1620) were stratified by the presence of five autoantibodies (online supplemental table 1) to determine the association with gene sets representing various IGS. Among autoantibody-stratified patients, GSVA enrichment scores showed an increase in the IGS in anti-dsDNA positive (anti-dsDNA+) patients compared with patients negative for the five measured autoantibodies (anti-dsDNA− anti-RBP−) (figure 1A). Notably, a significant increase in the IGS was also present in patients positive for anti-RNP (anti-RNP+) antibodies only when compared with the anti-dsDNA-anti-RBP− group and when compared with the anti-dsDNA+ group. This relationship was observed for type I, type II and core IFN signatures shared by both types I and II (online supplemental figure 1). To investigate whether these relationships could have been influenced by antibody titre, we correlated antibody levels with IGS enrichment scores in anti-dsDNA+ anti-RNP− patients and anti-dsDNA− anti-RNP+ patients, respectively, (online supplemental figure 2A). We observed a minimal but significant

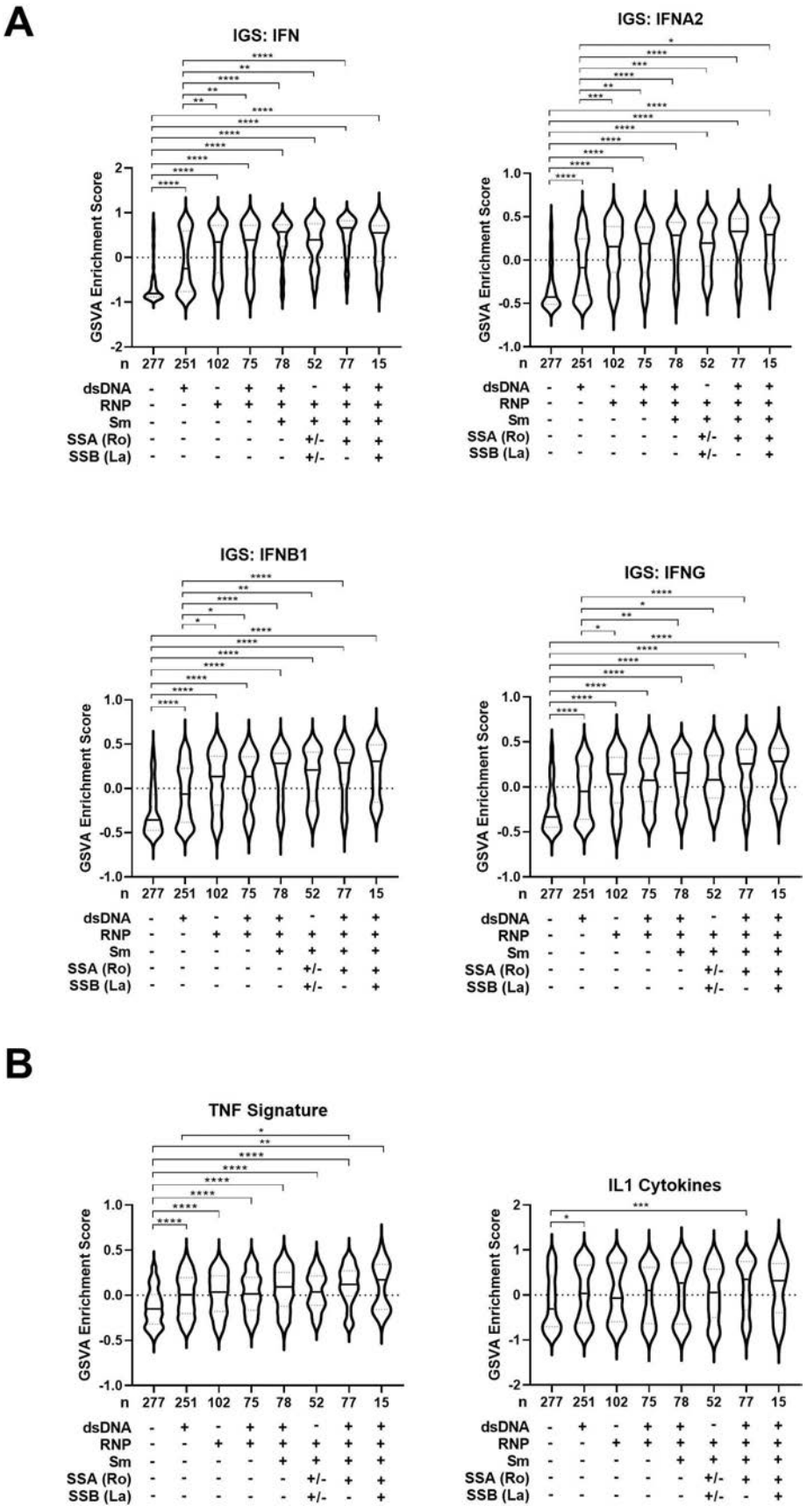

Figure 1 Overall, 1620 active, female SLE patients were stratified by the presence of five autoantibodies. GSVA was carried out on microarray data of these patients using various IGS (A) and cytokine signatures (B). Dunn's multiple comparisons test was performed to determine significant differences in IGS enrichment among the groups. Numbers of patients (n) in each of the comparator groups are annotated on the x-axis. Violin plots display median values (solid lines) and upper and lower quartiles (dashed lines). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

relationship between anti-dsDNA titre and the IGS (R^2=0.05, p<0.0001), whereas any amount of RNP appeared sufficient to be associated with the IGS (p=0.485). Furthermore, anti-dsDNA, anti-SSA and anti-SSB antibody titers were comparable among EA and AA groups, whereas anti-RNP and anti-Sm antibody levels were elevated in AA SLE (online supplemental figure 2B).

GSVA enrichment scores of other cytokine-induced signatures were similarly compared among the autoantibody-stratified groups (figure 1B). Whereas, anti-dsDNA+ and anti-RNP+ patients showed similar increases in the TNF signature compared with anti-dsDNA− anti-RBP− patients, a significant increase in the IL-1 signature was only present in anti-dsDNA+ patients.

The relationships between the IGS and complement levels were next evaluated, with linear regression identifying significant inverse relationships between C3 and C4 levels and IGS GSVA scores (figure 2A). For anti-dsDNA+ only patients (n=251), a significant relationship between C3/C4 and IGS GSVA scores was present (figure 2B). In contrast, no relationship between complement levels and the IGS was identified in the anti-RNP+ only patients (figure 2C). Furthermore, linear regression demonstrated significant relationships between increasing levels of anti-dsDNA antibody and decreasing C3 and C4 levels (figure 3A), but no such relationship between complement levels and anti-RNP levels (figure 3B).

We next stratified the anti-dsDNA+ only patients (figure 4A) and the anti-RNP+ only patients (figure 4B) in terms of low or high/normal C3 levels and by low or high/normal C4 levels; we then examined GSVA scores of the IGS in these groups. Again, significant increases in IGS expression were present in the low complement groups in the anti-dsDNA+ patients; whereas, in the anti-RNP+ patients, there were no significant differences in the IGS.

Since hydroxychloroquine (HCQ) is reported to inhibit TLR7 and TLR9 activity and thus block downstream induction of IFN,[45] we also analysed the IGS among patients taking HCQ (or an equivalent antimalarial drug) and those not taking HCQ. For both anti-dDNA+ anti-RNP− and anti-dsDNA− anti-RNP+ patients, no significant differences were observed in GSVA scores of the type I or type II IGS related to HCQ administration (online supplemental figure 3).

To assess the influence of ancestry on these interrelationships, we then analysed autoantibody stratification among EA and AA patients (online supplemental table 2). As these data indicate, over one-third of EA patients were anti-dsDNA− anti-RBP− and an almost equivalent proportion was positive for anti-dsDNA alone. In contrast, 18.2% of AA patients were anti-dsDNA− anti-RBP−; 14% were positive for only anti-dsDNA and 15.7% were anti-RNP+ only, compared with just 10.2% of EA patients. Overall, 18.2% of AA patients were both anti-dsDNA+ and anti-RNP+, while negative for anti-Sm, anti-SSA and anti-SSB, compared with just 6.19% of EA patients with the same autoantibody profile. Overall, 65.9% of EA patients expressed at least one of the five autoantibodies, whereas 81.8% of AA patients expressed one or more autoantibodies (p=8.08E−4, χ^2 with Yates correction). Notably, 34.6% of EA and 67.8% of AA SLE patients were anti-RNP+ (p<1.00E−5, χ^2 with Yates correction), whereas 52.2% of EA and 56.2% of AA SLE patients were anti-dsDNA+ (p=0.482, χ^2 with Yates correction).

In AA patients, IGS GSVA scores were significantly elevated in those that were anti-RNP+ or anti-RNP+ in combination with other autoantibodies, but not in anti-dsDNA+ only patients (figure 5). In contrast, in EA patients, GSVA scores were elevated in all autoantibody-positive groups compared with anti-dsDNA− anti-RBP− patients.

A unique IGS has previously been reported to be related to ancestry rather than disease activity.[40] We, therefore, assessed this relationship by GSVA to determine its association with autoantibodies. First, we confirmed its relationship to ancestry, where GSVA enrichment scores were higher among AA SLE patients compared with EA SLE (online supplemental figure 4A). Next, we examined this unique signature in SLE patients stratified by ancestry and autoantibody status (online supplemental figure 4B). Consistent with our previous results using different IGS, the ancestry-specific IGS was significantly enriched in autoantibody-positive subjects compared with anti-dsDNA− anti-RBP− patients, regardless of ancestry. Notably, GSVA enrichment scores of the ancestry-specific IGS were significantly higher in anti-dsDNA+ and anti-RNP+ EA patients and significantly more enriched in the anti-RBP+ group, whereas this signature was significantly enriched only in the anti-RBP+ group but not in anti-dsDNA+ AA SLE. These results indicate that enrichment of this ancestry-specific subset of the IGS follows the pattern of expression of the more global IGS, tracking more with the presence of anti-RNP antibodies, which are more frequent in AA SLE.

In both AA and EA anti-dsDNA+ anti-RNP− patients, we identified significant relationships between increasing anti-dsDNA levels and depression of C3 and C4 by linear regression (figure 6A). Interestingly, the regression coefficients were much stronger in the AA than EA cohort. In contrast, in anti-dsDNA− anti-RNP+ atients, anti-RNP levels did not exhibit relationships with C3 or C4 levels in either ancestral group (figure 6B).

Next, we used CART analysis to determine the most important predictors of the dependent variable, IGS enrichment. Status (positive, negative or borderline) of the five autoantibodies measured and C3 and C4 status (high, normal, low) were input as independent variables. Regression trees were visualised to display the hierarchy of importance of each variable on IGS GSVA Score.

In this analysis, anti-RNP was identified as the strongest predictor of IGS expression in both AA and EA cohorts (figure 7). Anti-dsDNA and anti-SSA/Ro antibodies were also identified as predictors of the IGS, but not anti-Sm or anti-SSB/La. Of note, whereas anti-dsDNA contributed to the IGS in global and EA SLE, it did not contribute to IGS enrichment in AA SLE. C3 was important after anti-RNP positivity in all SLE and in AA cohorts but appeared later in the hierarchy in the EA cohort. Only AA patients depended on C4 status for IGS enrichment secondary to having high or normal C3 levels.

Whereas anti-RNP and anti-dsDNA antibodies were associated with the IGS, we were not able to establish directional relationships. We, therefore, assessed autoantibody expression in IGS+ (defined as having an IGS GSVA enrichment score>0) and IGS− (defined as having as IGS GSVA enrichment score<0) patients. Autoantibodies were detected in IGS+ and IGS− samples. In the IGS+ group, 82.41% of patients were anti-dsDNA+ only, anti-RNP+ only or positive for both (online supplemental figure 5A), whereas 54.48% of IGS− patients were also positive for either anti-dsDNA only, anti-RNP only or both.

In contrast, the IGS was uncommon in subjects lacking autoantibodies, with 86.3% of anti-dsDNA− anti-RBP− SLE patients lacking the IGS. We did not detect an association with elevated serum IFN and anticardiolipin antibodies (online supplemental figure 6). There appeared to be a stepwise decrease in the frequency of the IGS related to the number of autoantibodies expressed (online supplemental figure 5B). This finding

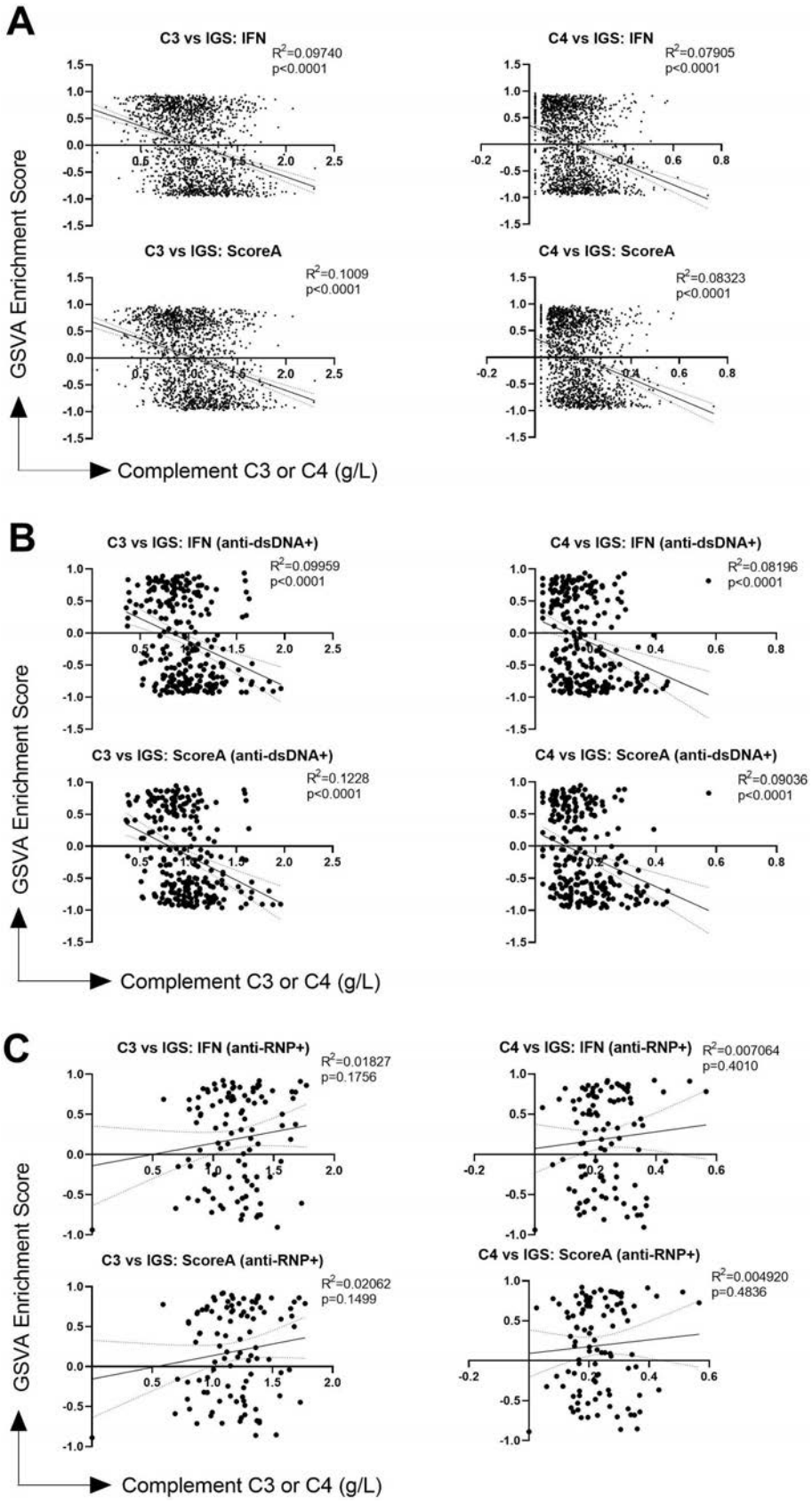

Figure 2 Linear regression analyses show relationships between complement levels and enrichment of interferon gene signature (IGS) in systemic lupus erythematosus (SLE) whole blood, where each dot on the scatterplots represents one patient sample. (A) Represents 1620 active, female SLE patients. (B) Represents 251 active, female SLE patients positive for anti-dsDNA and none of the other five autoantibodies measured. (C) Represents 102 active, female SLE patients positive for anti-RNP only and none of the other five autoantibodies measured. Dotted lines represent 95% confidence bands of the best-fit line.

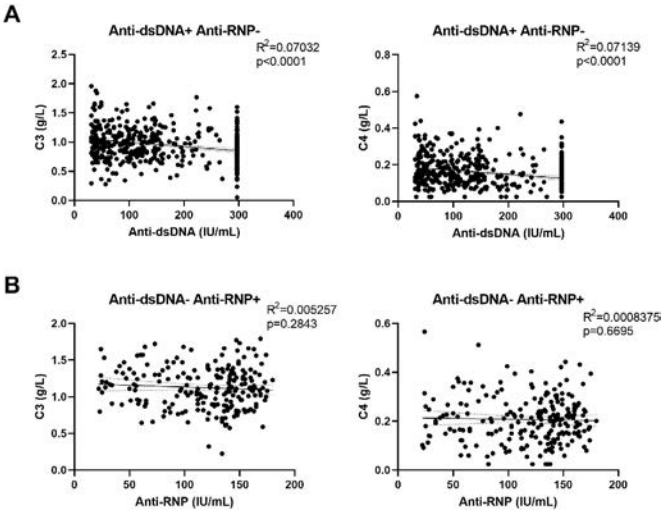

Figure 3 Linear regression analyses show relationships between autoantibody levels and complement levels in systemic lupus erythematosus (SLE) whole blood, where each dot on the scatterplots represents one patient sample. (A) Represents 480 active, female SLE patients positive for anti-dsDNA but negative for anti-RNP antibodies. (B) Represents 220 active, female SLE patients positive for anti-RNP but negative for anti-dsDNA antibodies. Dotted lines represent 95% confidence bands of the best-fit line.

suggests that patients can produce autoantibodies without the IGS whereas it is less likely that patients with the IGS are negative for autoantibodies.

Finally, we analysed relationships between complement levels, IGS expression and anti-SSA/Ro levels in patients positive for anti-SSA but negative for the other measured autoantibodies (anti-SSA+). Notably, a significant relationship between depressed C4 levels and IGS enrichment was identified (online supplemental figure 7A). Moreover, IGS GSVA scores were increased in anti-SSA+ patients stratified by low C3 and low C4 (online supplemental figure 7B). Despite these significant relationships with the IGS, neither C3 nor C4 levels were related to anti-SSA titre by linear regression (online supplemental figure 7C). Since only three patients were positive for anti-SSB antibodies alone, we were unable to similarly probe these relationships in anti-SSB+ patients further.

DISCUSSION

We employed a large clinical trial database to assess the relationship between autoantibody levels, complement consumption and the IGS. As previously reported, the presence of various autoantibodies differed among ancestral groups with anti-RNP especially prevalent in AA SLE and anti-RNP levels higher in AA SLE. Notably, the IGS was most prominently associated with the presence of anti-RNP antibodies, whereas it was associated with anti-dsDNA antibodies only in EA SLE. The apparent differences between anti-RNP and anti-dsDNA to associate with the IGS might relate to different signalling potentials of the intracellular TLRs engaged by their respective cargos in ICs.[21] Indeed, the anti-RNP assays employed used native RNP/Sm as antigens, and, therefore, would be likely to detect autoantibodies that incorporated RNA species into ICs that could engage endosomal TLRs. Alternatively, since anti-RNP can be present in larger amounts,[46] it is possible that this translates to a greater mass effect of anti-RNP versus anti-dsDNA ICs. This appears to be less likely, since the association of the IGS and anti-RNP antibodies did not appear to depend on titre, since no correlation was found between the IGS and anti-RNP levels. However, a significant but only a very modest correlation was detected between the anti-dsDNA titre and the IGS. The association of autoantibodies and complement levels was also complex, with anti-dsDNA levels inversely related to levels of C3 and C4, but not anti-RNP levels. Since ICs with anti-dsDNA or anti-RNP can induce IFN production,[12 47] these findings suggest that properties of ICs formed by various ANAs are functionally different, with ICs with anti-RNP likely unable to activate complement despite a capacity to induce IFN. In this regard, linear regression identified significant relationships between levels of anti-dsDNA and complement in both ancestral groups; however, regression coefficients were stronger in the AA cohort.

Few prior studies have addressed depression of complement by anti-RBPs because of the focus on anti-dsDNA in pathogenesis and the utility of anti-dsDNA antibodies as markers of disease activity especially in conjunction with complement.[48] In contrast to variable expression of anti-dsDNA, levels of antibodies to RBPs tend to remain relatively constant over time and they are therefore not routinely assessed especially with quantitative assays.[16 21] In addition, whereas anti-dsDNA antibodies are disease-specific, anti-RNP, anti-SSA/Ro and anti-SSB/La are disease-related, perhaps suggesting a lesser role in lupus pathology.[5]

Whereas simultaneous expression of multiple ANAs can limit assessment of the relationship between autoantibodies and complement, our study had a sufficiently large number of patients to allow analysis of samples that contained either anti-dsDNA only or anti-RNP only. In this way, we could show that anti-RNP, in contrast with anti-dsDNA, was not associated with depressed levels of either C3 or C4. Furthermore, we could show that, among patients with just anti-RNP, complement levels were not related to the IGS. These findings suggest that complexes formed by anti-RBPs, while associated with the IGS, appear to lack the requisite size or antigen distribution to activate complement. In this regard, previous studies indicated blood in patients with SLE contains ICs comprised of anti-RBPs.[49] Furthermore, studies on renal eluates have demonstrated the presence of anti-RBP antibodies.[50] It is possible, therefore, that anti-RBPs may form ICs that localise in the kidney, but they may not induce renal inflammation because, unlike anti-dsDNA, they do not activate complement.

While our results provide evidence that anti-dsDNA and anti-RBP antibodies differ in their ability to form ICs that likely activate complement, the basis of this difference is unclear. Previous studies have indicated that anti-RNP and other anti-RBP antibodies can activate complement when tested in in vitro assays such as a complement-fixing immunofluorescence assay (CFANA)[51–53]; the CFANA is similar to a classical ANA assay except for the use of immunofluorescence reagents to detect bound C3, C4 or properdin. In ANA assays of this kind, chemical fixation may alter the structure or distribution of the target antigens to facilitate antibody binding and complement fixation. Pending more information on the in vivo structure and location of nuclear antigens during disease, we can only speculate that the nature of DNA and RNP (and other RBPs) in patients differs with respect to antigen charge, density or size in ways that affect the binding of antibodies and complement engagement. Finally, the Ig heavy chain isotype of the autoantibodies may contribute to their biologic activity. In this regard, one study indicated that anti-RNP antibodies are primarily of the IgG2 subclass (which activate complement ineffectively),[54] whereas other studies have

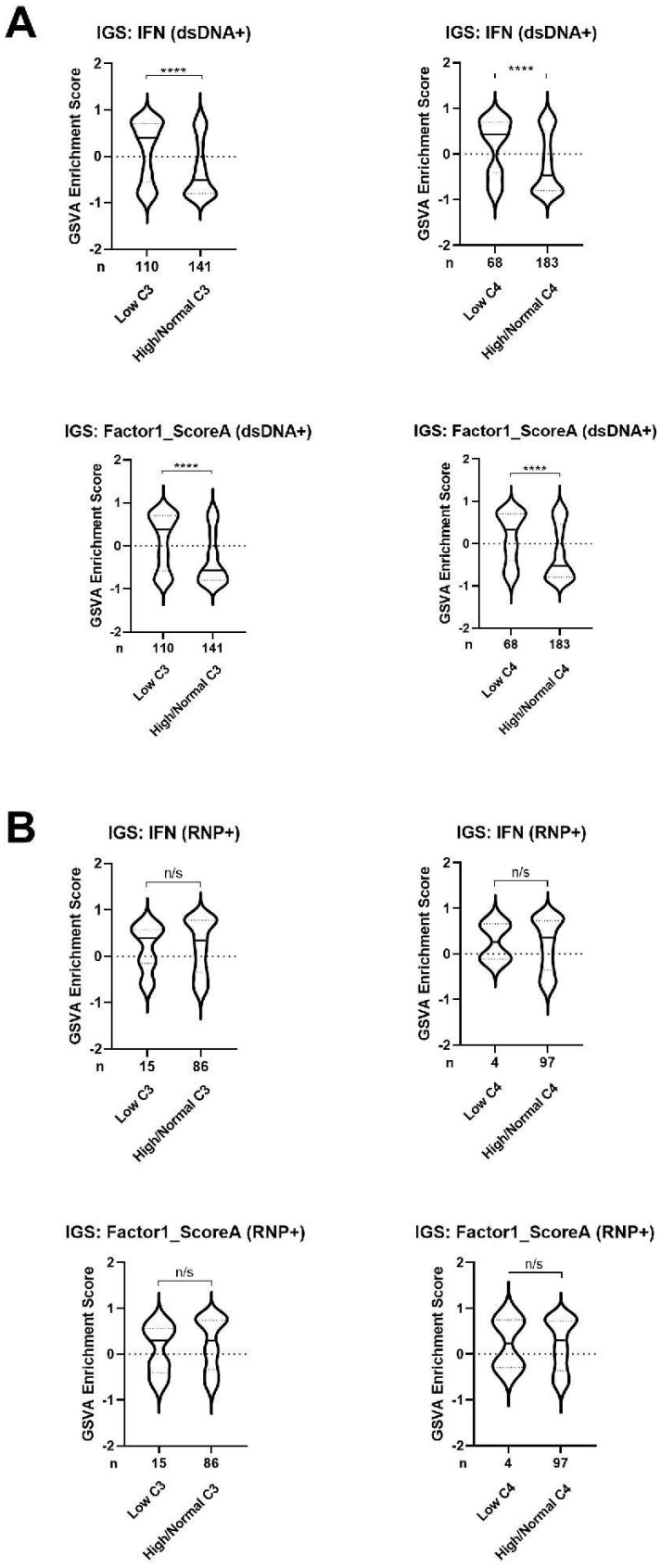

Figure 4 Gene set variation analysis (GSVA) was carried out on microarray data of systemic lupus erythematosus (SLE) patient whole blood, using various interferon (IFN) gene signatures (IGS). Subjects positive for anti-dsDNA only (A) or anti-RNP only (B) were stratified by the presence of low or normal/high complement C3 and C4 levels as shown. The Mann-Whitney U test was performed to determine significant differences in IGS enrichment between groups. Numbers of patients (n) in each of the comparator groups are annotated on the x-axis. Violin plots display median values (solid lines) and upper and lower quartiles (dashed lines). ****p<0.0001; n/s=not significant.

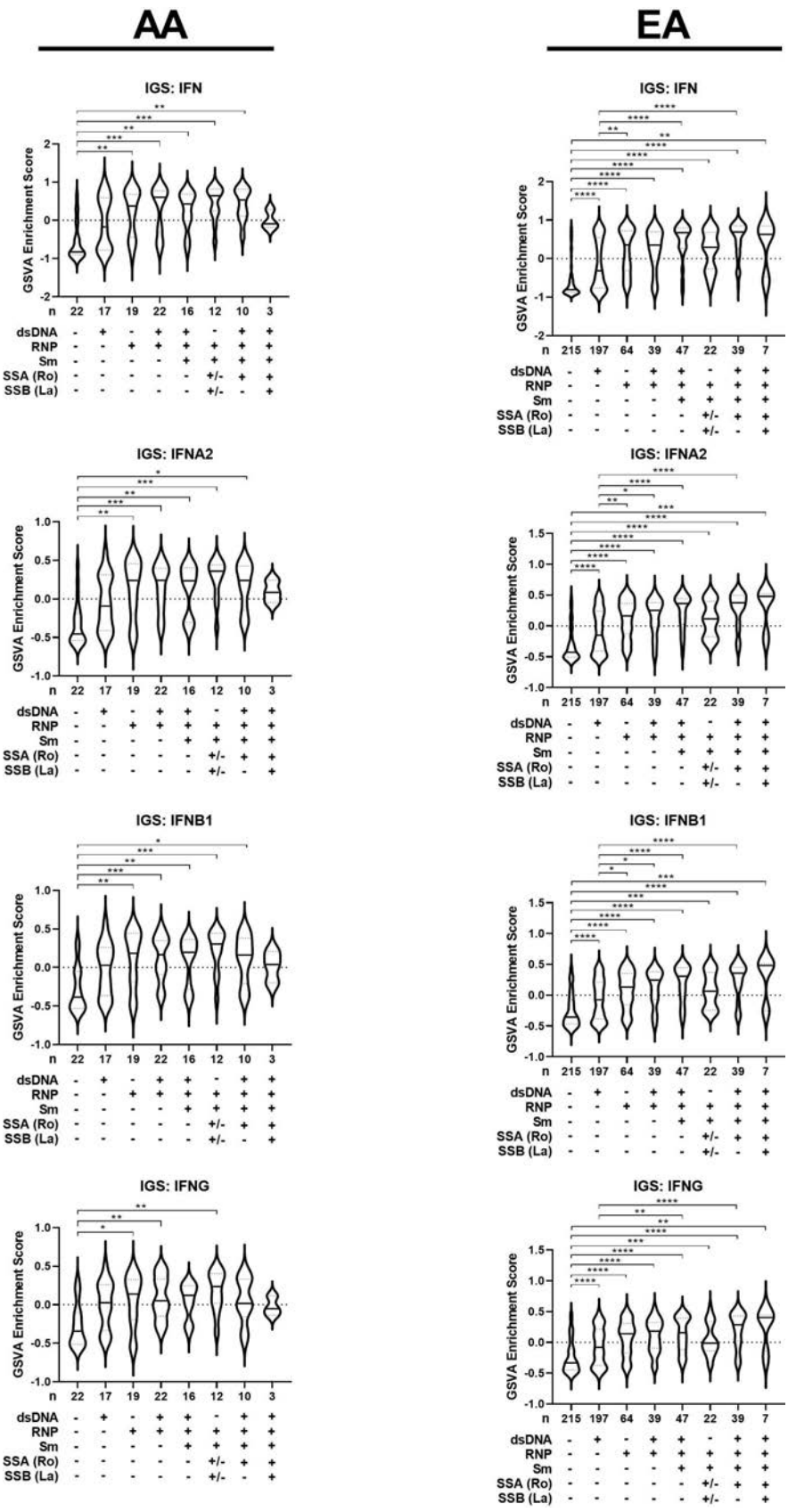

Figure 5 Overall, 121 African Ancestry (AA) and 630 European Ancestry (EA) active, female systemic lupus erythematosus (SLE) patients were stratified by the presence of five autoantibodies. Gene set variation analysis (GSVA) was carried out on microarray data of these patients using various interferon gene signatures (IGS). Dunn's multiple comparisons test was performed to determine significant differences in IGS enrichment among the groups. Numbers of patients (n) in each of the comparator groups are annotated on the x-axis. Violin plots display median values (solid lines) and upper and lower quartiles (dashed lines). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

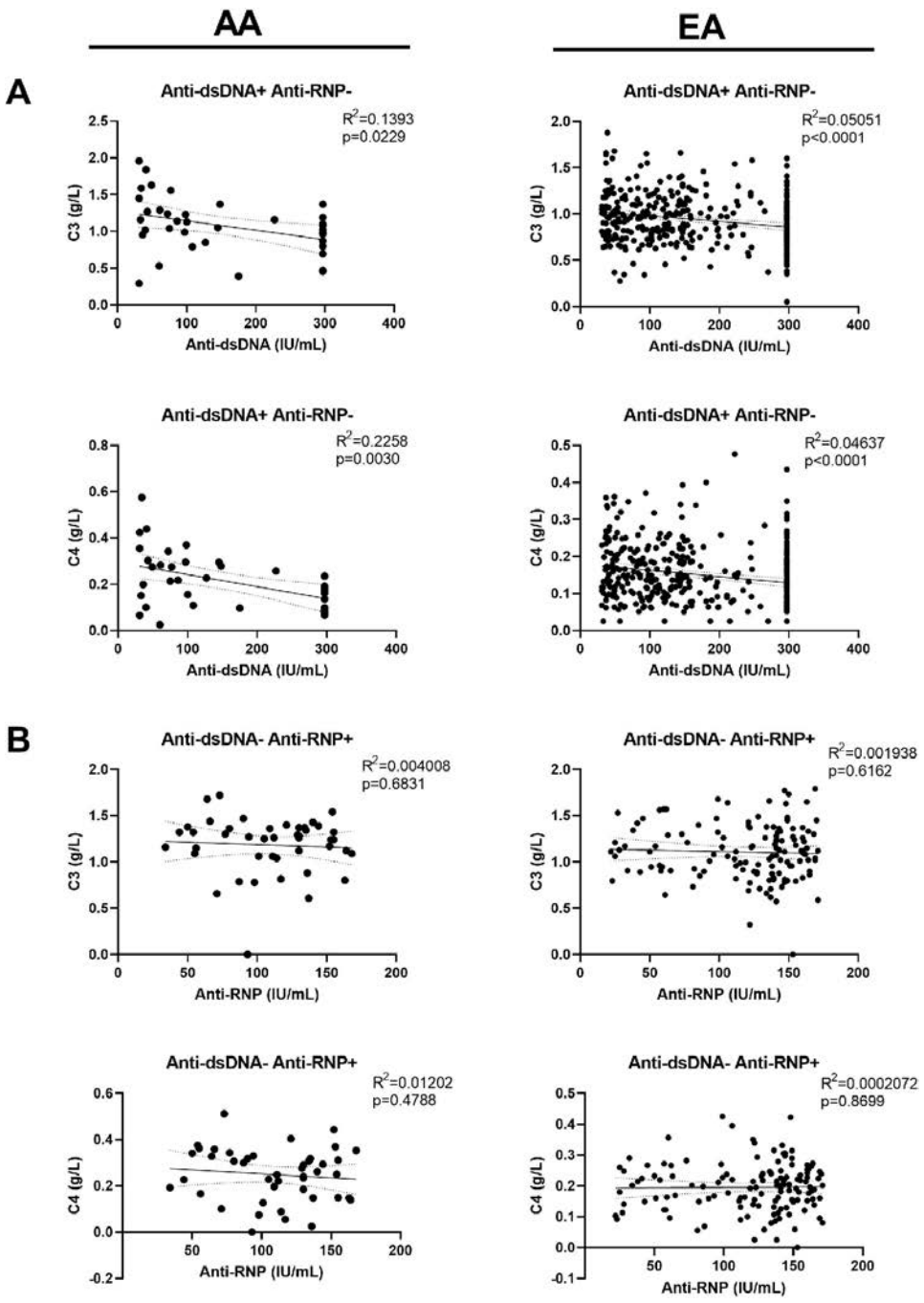

Figure 6 Linear regression analyses show relationships between autoantibody levels and complement levels in African Ancestry (AA) and European Ancestry (EA) systemic lupus erythematosus (SLE) patient whole blood, where each dot on the scatterplots represents one patient sample. (A) Represents 37 AA and 376 EA active, female SLE patients positive for anti-dsDNA but negative for anti-RNP antibodies. (B) Represents 44 AA and 132 EA active, female SLE patients positive for anti-RNP but negative for anti-dsDNA antibodies. Dotted lines represent 95% confidence bands of the best-fit line.

indicated that antibodies to RBPs are similar to anti-dsDNA and are predominantly IgG1, which engage complement efficiently.[55]

In characterising the impact of ancestry on serology, we showed that a much larger proportion of EA patients were anti-dsDNA− anti-RBP− or anti-dsDNA+ only, whereas AA SLE patients were more likely to be anti-RNP+, anti-dsDNA+ and anti-RNP+, or anti-dsDNA+, anti-RNP+ and anti-Sm+. Indeed, a significantly greater proportion of AA patients were positive for at least one autoantibody than EA patients. A larger proportion of AA patients were also anti-RNP+ compared to EA patients, whereas similar proportions of patients of each ancestry were anti-dsDNA+.

As has been previously reported,[15 22 23] we found a significantly stronger association between anti-RNP and the IGS in both AA and EA SLE. In AA, we noted no significant association between anti-dsDNA and the IGS, but a significant association with anti-RNP, whereas in EA SLE the association between the IGS and anti-RNP was significantly greater than that of anti-dsDNA and the IGS. This is consistent with a recent report noting an AA-specific IGS, characterised by greater enrichment in AA SLE

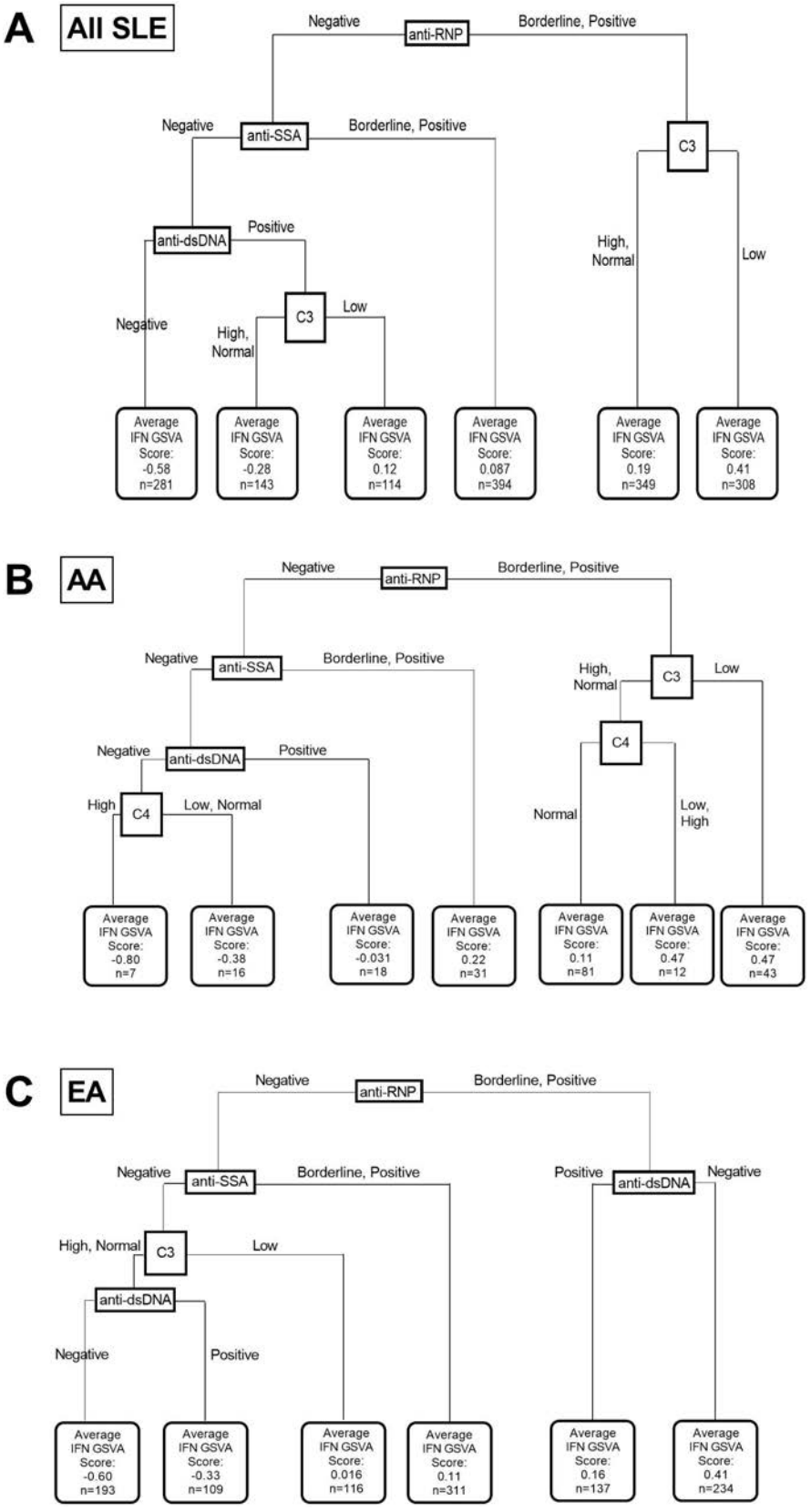

Figure 7 Classification and regression tree (CART) analysis was employed to determine the most important contributors to the core interferon gene signature (IGS) (shared by type I and II interferon (IFN)) as measured by gene set variation analysis (GSVA) enrichment scores. Resultant regression trees are visualised in (A) for all 1589 systemic lupus erythematosus (SLE) patients who had measurements of C3, C4 and all five autoantibodies, (B) 208/1589 SLE patients of African Ancestry (AA) and (C) 1100/1589 SLE patients of European Ancestry (EA). Autoantibody status was determined as follows: positive (<20 IU/mL), borderline (≥11 IU/mL and ≤20 IU/mL) or negative (<11 IU/mL). C3 levels were considered low if <0.9 g/L and normal if ≥0.9 g/L. C4 levels were considered low if <0.1 g/L and normal if ≥0.1 g/L.

patients than EA patients and enrichment occurring with lower frequency and magnitude in autoantibody-stratified EA than AA patients.[41] It is notable that we found that this subset of the IGS was more frequent in AA SLE, but it appeared to be more related to the presence of anti-RNP antibodies, which were more common in AA. Of note, CART also predicted anti-RNP antibodies to be the most important contributors of the IGS in AA SLE, with C3 and C4 the next most important contributors, in contrast with anti-dsDNA antibodies as the next most important contributors in EA SLE. These results suggest that there may be ancestral differences in the biologic functions of autoantibodies. Whereas anti-RNP was associated with the IGS in both ancestries, anti-dsDNA was only associated with the IGS in EA SLE.

We found that the measured autoantibodies can largely explain the presence of the IGS, since 86.3% of patients negative for all five autoantibodies lacked the IGS. We also observed a stepwise decrease in IGS enrichment with progression towards autoantibody negativity. Nevertheless, over half of patients expressing the IGS were positive for either anti-dsDNA or anti-RNP antibodies. These data suggest that, whereas the IGS is not required for autoantibody production, the presence of autoantibodies may be necessary for IGS induction. In this regard, we did not find differences in the IGS in patients related to HCQ use in anti-dsDNA+ anti-RNP− or anti-dsDNA− anti-RNP+ populations. These findings suggest either significant non-adherence among patients with antimalarial treatment at baseline or a lack of effect of these drugs on TLR signalling and IFN induction in treated patients. Still, 13.7% of patients lacking the five autoantibodies were IGS+, which may be explained by other mechanisms not involving ICs such as spontaneous MAVS oligomerisation as described by Buskiewicz *et al* or associations with other autoantibodies that were not measured in this study.[56]

While our findings involve large patient numbers, they may be limited by study of a clinical trial population. For entry, patients had to have active SLE but not active nephritis and no active CNS involvement; it is possible that other populations would show different biomarker relationships, thus these findings need not necessarily be generalised to the entire SLE population. Nevertheless, our findings indicate heterogeneity of ICs in SLE, thereby affecting the use of complement to infer the presence of ICs that induce IFN.

Acknowledgements The authors would like to acknowledge and thank Mary Artley for her assistance with the preparation of the manuscript. This work was funded by the RILITE Foundation. An abstract (https://acrabstracts.org/abstract/association-between-anti-rnp-antibodies-and-interferon-gene-expression-but-not-complement-consumption-in-sle/) describing this work was accepted for a poster presentation at the 2021 Annual Meeting of the American College of Rheumatology and is available online.

Contributors ELH analysed the data and wrote the manuscript. DSP and PEL contributed to the conception, design and oversaw conduct of the research, edited the manuscript and made the decision to publish; thus, DSP and PEL were the guarantors.

Funding This work was supported by a grant from the RILITE Foundation.

Competing interests The authors have no competing interests to disclose. ELH and PEL are salaried employees of AMPEL BioSolutions, LLC.

Patient consent for publication Not applicable.

Ethics approval This study does not involve human participants.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Data are available in a public, open access repository. Data were downloaded from Gene Expression Omnibus (GEO) under accession GSE88884.

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TRANSLATIONAL SCIENCE

Proteogenomic analysis of the autoreactive B cell repertoire in blood and tissues of patients with Sjögren's syndrome

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221604).

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Received 1 October 2021
Accepted 6 January 2022
Published Online First
10 February 2022

To cite: Broeren MGA, Wang JJ, Balzaretti G, *et al*. *Ann Rheum Dis* 2022;**81**:644–652.

ABSTRACT

Objective To comparatively analyse the aberrant affinity maturation of the antinuclear and rheumatoid factor (RF) B cell repertoires in blood and tissues of patients with Sjögren's syndrome (SjS) using an integrated omics workflow.

Methods Peptide sequencing of anti-Ro60, anti-Ro52, anti-La and RF was combined with B cell repertoire analysis at the DNA, RNA and single cell level in blood B cell subsets, affected salivary gland and extranodal marginal zone lymphomas of mucosa-associated lymphoid tissue (MALT) of patients with SjS.

Results Affected tissues contained anti-Ro60, anti-Ro52, anti-La and RF clones as a small part of a polyclonal infiltrate. Anti-Ro60, anti-La and anti-Ro52 clones outnumbered RF clones. MALT lymphoma tissues contained monoclonal RF expansions. Autoreactive clones were not selected from a restricted repertoire in a circulating B cell subset. The antinuclear antibody (ANA) repertoires displayed similar antigen-dependent and immunoglobulin (Ig) G1-directed affinity maturation. RF clones displayed antigen-dependent, IgM-directed and more B cell receptor integrity-dependent affinity maturation. This coincided with extensive intra-clonal diversification in RF-derived lymphomas. Regeneration of clinical disease manifestations after rituximab coincided with large RF clones, which not necessarily belonged to the lymphoma clone, that displayed continuous affinity maturation and intra-clonal diversification.

Conclusion The ANA and RF repertoires in patients with SjS display tissue-restricted, antigen-dependent and divergent affinity maturation. Affinity maturation of RF clones deviates further during RF clone derived lymphomagenesis and during regeneration of the autoreactive repertoire after temporary disruption by rituximab. These data give insight into the molecular mechanisms of autoreactive inflammation in SjS, assist MALT lymphoma diagnosis and allow tracking its response to rituximab.

Key messages

What is already known about this subject?

► Sjögren's syndrome features activated B cells in affected tissues, aberrancies in circulating B cell populations and autoantibodies, including anti-Ro60/SSA, antiRo52/SSA, anti-La/SSB and rheumatoid factors (RFs).

What does this study add?

► RF and antinuclear antibody (ANA) clones are enriched in affected tissues where they occur as a small part of a polyclonal repertoire. RF and ANA clones affinity maturate in divergent fashion, which increases during secondary RF lymphomagenesis and after temporary disruption by rituximab (RTX).

How might this impact on clinical practice or future developments?

► Analysis of RF clones in affected tissues may assist identification of mucosa-associated lymphoid tissue lymphomas and tracking of their response to RTX.

INTRODUCTION

Sjögren's syndrome (SjS) is a systemic autoimmune disease, principally affecting exocrine glands. It features activated B cells in affected tissues, aberrancies in circulating B cell populations and circulating autoantibodies (AutoAbs), including antinuclear antibodies (ANAs) anti-Ro60/SSA, anti-Ro52/SSA, anti-La/SSB and rheumatoid factors (RFs).[1–3] Although the precise role of autoreactive B cells in the pathogenesis of SjS is less well defined, the pathogenic role of these AutoAbs is suggested by animal experiments[4 5] and clinical observations.[6] Antibodies produced by lymphomas that develop in up to 10% of patients with SjS, most commonly mucosa-associated lymphoid tissue (MALT) lymphomas, express immunoglobulins (Igs) with RF activity.[7–10]

The generation of the ANA-specific and RF-specific B cell repertoires and how they breach self-tolerance checkpoints has not been precisely determined. In experimental animal models, autoreactive B cells affinity maturate in antigen-dependent fashion in germinal centres in lymphoid tissues or in extrafollicular sites such as the splenic marginal zone.[11–13] In some models, this involves stochastic selection of follicular B cells after an environmental stimulus in a genetically predisposed host. In other models, autoreactive B cells are generated from extrafollicular, polyreactive precursor B cells.[14]

The generation of antinuclear antigen-reactive B cells in affected tissues of patients with SjS has been related to positive selection of polyreactive precursor B cells.[15 16] Selection of these clones was enhanced by N-glycosylation sites in the B cell receptor variable region, resulting in activation by C-type lectins.[16] RF clones have been suggested to be selected from extrafollicular precursors.[17]

Evidence suggests that the same mechanisms operative in SjS might also contribute to generation of SjS-associated RF-derived MALT lymphomas. Lymphomagenesis of RF clones results from gradual accumulation of lymphoma driver mutations.[10] These lymphomas are clonally related to reactive B cell aggregates in the same salivary gland (SG). The latter are frequently organised as ectopic germinal center (GC)-like structures and display functional features.[18 19] High levels of somatic hypermutation (SHM) and intra-clonal variation in these lymphomas suggest that ectopic GCs allow RF B cell clones to proliferate and maturate, resulting in somatic mutations and MALT lymphoma development.[18 20–22] It is unknown why the RF repertoire is prone to oncogenic transformation compared with the ANA repertoire. MALT lymphomas can respond well to rituximab (RTX) mediated B cell depletion, but will eventually relapse.[8] Hypothetically, the ANA and RF repertoire regenerates in a different manner together with the lymphoma clones.

Herein, we combined mass spectrometry (MS) approach for serum antibody sequencing with methods to analyse the B cell repertoire at the RNA, DNA and single cell level to investigate the selection and affinity maturation of the autoreactive B cell repertoire in blood and tissues of patients with SjS.

METHODS

Study subjects

The autoreactive B cell repertoire was analysed in six patients with SjS, fulfilling the 2016 ACR-EULAR classification criteria, in comparison to 4 age-matched and 3 elderly healthy controls (table 1). Among patients with SjS, B005 and B007 developed MALT lymphoma and were treated with RTX. An overview of the blood and tissue sampling for all six patients is shown in online supplemental figure 1. Two additional patients, B012 and B013, were followed by B cell clonality testing of biopsies before and during diagnostic work up for MALT lymphoma diagnosis. The B cell repertoire in blood was analysed in 24 patients with SjS, fulfilling the 2016 ACR-EULAR classification criteria, 10 disease controls (systemic lupus erythematosus (LE), subacute cutaneous LE, systemic sclerosis and rheumatoid arthritis) that tested positive for anti-Ro and/or anti-La antibodies and 24 healthy controls (table 2). All study subjects gave written informed consent prior to inclusion in the study. For more details about the study subjects, see online supplemental methods.

Table 1 Patient characteristics at time of first study biopsy

Patient code	Sex	Age (years)	Duration since diagnosis (years)	IgM-RF titre (ie, /mL, cut-off: 10 mL)	IgG titre (g/L, cut-off: 16 g/L)	ESSDAI
B005	F	59	30	170	18	4
B007	M	63	7	4500	25	11
B008	M	61	1	48	19	6
B009	F	30	10	280	26	2
B010	F	74	21	15	15	2
B011	F	46	2	148	22	3
HC1	F	59	–	–	–	–
HC2	F	60	–	–	–	–
HC3	F	23	–	–	–	–
HC4	M	30	–	–	–	–
HC5	M	81	–	–	–	–
HC6	F	82	–	–	–	–
HC7	F	80	–	–	–	–

ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index.

Overview of analysis of Igs in blood and tissues

In six patients and four age-matched and three elderly healthy controls, the B cell repertoire was determined by heavy chain Ig mRNA analysis using next-generation sequencing (NGS; Ig-RNAseq) in blood and tissue samples. In blood samples, a comparison was made between whole blood samples and sorted B cell subsets. This was done to account for the bias toward abundant plasmablast/-cell reads in the blood samples. Sorting of B cells from tissues for clonality is challenging and cannot be performed on stored tissue samples. Therefore, in affected patient tissues, we made a comparison between Ig-RNAseq and NGS at the DNA level for Ig heavy and light chain (Ig-DNAseq) that we validated for clonality testing.[23] This protocol allows to determine the presence of clonal expansions, B cells with non-productive rearrangements and compensates for the mRNA abundance in plasma cells compared with memory B cells. We analysed two tissues at clinical relapse after RTX using a combination of bulk Ig-RNAseq and 10× Genomics general and Ig single cell RNA sequencing (sc-RNAseq and Ig-sc-RNAseq). Finally, we analysed the prevalence of autoreactive sequences in the overall B cell repertoire using MS sequence analysis (MS-seq). For this, we affinity-purified four generally prevalent AutoAbs in SjS from serum and analysed their complementarity determining region 3 (CDR3) amino acid sequences. The resulting MS sequences for anti-Ro60, anti-Ro52, anti-La and RF were aligned after blinding with all heavy chain Ig-RNAseq, Ig-DNA-seq and Ig-sc-RNAseq data from blood and tissue samples for the mapping of autoreactive peptide sequences of the same patient. The antigen-specific B cell clones in the circulation and tissues were then identified based on the matched CDR3 peptides. An overview of the methods performed for each sample is provided in online supplemental figure 1. Online supplemental figure 2 presents an overview of analyses performed in patients B005 and B007, the two patients with MALT lymphoma that were treated with RTX and followed in time. For more details about the experimental and bioinformatic methods, see online supplemental methods.

RESULTS

Memory B cell subsets in blood and tissues of patients with SjS contain expanded clones

First, we analysed the anti-Ro/anti-La and RF repertoire in blood and affected tissues from six patients with SjS. Ig-RNAseq showed that a higher fraction of Ig reads from affected tissues compared with blood mapped to MS proteomic sequences of AutoAbs ($p<0.01$; figure 1A).

To gain more insight in the B cell repertoire in blood of patients with SjS with anti-Ro/anti-La, we compared these (n=26) with patients with other anti-Ro/anti-La positive autoimmune disease (n=10) and healthy controls (n=24). Most Ig sequences detected in blood were low abundant clones, but a number of sequences was highly expressed (HES: >0.5% of total Ig reads, based on previous observations[24]) (figure 1B; online supplemental table 1 for total reads). The number of HESs did not differ significantly between the groups or in patients with RF, although there was a numerical trend toward a higher number of HESs in disease groups compared with healthy controls (table 2). Other

Table 2 Clinical and disease characteristics and B cell repertoire features in patient groups

	SjS (n=26)	Disease controls (n=10)	Healthy controls (n=24)
Female, %	73	70	46
Age in years, mean±SD	54.4±15.3	57±11.8	56.6±14.0
Diagnosis disease controls			
SLE, n	–	5	–
SCLE, n	–	3	–
Other, n	–	2	–
Individuals tested for virology, n	24	6	21
CMV-IgG positive, %	42	50	38
VCA-IgG positive, %	92	100	86
EBNA-IgG positive, %	79	67	76
Anti-SSB, n	22	8	–
Anti-SSA, n	26	9	–
RF, n	16	2	–
RF titres in IU/mL, median (IQR)	100 (41.3–207.5)	101 (11–191)	–
SLE antibodies, n	5	4	–
Anti-CCP, n	2	1	–
Individuals tested for serum IgG, n	23	3	–
IgG positive, %	60.9	66.7	–
IgG titres in IU/mL, mean±SD	20.9±4.8	19±7.8	–
Serum cryoglobulins, n	3	1	–
Serum M protein, n	1	0	–
Leucopenia, n	9	2	–
Neutropenia, n	3	1	–
Lymphopenia, n	7	4	–
ESSDAI, median (IQR)	2 (1–2.3)	1 (0–2)	–
Maximal past ESSDAI, median (IQR)	3 (2–5.3)	1.5 (0.3–8)	–
ESR in mm/hour, median (IQR)	20 (7.5–28.5)	8 (2–43)	
Concomitant Raynaud, n	15	5	–
Concomitant arthritis, n	4	3	–
Organ involvement, n	5	3	–
WB HES, median (IQR)	2.5 (0.8–6.5)	3.5 (0–7.8)	0.5 (0–4.8)
Percentage of clones with SHM >0 in WB, median (IQR)	60.5 (50.8–75.6)	69.2 (57.6–81.3)	60.1 (52.7–65)
Percentage of clones with N-glycosylation sites >0 in WB, median (IQR)	6 (5–7.6)	7.8 (5.7–9.5)	5.6 (5.2–6.5)
CDR3 length in WB, mean±SD	24±0.4	23.8±0.4	24.3±0.1

CCP, cyclic citrullinated peptides; CDR3, complementarity determining region 3; CMV, cytomegalovirus; EBNA, EBV nuclear antigen; ESR, erythrocyte sedimentation rate; ESSDAI, EULAR Sjögren's Syndrome Disease Activity Index; HES, highly expressed sequences; Ig, immunoglobulin; RF, rheumatoid factor; SCLE, subacute lupus erythematosus; SHM, somatic hypermutation; SjS, Sjögren's syndrome; SLE, systemic lupus erythematosus; VCA, virus capsid antigen; WB, whole blood.

repertoire features, that is, the extent of SHM, CDR3 characteristics and the number of potential N-glycosylation sites, were also similar between groups.

We compared the repertoire in blood and affected tissues in the six patients with SjS in whom biopsies were acquired. HESs detected by Ig-RNAseq are predominantly expanded plasmablasts/-cell clones, since these produce large amounts of Ig compared with memory B cells. Therefore, we compared HESs in whole blood samples to sorted memory B cell subsets. HESs were detected in unswitched memory (UM), switched memory (SM) and double-negative (DN) B cells in contrast to naïve B cells (figure 1B).

Tissues contained a similar number of HESs compared with circulating memory B cell subsets. The SHM load was similar between circulating memory B cell subsets and affected tissues (online supplemental figure 4A). The number and SHM load of HESs were lower in whole blood in line with a larger proportion of naïve B cells in those samples. The overlap between the clones in the tissues and in the circulating subsets was low, both for the complete set of Ig sequences and for the HESs (online supplemental figure 4B,C). The overlap in HESs was highest between tissue and whole blood, possibly because of circulating plasma cells in the latter samples. In only one patient, there was 30% overlap between HESs in SG tissue and circulating SM B cells. In summary, circulating memory B cell subsets contain expanded clones, but these only occasionally concern clones from affected tissues.

Tissues are enriched for autoreactive clones

We analysed the relationship between HESs and AutoAbs in blood. A small proportion of the B cell repertoire in whole blood and sorted B cell subsets of patients with SjS mapped to amino acid sequences of AutoAbs (figure 1A). RFs were present in the UM and DN populations and ANAs in SM and DN populations. None of the HESs in whole blood or sorted B cell subsets mapped to AutoAbs. AutoAb titres were not correlated to the number of HESs or AutoAb reads. Taken together, memory B cell subsets in blood of patients with SjS contain expanded clones, but these do not concern anti-Ro52, anti-Ro60, anti-La or RF clones.

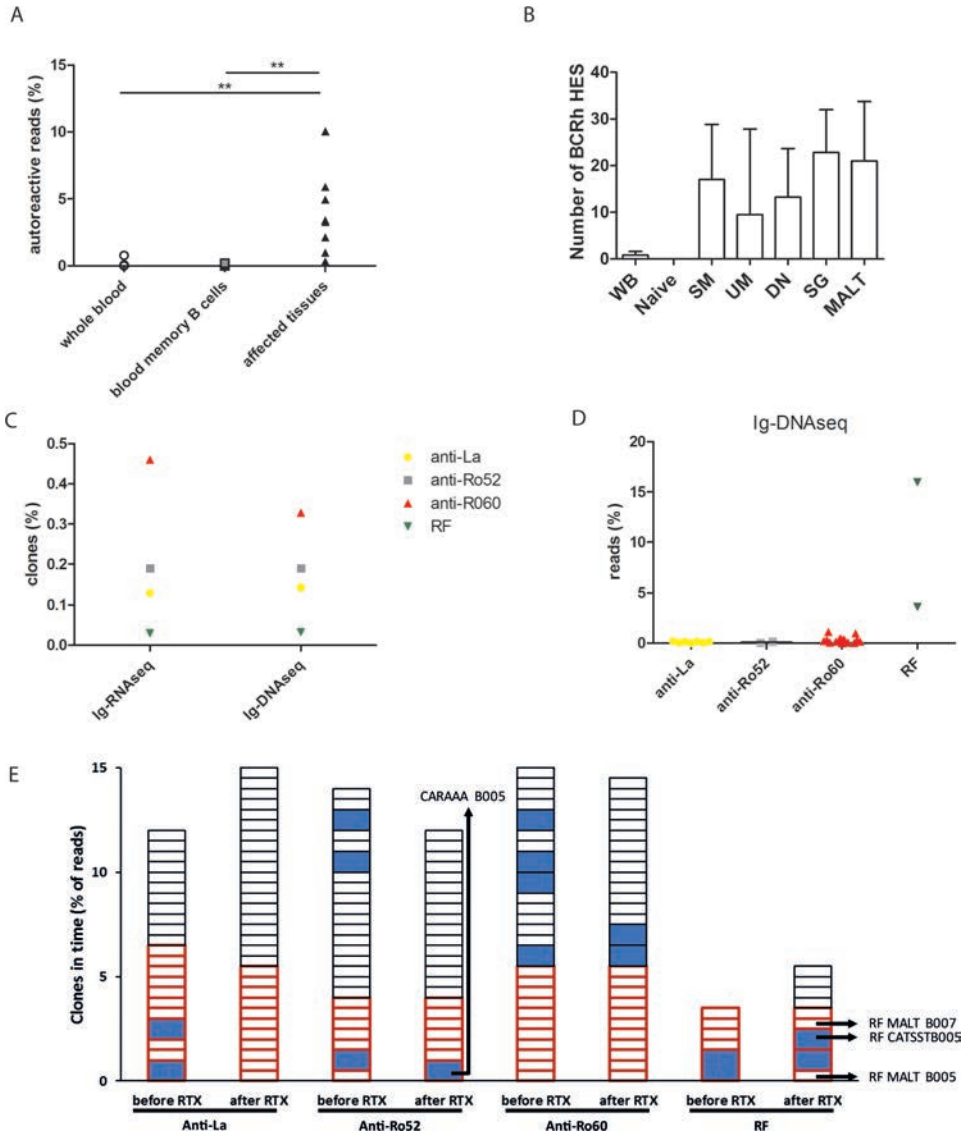

Figure 1 Analysis of autoreactive B cell clones in patients with SjS. (A) The presence of autoreactive B cell clones in blood versus tissues of six patients with SjS. The percentage of autoreactive Ig-RNA reads of all Ig from the same compartment per patient is shown. WB and sorted memory B cell subsets from blood were compared with affected tissues. Pooled analyses are shown for the sorted UM, DN and SM B cell subsets and for collected tissues (SG and MALT lymphoma); (B) the presence of HESs in WB, naive and memory B cell subsets sorted from blood and SG and MALT tissues. Sorted cells were UM B cells (CD19 +, CD38−, IgD+ and CD27+), DN B cells (CD19 +, CD38−, IgD− and CD27−) and SM B cells (CD19 +, CD38−, IgD− and CD27+); (C) mean percentage of clones in affected tissues that mapped to proteomic sequences of serum anti-Ro52, anti-La, anti-Ro60 and RF AutoAb per patient comparing Ig-RNAseq and Ig-DNAseq; (D) Ig-DNAseq analysis of the percentage of autoreactive reads of all Igs from the same tissue in MALT lymphoma tissues; (E) the presence of autoreactive clones in SG and MALT tissues before and after RTX for patients B005 and B007 in Ig-RNAseq. Small squares indicate clones of up to 0.5% of reads. Larger squares (in blue) depict larger clones with number of reads rounded off to multiples of 0.5%. The legend depicts the first amino acids of the CDR3 sequence of large clones of interest. Squares with red bold border line indicate clones that are detected before and after RTX. Clones were defined by sequences with the same V and J segments and the same CDR3 region. Yellow dots show anti-La, grey squares anti-Ro52, red triangles anti-Ro60, green squares RF and dark-green MALT. Panels A shows medians and IQRs. **p<0.01. AutoAb, autoantibodies; CDR3, complementarity determining region 3; DN, double-negative; HESs, highly expressed sequences; Ig, immunoglobulin; MALT, mucosa-associated lymphoid tissue; RF, rheumatoid factor; RTX, rituximab; SG, salivary gland; SjS, Sjögren's syndrome; SM, switched memory; UM, unswitched memory; WB, whole blood.

Affected tissues contain a polyclonal repertoire, including ANA and RF clones and monoclonal RF expansions in MALT lymphoma tissues

To gain more insight in the autoreactive repertoire in affected tissues, we comparatively analysed clones with immunohistochemistry (IHC), Ig-RNAseq and Ig-DNAseq using a protocol that we developed and validated for detection of B cell clonality by NGS in stored tissues.[23] HESs in Ig-DNAseq are equally likely to be memory B cell or plasma cell clones. IHC with B cell and plasma cell markers aids differentiation between B cell and plasma cell expansions.

Morphology/IHC for CD20, CD79a, kappa and lambda showed a variable infiltration by B cell follicles (CD20+) and plasma cells (CD79a+/CD20−) in affected SG tissues (online supplemental figure 3). The MALT lymphoma tissues showed more than 50% CD20+ B cells and variable plasma cell infiltration.

Both Ig-RNAseq and Ig-DNAseq showed the presence of a polyclonal repertoire with a variable extent of HESs in all tissues. ANA and RF clones constituted a small fraction of clones (figure 1C). MALT lymphoma tissues of B005 and B007 showed a highly expanded B cell clone in Ig-DNAseq based on the presence of clonal Ig heavy and light chain rearrangements. The most abundant Immunoglobulin Heavy Locus framework 3 (IGH-FR3) clonotype in both MALT lymphoma tissues was detected at 3% and 11% of all IGH clonotypes (B005 and B007, respectively (figure 1D; online supplemental data)), which is less than the estimated tumour load. This was probably caused by impaired primer annealing because of SHM of the IGHV–IGHJ rearrangements. In both MALT lymphomas, clonal light chain rearrangements were detected in higher abundance than the IGH-FR3 clonal rearrangement (B005: 64% and B007: 28% (online supplemental data)), which can be explained by the absence of SHM in the Immunoglobulin kappa (IGK) locus: clonal IGKV–IGKJ rearrangements (in B007) do hardly and an intron K-deleting element rearrangements (in B005) do not undergo SHM. The expanded clones mapped to serum MS-seq RF in both patients. In summary, ANA and RF clones are enriched in affected tissues as a small fraction of a polyclonal infiltrate. The analysed MALT lymphomas were monoclonal RF expansions.

At relapse after RTX treatment, RF clones can occur as a mix of small and large clones

We analysed the regeneration of the autoreactive repertoire in affected tissues after temporary perturbation with RTX monotherapy in two patients with MALT lymphoma (see online supplemental methods). Ig-RNAseq analysis at relapse after RTX showed that the RF lymphoma clone in B005 was only detectable as a small clone (figure 1E). Seven other RF clones occurred, two of which were large. Four of the RF clones were new, three were already detectable before RTX (figure 1E). In B007, the single RF lymphoma clone persisted as a single RF clone.

Similarly, the ANA clones persisted or disappeared after RTX and new clones appeared (figure 1E). Similar to affected tissues before RTX, most ANA clones were small clones and some large, occurring as a small proportion of a polyclonal infiltrate.

RF and ANA repertoires show stochastic selection

We next analysed gene segment usage of ANA and RF clones in pooled samples. The number of anti-Ro60, anti-La and anti-Ro52 clones was higher than the number of RF clones (figure 2A; online supplemental figure 4D)($p<0.05$). All ANA clones of which the isotype could be determined used an IgG1 constant domain, with the exception of two anti-La IgM and two anti-Ro60 IgA1 clones (figure 2B). In contrast, all RF antibodies were IgM.

Analysis of IGHV gene use revealed that RF clones used a limited number of IGHV gene segments with predominant use of IGHV1-69, combined with IGHJ4 and an IGKV3-20 light chain (figure 2C). IGHV1-69, IGHJ4 and IGKV3-20 were used by the MALT lymphomas and the large RF clones that expanded after RTX. This is a stereotypic RF clonotype for SjS-associated MALT lymphoma.[25] In mice, RFs were generated from extrafollicular B cells from the splenic marginal zone that circulate as UM B cells.[13 26] In our cohort, expanded clones in UM B cells were not shared with other memory B cell subsets, indicating a unique origin (online supplemental figure 5B). However, shared stereotypic RF sequences were not enriched in UM B cells or their HESs in blood of healthy controls or the same patients with SjS (online supplemental figures 7 and 8). This implies that stereotypic RF selection is driven stochastically by tissue-specific factors.

Earlier studies suggest that ANA may be derived from polyreactive B cell precursors that share sequence motifs.[14–16] In line with a polyreactive nature, 13% of anti-Ro60, anti-Ro52 and anti-La clones precipitated with 2 out of 3 ANA (online supplemental table 2). ANA clones shared a preference for IGHV3-23, IGHV1-18, IGHV3-74 and IGHV4-61 usage in their variable domains, in line with our previous studies.[27 28] This preference diverged from RF and the IGHV usage in whole blood samples (figure 2C). The IGHV segments preferred by ANA were not enriched in naïve or memory B cell subsets or expanded clones in blood of patients with SjS vs healthy controls (online supplemental figures 4–6). Taken together, ANA display signs of polyreactivity and similar stochastic tissue-restricted selection that differs from RF clones, despite recognising different antigens.

RF MALT lymphoma clones and large non-MALT RF clones at relapse after RTX show continuing SHM and high intra-clonal diversification

Analysis of affinity maturation showed that the SHM load of RF clones was relatively low compared with ANA clones (figure 2D). The SHM load was somewhat higher in MALT RF compared with other RF clones. After RTX, large RF and MALT RF clones accumulated additional SHM, while the SHM load of ANA clones decreased (figure 2D). This indicates that SHM continues in MALT lymphoma clones and large non-MALT RF clones after RTX.

Before RTX intra-clonal diversification was similar between ANA and RF clones (figure 2E). RF MALT clones showed large intra-clonal diversity compared with other RF clones (figure 2E), suggesting a role for aberrant intra-clonal diversification and increased cell survival in MALT lymphomagenesis from RF clones. After RTX large non-MALT RF clones displayed increased intra-clonal diversification (figure 2E). To allow analysis of Ig expression at the individual cell level, Ig-sc-RNAseq was performed in the tissues, obtained freshly at relapse after RTX (online supplemental figures 1 and 2). In advantage to bulk Ig-RNAseq, sc-Ig-RNAseq allows a precise analysis of intra-clonal diversification, since many Ig reads are sequenced per cell. In addition, it allows parallel analysis of general RNA expression per cell. Ig-sc-RNAseq confirmed that in both patients the most expanded RF clones at relapse after RTX displayed extensive intra-clonal diversification (figure 3A,B). The intra-clonal diversity of ANA clones was unaltered after RTX (figure 2E). The largest ANA clone displayed little intra-clonal diversification (figure 3C). General sc-RNAseq showed that RF clones consisted of a mixture of memory B cells, plasma cells and proliferating germinal centre-like cells (figure 2F). In contrast, ANA clones mainly concerned plasma cells. Proliferating B cells, including RF and ANA B cells, could be discerned as a separate cluster in principal component analysis. These expressed proliferation markers, such as MKI67, CDK1 and CDC20. A proportion expressed AICDA and BCL6 indicative of T cell dependent activation. Mutation analysis confirmed AICDA mediation (figure 3E,F). Taken together, during regeneration of disease manifestations after therapeutic B cell depletion, both MALT and non-MALT RF clones can regenerate as large clones in affected tissues and show continuous affinity maturation, accompanied by marked intra-clonal diversification.

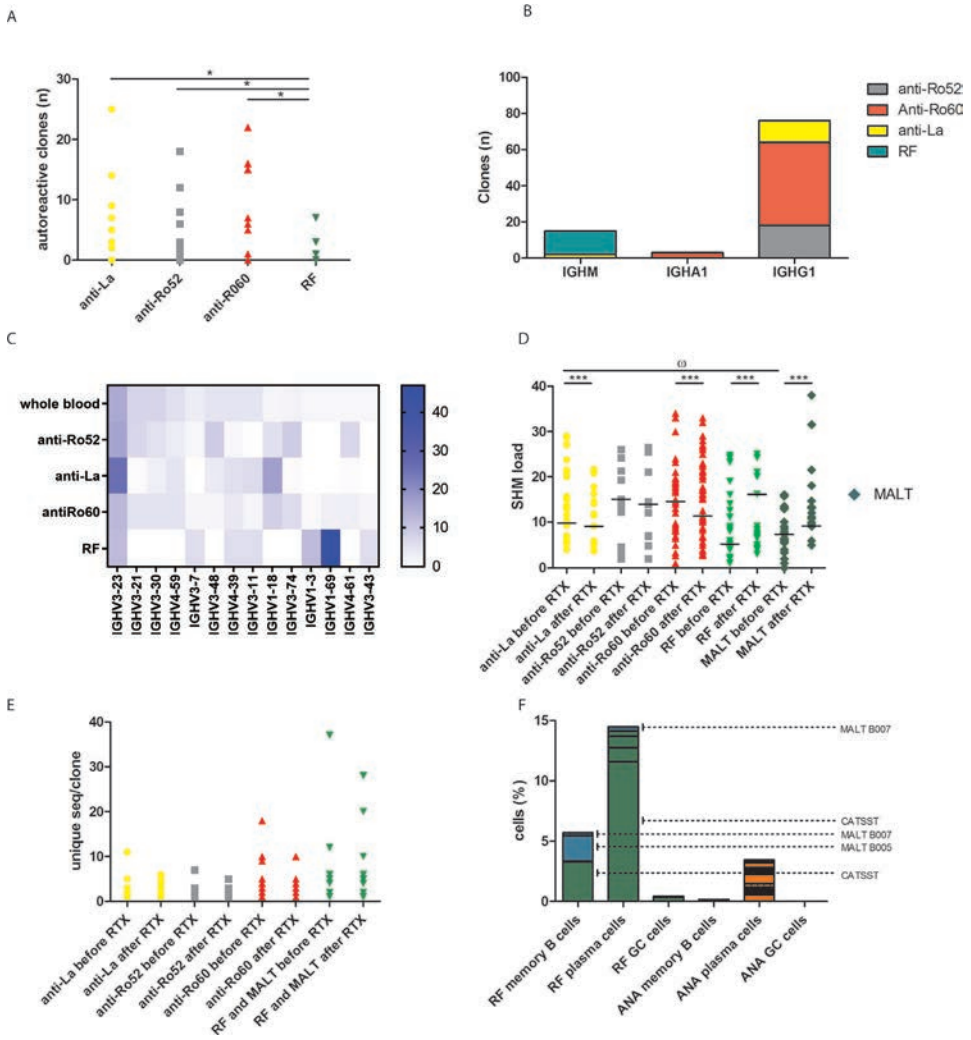

Figure 2 Analysis of selection and affinity maturation of autoreactive B cell clones. Yellow dots show anti-La, grey squares anti-Ro52, red triangles anti-Ro60, green squares RF and dark-green MALT. (A) The number of clones that mapped to sequences of serum anti-Ro52, anti-La, anti-Ro60 and RF AutoAb in all tissue samples before and after RTX. *p<0.05.; (B) isotype of the autoreactive clones of which the constant domain could be successfully attributed for pooled samples; (C) heatmap of all IGHV genes of whole blood samples versus anti-Ro52, anti-Ro60, anti-La and RF clones obtained from pooled samples; (D) SHM load of autoreactive clones before versus after RTX in pooled samples in Ig-RNAseq. Bars show medians: $^{\omega}$p<0.05 for post-hoc Bonferroni-corrected comparisons between all groups before RTX. Medians are shown as: ***p<0.001. (E) Intra-clonal variation of autoreactive clones before versus after RTX in pooled samples in Ig-RNAseq. Unique sequences per clonotype are defined by sequences with the same V and J segments and the same CDR3 region, allowing for two mismatches in CDR3 in Ig-RNAseq. (F) sc-RNAseq was performed in duplo in unselected cells retrieved from biopsies of tissues affected by MALT lymphoma in patients B005 (SG) and B007 (lymph node). Samples were obtained at disease relapse after RTX. T-distributed stochastic neighbor embedding (t-SNE) mapping was performed that identified memory B cells (CD79A+ and CD138–), plasma cells (CD79A+ and CD138+) and germinal center (GC)-like B cell (CD79A+ and MKI67+) clusters. Depicted are the number of cells for each autoreactive clone in these clusters of the pooled samples. Green panels show data for RF clones, blue for MALT RF clones and orange for ANA clones. The largest RF clone in the sample of B005 is shown in the legend with the first amino acids of its CDR3 (CATSST). ANA, antinuclear antibody; AutoAb, autoantibodies; CDR3, complementarity determining region 3; Ig, immunoglobulin; MALT, mucosa-associated lymphoid tissue; RF, rheumatoid factor; RTX, rituximab; sc-RNAseq, single cell RNA sequencing; SG, salivary gland; SHM, somatic hypermutation.

ANA clones display signs of similar antigen-driven affinity maturation that differs from RF clones

We analysed the antigen dependence of affinity maturation in ANA and RF clones using the BASEline tool (see online supplemental methods). Analysis of SHM patterns showed that all autoreactive clones displayed a similar antigen-dependent selection pressure (figure 3D). However, RF clones displayed a stronger negative selection pressure on the framework region (FR) compared with ANA clones, indicating that structural integrity of the variable gene segments is most important for positive selection of RF clones.

In an earlier study, selection of recombinantly expressed Igs from SjS tissues was enhanced by N-glycosylation sites in the B cell receptor variable region FR1, resulting in activation by C-type lectins.[16] Few ANA and RF clones had N-glycosylation sites in FR1 (0%–10%). Most were present in FR3 or CDR2 without clear-cut differences in number between AutoAb specificities.

Potential clinical utility of Ig-seq to assist lymphoma diagnosis

Finally, we analysed the potential utility of Ig-seq to assist lymphoma diagnosis in patients with SjS. For this, Ig clonality assessment was

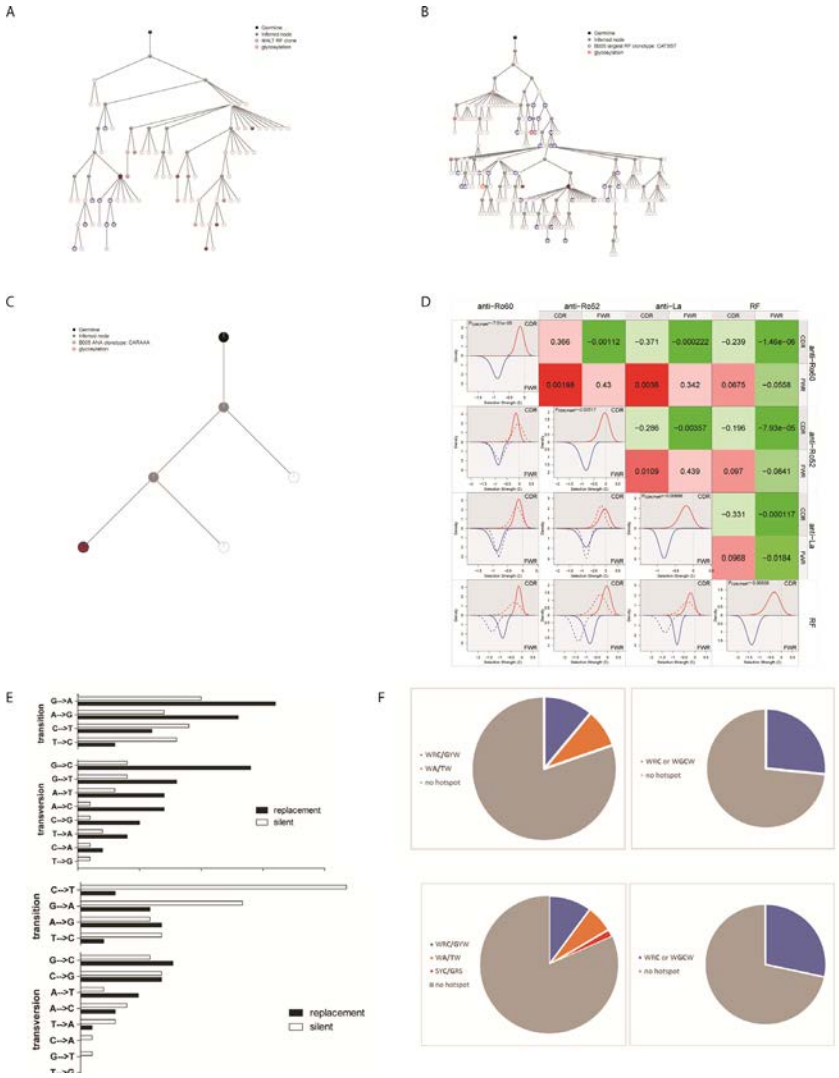

Figure 3 Mutation patterns of affinity maturation of autoreactive B cell clones. (A–C) Lineage tree analysis of the B007 MALT RF clonotype (CAREMD, 86 cells (A)), the B005 largest RF clonotype (CATSST, 519 cells (B)) and the B005 single large ANA clonotype (CARAAA anti-Ro52, 79 cells (C)) in the sc-Ig-RNAseq of patients B005 and B007 tissues at clinical relapse after RTX. Clonotypes are defined as clones with a similar V(D)J assignment to the heavy and light chains and a maximum of two different amino acid mutations. The upper node (germline) depicts the putative germline. The analysis is based on complete Ig sequences. Grey nodes (inferred node) are inferred sequences not observed in the dataset. Blue circles (clonotype node) are all nodes which are assigned to the same clonotype. The colour gradient of the inside of clonotype nodes is a gradient that reflects the number of sequences observed which this exact receptor sequence. The lightest colour (white) in the range represents 1 sequence and the darkest colour represents the maximum number of sequences for the clone (13 sequences for (A), 74 sequences for (B) and 163 sequences for (C)). In addition, Ig sequences with predicted glycosylation sites are shown in a red circle. (D) BASEline analysis of the selection strength of anti-Ro60, anti-Ro52, anti-La and RF clones. All fully sequenced autoreactive clones from B005 and B007 were included in the analysis. The CDR3 sequences were excluded. The lower left graphs depict comparisons of the selection strengths of the CDR and FWR. Each panel shows a comparison between two AutoAbs groups. A low selection strength can result from a high quantity of silent mutations compared with amino acid-changing mutations. The values in coloured fields on the upper right indicate p values for the comparisons in selection strength on the CDR and FWR between AutoAb groups. (E) Total quantities of silent and replacement transitions and transversions in the largest CATSST RF clonotype Ig sequences of B005 (top) and the large MALT CAREMD RF clone of B007 (bottom). The total transitions vs total transversions, silent transitions vs silent transversions and replacement transitions vs replacement transversions were compared using a T test. P values <0.05 were regarded as significant. (**F**) Assessment of the occurrence of typical AID hotspots WRC/GYW (W=A/T, R=A/G, Y=T/C), WA/TW, or hotspots WRC and/or WGCW for the CATSST RF clone of B005 (top) and the MALT RF clone of B007 (bottom). ANA, antinuclear antibody; AutoAb, autoantibody; CDR3, complementarity determining region 3; FWR, framework region; Ig, immunoglobulin; RF, rheumatoid factor; RTX, rituximab.

performed in affected tissues of two additional patients with SjS (B012 and B013), who had been referred from other hospitals because of challenging lymphoma diagnostics (detailed case descriptions in online supplemental methods). In both cases Ig-seq helped to establish a diagnosis of MALT lymphoma.

In B012, Ig-DNA-seq showed a monoclonal stereotypic RF expansion in biopsies of a liver and lymph node that had earlier received a diagnosis of primary biliary cirrhosis and autoimmune inflammation. Of interest, the same clonotype as in the lymphoma was detected in the top 40 of most abundant clonotypes present in a labial biopsy that had been performed for SjS diagnosis 2 years earlier before lymphoma onset.

In B013, Ig-seq showed that two suspected mass lesions in different tissues consisted of two different MALT lymphomas

DISCUSSION

This study shows that RF and ANA B cells are enriched in affected tissues of patients with SjS, where they occur as a small part of a polyclonal repertoire. RF and ANA clones affinity maturate in divergent fashion, which increases in patients with secondary RF lymphomagenesis. The RF repertoire displays IgM and antigen-dependent affinity maturation that coincides with intra-clonal diversification associated lymphomagenesis. Regeneration of clinical disease manifestations after RTX coincides with large RF clones, which not necessarily belong to the lymphoma clone, that display continuous affinity maturation and intra-clonal diversification.

In SjS, experimental and translational studies have suggested that autoreactive clones are generated from disturbed circulating B cell populations. Circulating naïve B cells in patients with SjS are enriched for polyreactive and nuclear antigen-reactive cells and circulating memory B cells contain anergic autoreactive clones.[16 29–31] Here, we show that circulating memory B cell populations contain expanded B cell clones. However, the autoreactive repertoire is not associated with a restricted repertoire in a circulating B cell population. Instead, it is enriched in affected tissues, displaying antigen-dependent affinity maturation that associates with a preference for shared sequence motifs.

The clones producing Ig against nuclear proteins Ro60, Ro52 and La displayed features consistent with antigen-dependent selection of high Ig affinity clones: they were mostly small plasma cell clones, consistently expressed IgG1, had a relatively high SHM load and exhibited limited intra-clonal diversification. A proportion of ANA clones precipitated with multiple antinuclear antigens, suggesting a polyreactive nature. The large number of ANA clones displaying similar affinity maturation suggests that these evolved as a result of intramolecular and intermolecular epitope spreading. In contrast, RF clones displayed features of suboptimal affinity maturation: they occurred less often, expressed IgM, had a relatively low SHM load and depended on specific variable gene segments. After RTX, the SHM load and the intra-clonal diversification of the most dominant RF clones increased. This supports continuing affinity maturation of RF clones.

The predominant expression of IgM by RF clones versus IgG1 by ANA clones in the context of a similar antigen-dependent selection pressure suggests that their isotype use determines their susceptibility to lymphomagenesis. Class-switching to IgG1 induces a preference toward plasma cell differentiation.[32] The continued expression of IgM by all detectable RF clones may be caused by the dependence of optimal RF B cell receptor stimulation by immune complexes that can cross-link multiple IgM isotype RF BCRs on the clonal membrane.[33] Such a required stereochemistry to pass the threshold for sufficient B cell receptor activation would explain the observed selection dependence on the use and structural integrity of a restricted set of Ig variable regions. Continued expression of IgM, together with less access to co-stimulatory signals, may result in suboptimal affinity maturation. A proportion of RF clones exhibited continuing proliferation and extensive intra-clonal diversification. These observations are in line with gradual accumulation of lymphoma driver mutations in germinal-centre-like cells.[10]

In both patients that were treated with RTX, clinical relapse coincided with expansion of autoreactive clones. The extensive depletion of B cells after RTX induces a reciprocal increase in B cell activating factor (BAFF) levels, which may facilitate clonal expansion of new and persisting clones.[34 35] In mice induced with a T–B cell dependent form of experimental encephalomyelitis, part of autoreactive memory B cells persisted after RTX in lymphoid tissue and disease flare was associated with expansion of these cells within a restricted repertoire.[36] The observed increase in SHM load of dominant RF clones after RTX may be explained by increased positive selection of RF clones because of increased BAFF levels. Moreover, in both patients, RF clones proliferated in the affected tissues at relapse after RTX. This was the solitary lymphoma clone in B007 and multiple non-lymphoma RF clones in B005. Intriguingly, B007 experienced a prolonged clinical response of 2 years, while in B005 the disease relapsed within 6 months. In patient, B005 new RF clones proliferated quickly after RTX and likely contributed to the increase in SG swelling after 6 months.

Finally, from a clinical perspective, we found that RTX did not succeed in abrogating lymphomatous B cell clones. Possibly, other B cell depletive treatments may have superior efficacy. Besides this, it can be challenging to discriminate MALT lymphoma from SjS-associated inflammation, determine the best treatment regimen and assess response to treatment. A diagnosis is made by assessing the combination of clinical presentation, histology, phenotype and sometimes clonality analysis and/or genetic studies. Also in the study patients, the diagnosis of MALT lymphoma had been challenging. Ig-seq retrospectively could have assisted in establishing a diagnosis earlier and more precisely. Future prospective investigations should investigate in more patients in more detail the added value of Ig-seq for diagnostic problems in patients with SjS with one or more mass lesions and for detection of small (pre-)lymphomatous clones in major SGs.

To summarise, we used for the first time an integrated omics workflow to analyse the generation of the autoreactive repertoire in circulating B cell populations and affected tissues of patients with SjS, and demonstrated tissue restricted, aberrant affinity maturation of RF clones compared with ANA clones in inflamed tissues. These data give insight into the molecular mechanisms of autoreactive inflammation and MALT lymphoma, and help to analyse the clinical response to RTX treatment in individual patients.

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Acknowledgements We would like to thank Sjoukje Mulder for monitoring the study and Amrah Weijn for analytical support.

Contributors Experiments: MGAB, GBalzaretti, JJW, PJTAG, BDCvS, GBounova and CK. Data analysis: MGAB, GBalzaretti, JJW, PJTAG, BDCvS, KMH, GBounova, CK, SB, GJMP, TPG, NDV and RT. Manuscript: MGAB, GBalzaretti, JJW, PJTAG, KMH, CK, SB, GJMP, TPG, NDV and RT. Study design: RT. Guarantor: RT.

Funding This project was executed based on a Clinical Fellowship grant from the Netherlands Organisation for Health Research and Development (ZonMw; 90714540 and 435005039). Part of this work was carried out on the Dutch national e-infrastructure with the support of SURF Cooperative. JJW was supported by an Australian National Health and Medical Research Council Early Career Fellowship (1090759) and a Flinders University Research Collaboration grant.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by the Medical Ethical Committee of the Radboud University Medical Center, Nijmegen, the Netherlands (cmo number: 2015-1721). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Single-cell FASTQ files were deposited at the NCBI as BioProject ID PRJNA742201 and PRJNA7883525.

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CLINICAL SCIENCE

Efficacy and safety of mavrilimumab in giant cell arteritis: a phase 2, randomised, double-blind, placebo-controlled trial

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221865).

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For 'Presented at statement' see end of article.

Received 16 November 2021
Accepted 4 February 2022
Published Online First 9 March 2022

Check for updates

To cite: Cid MC, Unizony SH, Blockmans D, *et al*. *Ann Rheum Dis* 2022;**81**:653–661.

ABSTRACT

Objectives Granulocyte-macrophage colony-stimulating factor (GM-CSF) is implicated in pathogenesis of giant cell arteritis. We evaluated the efficacy of the GM-CSF receptor antagonist mavrilimumab in maintaining disease remission.

Methods This phase 2, double-blind, placebo-controlled trial enrolled patients with biopsy-confirmed or imaging-confirmed giant cell arteritis in 50 centres (North America, Europe, Australia). Active disease within 6 weeks of baseline was required for inclusion. Patients in glucocorticoid-induced remission were randomly assigned (3:2 ratio) to mavrilimumab 150 mg or placebo injected subcutaneously every 2 weeks. Both groups received a 26-week prednisone taper. The primary outcome was time to adjudicated flare by week 26. A prespecified secondary efficacy outcome was sustained remission at week 26 by Kaplan-Meier estimation. Safety was also assessed.

Results Of 42 mavrilimumab recipients, flare occurred in 19% (n=8). Of 28 placebo recipients, flare occurred in 46% (n=13). Median time to flare (primary outcome) was 25.1 weeks in the placebo group, but the median was not reached in the mavrilimumab group (HR 0.38; 95% CI 0.15 to 0.92; p=0.026). Sustained remission at week 26 was 83% for mavrilimumab and 50% for placebo recipients (p=0.0038). Adverse events occurred in 78.6% (n=33) of mavrilimumab and 89.3% (n=25) of placebo recipients. No deaths or vision loss occurred in either group.

Conclusions Mavrilimumab plus 26 weeks of prednisone was superior to placebo plus 26 weeks of prednisone for time to flare by week 26 and sustained remission in patients with giant cell arteritis. Longer treatment is needed to determine response durability and quantify the glucocorticoid-sparing potential of mavrilimumab.

Trial registration number ClinicalTrials.gov number: NCT03827018, Europe (EUdraCT number: 2018-001003-36), and Australia (CT-2018-CTN-01865-1).

Key messages

What is already known about this subject?

- Currently available treatments for giant cell arteritis have important limitations. Most patients with giant cell arteritis treated with glucocorticoids alone experience disease relapse and/or develop glucocorticoid-related toxicity, and a significant proportion of patients treated with tocilizumab cannot achieve sustained remission or must discontinue this medication due to adverse events.
- Translational research has implicated granulocyte-macrophage colony-stimulating factor (GM-CSF) in the pathogenesis of giant cell arteritis, with studies showing upregulation of the GM-CSF and TH1/TH17 pathways in temporal arteries of patients with giant cell arteritis and amelioration of the abnormal immune response (eg, inflammatory cell infiltration and expression of interferon-γ and interleukin-6) on GM-CSF signalling blockade with mavrilimumab.

What does this study add?

- This study demonstrated that mavrilimumab in combination with a 26-week prednisone taper was superior to placebo with a 26-week prednisone taper in reducing the risk of flare and maintaining sustained remission and was well tolerated.

How might this impact on clinical practice or future developments?

- The study findings support the hypothesis that GM-CSF signalling activates important pathways in the pathogenesis of giant cell arteritis, and that inhibition of these pathways by GM-CSF receptor blockade with mavrilimumab might maintain remission of the disease.
- These phase 2 results are encouraging for the further development of mavrilimumab as a potential treatment for giant cell arteritis.

INTRODUCTION

Giant cell arteritis (GCA) is the most prevalent form of systemic vasculitis in adults.[1] The disease is driven by $CD4^+$ T-cells (T helper (T_h) 1 and 17 cells) and macrophages that infiltrate large-sized and medium-sized arteries.[2 3] Clinical manifestations include headaches, jaw claudication, ocular ischaemia, polymyalgia rheumatica and constitutional symptoms.[1 4] Possible complications include blindness and aortic aneurysms.[1] Most patients with active GCA exhibit elevated acute-phase reactants, including erythrocyte sedimentation rate (ESR) and serum C reactive protein (CRP) levels,[5] that, along with serial assessment of clinical manifestations, are useful in monitoring disease activity.[1]

Therapeutic options that safely maintain disease remission in patients with GCA are limited.[6] When treated with glucocorticoids alone, approximately 34%–75% of patients experience disease flare on dose reduction or drug discontinuation.[4 7 8] Moreover, the prolonged treatment with glucocorticoids required to control the disease, usually more than 12–18 months, causes significant glucocorticoid-related toxicity in the majority of patients.[9 10] Tocilizumab in combination with ≥6 months of glucocorticoids has demonstrated efficacy in maintaining disease remission and sparing the use of glucocorticoids and is the only approved adjuvant treatment for GCA patients. Unfortunately, 24%–30% of patients receiving tocilizumab flare within 1 year, and approximately 5%–8% of them must discontinue treatment because of side effects.[11 12] Also, given the direct suppression of hepatic acute-phase reactant synthesis, tocilizumab renders ESR and CRP unreliable for monitoring of disease activity and potential intercurrent infectious complications.[13 14] Other medications which have been tried for GCA, such as methotrexate and abatacept, have demonstrated modest benefits at best or need confirmation.[15–17] Therefore, novel treatments that safely maintain remission of GCA while allowing for acute-phase reactant monitoring are needed.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multifunctional cytokine that modulates the biology of dendritic cells, $CD4^+$ T-cells and macrophages.[18] Preclinical research has implicated GM-CSF in the pathogenesis of GCA.[19–22] GM-CSF, its receptor, and downstream signalling molecules are expressed by immune and endothelial cells in temporal arteries from patients.[19–22] Furthermore, GM-CSF receptor blockade in cultured temporal arteries resulted in decreased expression of dendritic cell, T-cell, and macrophage markers along with down-regulation in transcription of genes associated with the T_h1 and T_h17 immune responses (eg, interferon-γ and interleukin-6).[20 22] In a mouse model of vascular inflammation, GM-CSF inhibition was associated with reduced arterial inflammation and remodelling.[23] Mavrilimumab, an immunoglobulin G4 monoclonal antibody with demonstrated efficacy in phase 2 studies of rheumatoid arthritis,[24 25] blocks GM-CSF signalling by binding to the alpha chain of the receptor.

We conducted a proof-of-concept, randomised, double-blind, placebo-controlled trial to investigate whether mavrilimumab reduced the risk of GCA flare compared with placebo, during a 26-week glucocorticoid taper.

METHODS

Study design

This randomised, double-blind, placebo-controlled phase 2 trial was conducted in 50 centres across 15 countries in North America, Europe, and Australia.

Patients

Patients age 50–85 years with new-onset (diagnosis ≤6 weeks before baseline) or relapsing/refractory (diagnosis >6 weeks before baseline) GCA and active disease within 6 weeks of randomisation were eligible. Active disease was defined as the presence of one or more clinical manifestations, including cranial (eg, headache, scalp or temporal artery tenderness, new/worsening ischaemia-related visual impairment or jaw claudication) or extracranial (eg, new/worsening extremity claudication or polymyalgia rheumatica) signs or symptoms, plus Westergren ESR ≥30 mm per hour or a CRP level ≥1 mg per decilitre. Isolated ESR or CRP elevation was not considered active disease for patient enrolment. GCA diagnosis was confirmed based on a temporal artery biopsy showing GCA features or by findings indicative of vasculitis on temporal artery ultrasonography or large-vessel imaging including magnetic resonance angiography, computed tomography (CT) angiography or positron emission tomography/CT. Complete eligibility criteria are detailed in online supplemental methods.

Procedures

Following a screening period (≤6 weeks), eligible patients were randomly assigned in a 3:2 ratio to mavrilimumab 150 mg or placebo subcutaneously every other week with a 26-week prednisone taper and entered a double-blind, placebo-controlled treatment period (26 weeks), which was followed by a safety follow-up period (12 weeks) (figure 1). Given that in prior 1-year trials with 26-week steroid tapers[11 16] the majority of disease flares occurred within the first 6 months, we limited the treatment period of this proof-of-concept trial to 26 weeks to expedite the generation of efficacy results. Randomisation was stratified by disease type (new onset or relapsing/refractory) at baseline. At baseline, patients were required to be in glucocorticoid-induced remission and on an oral prednisone dose between 20 and 60 mg daily. Remission at baseline was defined as the absence of disease signs and symptoms and ESR<20 mm per hour or serum CRP concentration <1 mg per decilitre. From baseline, the prednisone dose was tapered weekly in both groups as stipulated by the protocol. Additional details can be found in online supplemental file.

Outcomes

Efficacy

Patients were assessed at planned study visits and during unscheduled visits to determine disease remission status and whether the protocol prednisone taper could continue. It was recommended that the investigator evaluate signs and symptoms of GCA before reviewing laboratory or imaging results to minimise potential bias. ESR and/or CRP levels were measured locally. Patients requiring treatment for flare during the double-blind period discontinued study drug and received standard treatment, including glucocorticoids, as per the investigators' clinical judgement. After the 26-week treatment period, patients discontinued study drug and transitioned to standard of care, which could include glucocorticoids, during a 12-week washout period. Patients were closely monitored for safety and flare through week 38.

The primary efficacy end point was time to first GCA flare by week 26. Flare was centrally adjudicated by an independent, blinded clinical end point adjudication committee and defined as elevation of ESR (≥30 mm/hour) and/or CRP (≥1 mg/dL) along with either the presence of unequivocal cranial or extracranial signs or symptoms or the occurrence of new or worsening

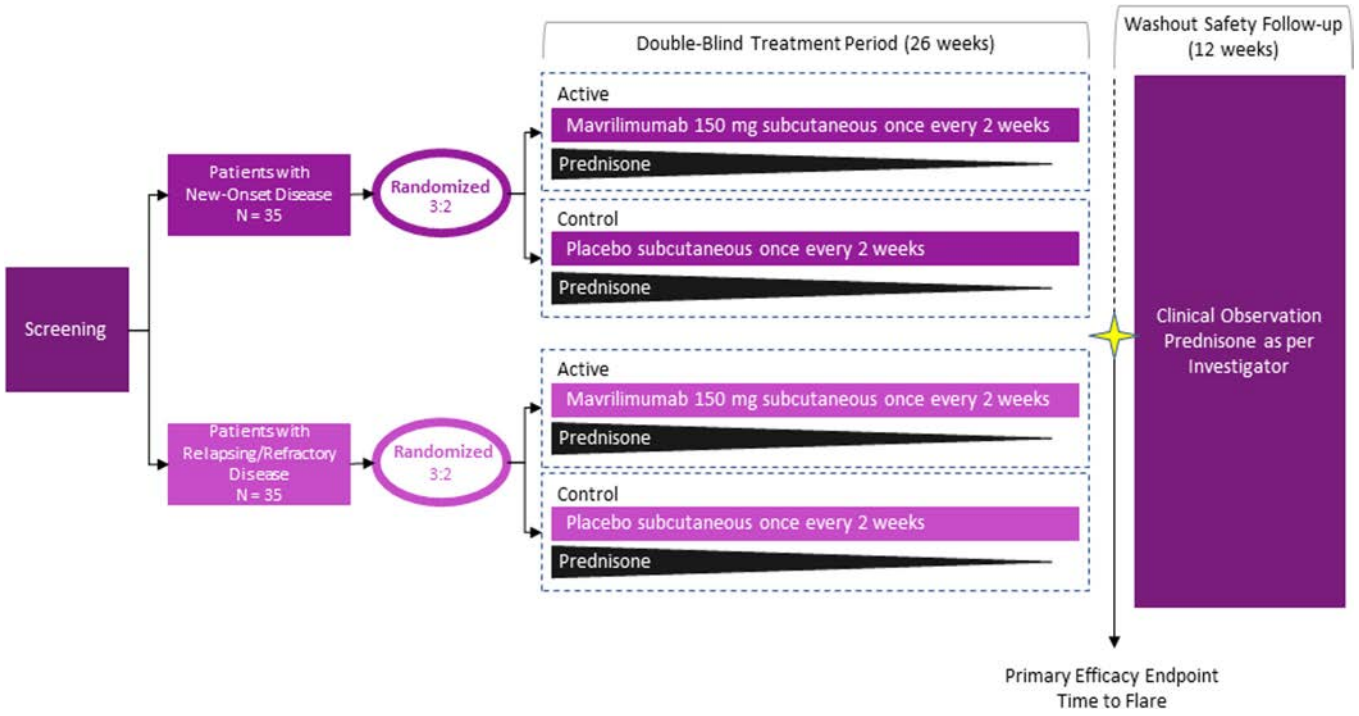

Figure 1 Trial design. Patients were randomised in a 3:2 ratio to mavrilimumab or placebo using disease type (new onset or relapsing/refractory) as a stratification factor. Prednisone was tapered over the 26-week study as specified in the protocol.

imaging abnormalities suggestive of active vasculitis. ESR or CRP elevation was not considered disease flare in the absence of signs, symptoms or imaging abnormalities suggesting disease activity. Further details of flare adjudication are included in online supplemental methods.

A key prespecified secondary efficacy end point was sustained remission rate at week 26 using Kaplan-Meier estimation, which was defined as the absence of flare from randomisation through week 26. Time to flare and sustained remission by week 26 were also assessed in the subgroups of patients with new-onset and relapsing/refractory disease at baseline. Cumulative prednisone dose by treatment arm was assessed. The proportion of patients with elevated ESR or CRP but without giant-cell arteritis flare was assessed in a post hoc analysis. Additional secondary end points and their hierarchy are described in online supplemental methods.

Safety

Safety was assessed through week 38 for all patients who received at least one mavrilimumab or placebo dose. Incidence, severity, and relationship of adverse events to study drug were summarised by treatment group. A data-monitoring committee periodically reviewed all safety data during the trial. Patients underwent serial pulmonary function testing and completed the modified Borg Dyspnoea Scale[26] at regular intervals. An independent committee adjudicated pulmonary adverse events of special interest including the potential occurrence of pulmonary alveolar proteinosis.[27]

Statistical analyses

A sample size of approximately 70 patients was determined based on an assumption, consistent with literature data, that 50% of placebo recipients and 15% of mavrilimumab recipients would flare by week 26, with a median time to flare of approximately 26 weeks in placebo group and 111 weeks in mavrilimumab group, corresponding with an HR of approximately 0.234. Using a time-to-flare model and a 3:2 randomisation ratio, we calculated that 20 flares would give the trial 87% power to detect a significant difference between treatment groups with a two-sided alpha level of 0.05. The analysis of the new onset and relapsing/refractory subgroups, while prespecified, was not powered for significance. The efficacy end point analysis was performed in the modified intention-to-treat population, which included all randomised patients who had received at least one dose of study treatment and had at least one assessment in the double-blind treatment period. The primary end point and other time-to-event end points were summarised with percentiles and 95% CIs using the Kaplan-Meier method. Patients without a flare were censored at the last assessment by week 26 or by end of treatment visit, in case of early treatment discontinuation, for calculation of the time to flare. A log-rank test stratified by disease type (new onset vs relapsing/refractory) at baseline was used to compare mavrilimumab with placebo. The number and percentage of patients who had a flare during the 26-week double-blind period were summarised for each treatment group. A Cox proportional hazards model was used to calculate hazard ratios and 95% CIs. Sustained remission at week 26 was derived by Kaplan-Meier curve analysis.

All secondary outcomes based on proportions were assessed using the Cochran-Mantel-Haenszel test.

A gatekeeping multiplicity-adjustment procedure in combination with the Hochberg method was applied for prespecified stepwise testing of the primary end point and the secondary end points. If the two-sided p value for an end point (highest in hierarchy) was no more than 0.05, the next prespecified end point in the hierarchy would be tested at the same alpha level. Details of hierarchy are provided in online supplemental methods.

RESULTS

Patients

Of 112 patients assessed for eligibility, 70 were enrolled in the trial between 20 September 2018 and 27 January 2020.

Table 1 Baseline characteristics of the intention-to-treat population†

	Mavrilimumab‡ (n=42)	Placebo (n=28)
Age (years)	69.7 (7.0)	69.7 (8.3)
Sex		
Male	10 (24%)	10 (36%)
Female	32 (76%)	18 (64%)
Race		
White	40 (95%)	28 (100%)
Other	2 (5%)	0
Hispanic or Latino ethnicity	1 (2%)	2 (7%)
Weight (kg)	70.9 (18.7)	71.1 (12.0)
Body mass index (kg/m^2)	26.2 (6.8)	26.1 (3.6)
Prior treatment		
Glucocorticoids	42 (100%)	27 (96%)
Methotrexate	0	1 (4%)
Diagnostic confirmation		
By positive temporal artery biopsy	22 (52%)	9 (32%)
By positive imaging	29 (69%)	22 (79%)
Time since diagnosis (months)	7.9 (15.4)	9.8 (21.8)
Giant-cell arteritis		
New onset*	24 (57%)	11 (39%)
Relapsing/refractory*	18 (43%)	17 (61%)
Giant-cell arteritis type		
Cranial signs or symptoms	32 (76%)	21 (75%)
Extracranial signs or symptoms	9 (21%)	6 (21%)
C reactive protein level (study eligibility value) (mg/dL)	4.7 (4.7)	3.6 (3.2)
Erythrocyte sedimentation rate (study eligibility value) (mm/hour)	57.0 (24.6)	55.1 (30.2)
Prednisone starting dose		
≤30 mg	16 (38.1)	14 (50.0)
>30 mg	26 (61.9)	14 (50.0)

Data are n (%) or mean (SD).
*Seven patients were misstratified due to investigator error (new onset vs relapsing/refractory misclassification) at study entry. For the efficacy analysis, these patients were included in the appropriate protocol-defined subgroups, leading to a proportion of 57% of patients with new-onset disease in the mavrilimumab group (43% relapsing/refractory) and 39% of patients with new-onset disease in the placebo group (61% relapsing/refractory).
†Baseline is last assessment within 3 days before the first dose of mavrilimumab or placebo.
‡150 mg subcutaneously every 2 weeks.

Figure 1 shows the clinical trial schema. A total of 42 patients were randomly assigned to mavrilimumab and 28 to placebo. The demographic and baseline characteristics of the treatment groups are displayed in table 1. GCA diagnosis was confirmed by biopsy in 31 (44%) patients and by imaging in 51 (73%) patients. A total of 66 patients completed the 26-week study period (figure 2).

Primary and key secondary efficacy outcomes

During the 26-week placebo-controlled period, 21 patients developed an adjudicated flare: eight (19%) mavrilimumab recipients and 13 (46.4%) placebo recipients. GCA signs or symptoms were present in 20 of the 21 patients with flare; in the one other patient, flare was determined based on presence of active vasculitis on ultrasound imaging. Median time to flare (primary end point) in placebo recipients was 25.1 weeks (95% CI 16.0 to not estimable (NE)). The median time to flare among mavrilimumab recipients was not reached within the 26-week follow-up period. Mavrilimumab reduced the risk of flare vs placebo (HR, 0.38; 95% CI 0.15 to 0.92; p=0.026) (figure 3). Sustained remission at week 26 (key secondary end point) was reached in 83.2% of mavrilimumab recipients and 49.9% of placebo recipients (33.3 percentage points difference; p=0.0038) (figure 4, table 2). Detailed flare information is provided in online supplemental table S1.

New-onset and relapsing/refractory disease

Among the subgroup of patients with new-onset GCA at baseline, flare occurred in 12.5% of mavrilimumab recipients and 36.4% of placebo recipients (HR, 0.29; 95% CI, 0.06 to 1.31) (table 2; online supplemental figure S1A); 91.3% of mavrilimumab recipients and 62.3% of placebo recipients had sustained remission at week 26 (table 2, online supplemental figure S2A). Among the subgroup of patients with relapsing/refractory disease at baseline, flares occurred in 27.8% of mavrilimumab recipients and 52.9% of placebo recipients (HR, 0.43; 95% CI 0.14 to 1.30) (table 2; online supplemental figure S1B); sustained remission at week 26 was observed in 72.2% of mavrilimumab recipients and 41.7% of placebo recipients (table 2, online supplemental figure S2B).

Cumulative prednisone dose

The mean cumulative prednisone dose by week 26 was 2074 mg in mavrilimumab recipients and 2403 mg in placebo recipients (nominal p=0.067); least-squares mean difference (nominal 95% CI) was –326 mg (–676 mg to 23 mg). Additional secondary end points assessed at week 26 are reported in table 3 and the online supplemental results.

Acute-phase reactants

Among the 21 patients who had a flare, all had increased ESR or CRP values at the time of flare (by pre-specified flare definition); the median (IQR) CRP level was 1.8 (1.4–6.3) mg per decilitre in mavrilimumab recipients and 1.8 (1.2–2.8) mg per decilitre in placebo recipients. Corresponding ESR values were 40 (33 –73) mm per hour in mavrilimumab recipients and 49 (33–51) mm per hour in placebo recipients (online supplemental table S2). Among 34 mavrilimumab recipients who did not have a flare, 47.1% had at least one elevated ESR (≥30 mm/hour) and 29.4% had at least one elevated CRP (≥1 mg/dL) value through week 26. Among 15 placebo recipients who did not have a flare, 66.7% had at least one elevated ESR and 73.3% had at least one elevated CRP value through week 26 (online supplemental table S2).

Safety

Adverse events were reported in 78.6% of mavrilimumab recipients and 89.3% of placebo recipients (table 4). Serious adverse events, all unrelated to study drug, were reported in 4.8% of mavrilimumab recipients (one case each of hypertrophic cardiomyopathy and dementia) and 10.7% of placebo recipients (one case each of gastrointestinal haemorrhage, peripheral oedema and pulmonary fibrosis). No adverse event resulted in permanent vision loss or death in either treatment group. Adverse events leading to study drug discontinuation occurred in one patient in each treatment group: dementia in a mavrilimumab recipient and chest pain in a placebo recipient.

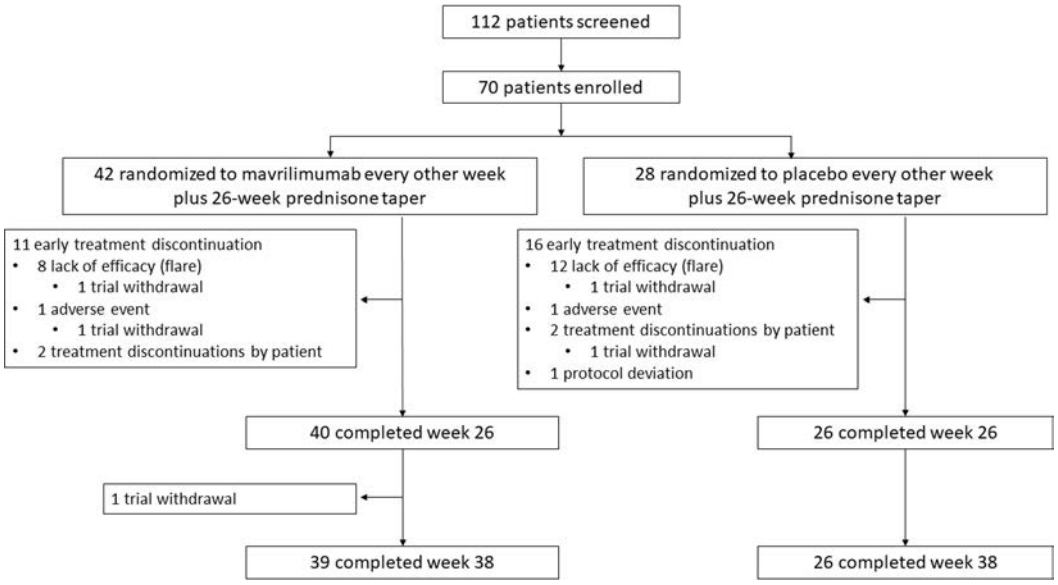

Figure 2 Trial profile. Not all patients who discontinued treatment withdrew from the trial; two patients receiving mavrilimumab and two patients receiving placebo withdrew before week 26, and one patient receiving mavrilimumab withdrew between week 26 and week 38.

The most frequent non-serious adverse events in mavrilimumab recipients were non-specific headache, nasopharyngitis and neck pain. Infections were reported in 42.9% of mavrilimumab recipients and 35.7% of placebo recipients. No serious or severe infections occurred during the trial. Respiratory adverse events were reported in similar proportions in the treatment groups (mavrilimumab, 11.9%; placebo, 10.7%). In mavrilimumab recipients, these included mild cough, mild dyspnoea and mild vasomotor rhinitis. There were no substantive differences between treatment groups in pulmonary function tests, including diffusing capacity of the lung for carbon monoxide, and no cases of pulmonary alveolar proteinosis occurred.

DISCUSSION

This trial provides the first evidence of the efficacy and safety of mavrilimumab in patients with GCA. Mavrilimumab with a 26-week prednisone taper was superior to placebo with a 26-week prednisone taper in reducing the risk of flare and maintaining sustained remission. Consistent efficacy trends were observed in new-onset and relapsing/refractory disease subgroups, although this analysis was not powered for statistical significance. Mavrilimumab was well tolerated, and the overall incidence of adverse events and serious adverse events was similar between groups.

GCA treatments that safely maintain disease remission are lacking.[6] The clinical course of patients treated exclusively with

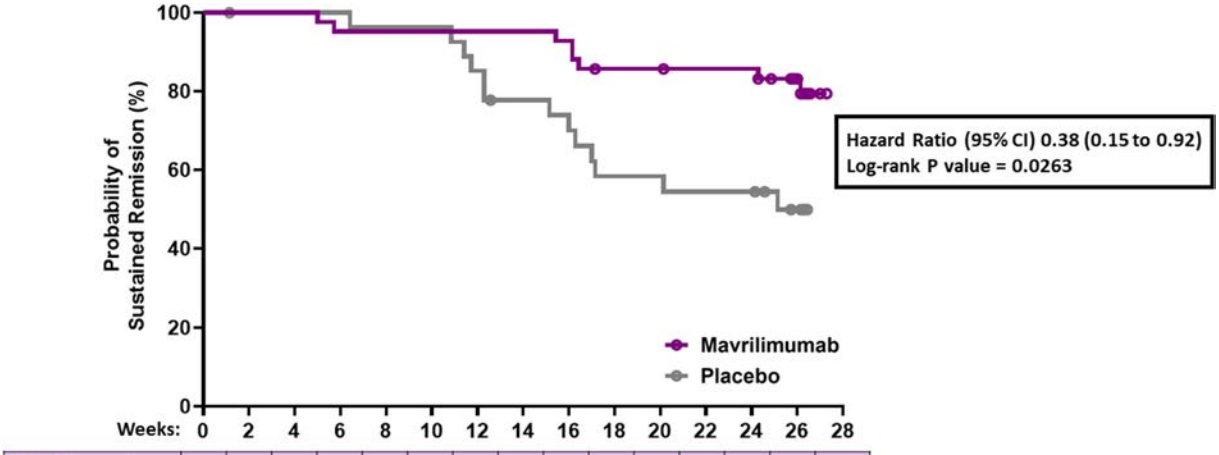

Figure 3 Time to first flare of giant-cell arteritis in all patients. At baseline, patients had to be in remission (defined as the absence of giant-cell arteritis signs and symptoms and erythrocyte sedimentation rate <20 mm/hour or C reactive protein level <1 mg/dL) and receiving an oral prednisone dose between 20 mg and 60 mg daily. Patients who discontinued treatment for reasons other than flare were censored for the calculation of time to flare. The median time to flare could not be calculated for patients receiving mavrilimumab because fewer than 50% of patients experienced a flare during the 26 weeks study period.

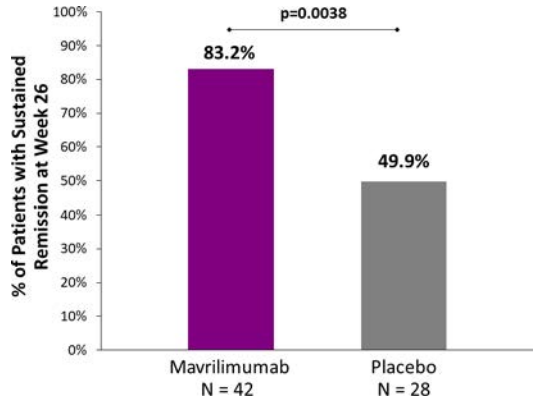

Figure 4 Sustained remission rate of giant-cell arteritis in all patients at week 26. The difference in sustained remission at week 26 (key secondary endpoint) was statistically significant (33.3 percentage points; p=0.0038). Sustained remission was defined as the absence of flare from randomisation through week 26. Sustained remission rate was derived by Kaplan-Meier curve analysis.

glucocorticoids is complicated by high rates of disease flare and increased incidence of glucocorticoid-related toxicity.[4 7 8] Tocilizumab is the only GCA medication with confirmed, clinically meaningful efficacy in terms of remission maintenance and glucocorticoid-sparing.[11] However, 24%–30% of patients receiving tocilizumab flare within 1 year, and approximately 5%–8% of them must discontinue treatment because of side effects.[11–13] In this study, mavrilimumab reduced the risk of flare without adverse events of serious infection or pulmonary alveolar proteinosis,[28] becoming a promising option for further development in a field in which alternative treatments are a great unmet need.

It is well recognised that the elevation of ESR or serum CRP is not completely sensitive or specific for the diagnosis of GCA flare.[5 13] However, these acute-phase reactants have been widely used by clinicians as one of several practical elements for monitoring disease activity status in steroid-treated patients. Because tocilizumab reduces IL-6 activity in the liver, it directly inhibits hepatic synthesis of acute-phase reactants and reduces ESR and CRP independently of its immunomodulatory action,[13] rendering these biomarkers unreliable for monitoring disease activity.[13] The fact that flares in this trial were associated with increased acute-phase reactants regardless of whether patients were on mavrilimumab or only glucocorticoids suggests that ESR and CRP retained their clinical diagnostic value during GM-CSF blockade.

The safety profile of mavrilimumab was consistent with that observed in larger, long-term studies of patients with rheumatoid arthritis.[25 29] In this phase 2 trial, mavrilimumab was well tolerated, and most adverse events were mild or moderate. Because GM-CSF plays an important role in lung homeostasis by promoting alveolar macrophage-induced surfactant clearance,[27 28] respiratory adverse events, including changes in lung function, were assessed by an independent pulmonary evaluation committee. Of note, there were no differences in pulmonary function tests between treatment groups and no cases of pulmonary alveolar proteinosis occurred during the trial.

The design of this phase 2 study incorporated strategic development-phase-specific trade-offs in strengths and limitations as well as guidance provided by regulatory agencies during review of the protocol. On the one hand, informed by the timing of disease flare in other trials,[11 16] the proposed 26-week placebo-controlled treatment period allowed for expedited generation of proof-of-concept data. The time-to-event variable of time-to-flare was chosen for the primary end point (as opposed to disease remission at a given timepoint) because it would allow for a more comprehensive interpretation of the results by adding the domain of time and the event cadence to the cumulative crude event rates. On the other hand, a period longer than 26 weeks would have been ideal to properly assess long-term remission maintenance and glucocorticoid sparing, important treatment objectives for this chronic, relapsing disease. In this trial, the mean cumulative prednisone dose was lower in mavrilimumab recipients than in placebo recipients, due to higher disease flare and glucocorticoid rescue rates in patients in the placebo group.

Table 2 Primary end point and key secondary end points

End point	Mavrilimumab**	Placebo	HR or difference	P value*
All study patients†	(N=42)	(N=28)	–	–
Patients with flare	8 (19.0%)	13 (46.4%)	–	–
Time to flare (primary end point)—week	NE (NE, NE)	25.1 (16.0 to NE)	0.38 (0.15 to 0.92)‡	0.026
Sustained remission§—%	83.2 (67.9 to 91.6)	49.9 (29.6 to 67.3)	33.3 (10.7 to 55.8)¶	0.0038
Patients with new-onset† giant-cell arteritis at baseline	(N=24)	(N=11)	–	–
Patients with flare	3 (12.5%)	4 (36.4%)	–	–
Time to flare—week	NE (NE to NE)	NE (11.7 to NE)	0.29 (0.06 to 1.31)‡	–
Sustained remission§—%	91.3 (69.3 to 97.7)	62.3 (27.7 to 84.0)	28.9 (–2.7 to 60.5)¶	–
Patients with relapsing/refractory† giant-cell arteritis at baseline	(N=18)	(N=17)	–	–
Patients with flare	5 (27.8%)	9 (52.9%)	–	–
Time to flare—week	NE (16.4 to NE)	22.6 (16.0 to NE)	0.43 (0.14 to 1.30)‡	–
Sustained remission§—%	72.2 (45.6 to 87.4)	41.7 (17.4 to 64.5)	30.6 (–2.1 to 63.2)¶	–

Data are n (%) or median (95% CI), except as indicated.
*P values are two sided.
†Modified intention-to-treat (mITT) population.
‡Calculated using a Cox proportional hazards model with treatment as covariate.
§The Kaplan-Meier method was used to estimate event rates. In some cases, results were NE because the event rates were too low.
¶Calculated as the difference in sustained remission between the two groups using normal approximation with placebo as the reference.
**150 mg subcutaneously every 2 weeks.
NE, not estimable.

Table 3 Other secondary end points

End point	Mavrilimumab* (N=42)	Placebo (N=28)	P value
Time to elevated erythrocyte sedimentation rate by week 26,† median (95% CI) weeks‡	26.1 (16.1, NE)	12.1 (8.1, 16.6)	0.028§
Time to elevated C reactive protein level by week 26,¶ median (95% CI) weeks‡	NE (8.1, NE)	12.3 (3.3, 24.1)	0.038§
Time to signs and symptoms of giant-cell arteritis or new or worsening vasculitis by imaging by week 26, median (95% CI) weeks‡	NE (NE, NE)	25.1 (15.1, NE)	0.065§
Cumulative prednisone dose at week 26, mean (SD) mg	2074 (708)	2403 (1014)	0.067**
Percentage of patients completing glucocorticoid taper†† and with normal erythrocyte sedimentation rate by week 26	19 (45.2%)	4 (14.3%)	0.020**
Percentage of patients completing glucocorticoid taper†† and with normal C reactive protein level by week 26	10 (23.8%)	4 (14.3%)	0.55**
Percentage of patients completing glucocorticoid taper†† and with no signs or symptoms of giant-cell arteritis by week 26	30 (71.4%)	9 (32.1%)	0.0031**
Cumulative prednisone dose at week 38‡‡, mean (SD) mg	2465 (1107)	2845 (1320)	0.16**

Data are n (%) except as indicated.
*150 mg subcutaneously every 2 weeks.
†Elevated erythrocyte sedimentation rate is defined as the first rate greater than or equal to 30 mm/hour; patients with an elevated rate within 3 days of the first dose of study drug were excluded from the analysis.
‡Kaplan-Meier method.
§Log-rank test stratified by randomisation strata.
¶Elevated C reactive protein level is defined as the first level greater than or equal to 1.0 mg/dL; patients with an elevated level within 3 days of the first dose were excluded from the analysis.
**Analysed by Cochran-Mantel-Haenszel test stratified by randomisation strata. Nominal p value.
††Patients were considered to have completed glucocorticoid taper if by week 26 they were receiving 1 mg/day for patients who had a starting dose of 60 mg/day, or 0 mg/day for patients who had a starting dose of less than 60 mg/day.
‡‡After the 26-week treatment period, investigators could manage disease in patients at their discretion, including use of glucocorticoids.

The difference between groups through week 26, however, did not reach statistical significance, likely because of the late time-to-flare (median 25.1 weeks) in the placebo group relative to the 26-week time point at which the assessment of cumulative prednisone dose ended.

Table 4 Treatment-emergent adverse events

Adverse events	Mavrilimumab* (N=42)	Placebo (N=28)
Patients with ≥1 adverse event	33 (78.6%)	25 (89.3%)
Serious adverse event	2 (4.8%)	3 (10.7%)
Serious adverse event related to study drug	0	0
Adverse event resulting in death	0	0
Adverse event leading to study drug discontinuation	1 (2.4%)	1 (3.6%)
Adverse events by maximum severity†		
Mild	18 (42.9%)	13 (46.4%)
Moderate	14 (33.3%)	11 (39.3%)
Severe	1 (2.4%)	1 (3.6%)
Most common adverse events‡		
Headache	6 (14.3%)	7 (25.0%)
Nasopharyngitis	5 (11.9%)	3 (10.7%)
Neck pain	4 (9.5%)	2 (7.1%)
Arthralgia	2 (4.8%)	4 (14.3%)
Hypertension	1 (2.4%)	4 (14.3%)
Back pain	3 (7.1%)	3 (10.7%)
Muscle spasms	3 (7.1%)	3 (10.7%)
Upper respiratory tract infection	3 (7.1%)	2 (7.1%)
Constipation	3 (7.1%)	0
Diarrhoea	0	3 (10.7%)
Fall	2 (4.8%)	5 (17.9%)

Data are n (%).
*150 mg subcutaneously every 2 weeks.
†Each patient is represented only with maximum severity.
‡Reported in >2 patients in either treatment group.

A slight imbalance in the number of patients with new-onset and relapsing/refractory disease between groups could have influenced the results to some extent and may represent a limitation of the study. Although, such possibility seems unlikely in view of prior research demonstrating that duration of disease and the status of newly diagnosed vs relapsing disease do not independently predict treatment failure,[30] confirmation of these phase 2 results in a larger trial with well-balanced baseline features is required.

Current medications for GCA (eg, glucocorticoids and tocilizumab) target primarily the $CD4^+$ T_h17 immune response, possibly leaving residual $CD4^+$ T_h1 pathway activity, which may explain why a sizeable proportion of patients flare with these treatments. In contrast, GM-CSF blockade with mavrilimumab may address the pathogenic mechanisms of GCA more comprehensively via its demonstrated suppressive effects on macrophages, $CD4^+$ T_h17 cells, and $CD4^+$ T_h1 cells, including downregulation of IFNγ expression.[22 23] However, further mechanistic research linked to clinical outcomes is needed before firm conclusions can be drawn.

In summary, mavrilimumab given with a 26-week prednisone taper significantly reduced the risk of flare and improved the sustained remission rates compared with placebo with a 26-week prednisone taper in patients with GCA. Mavrilimumab was well tolerated, and no new safety signals emerged in this clinical trial. These results are supportive of further clinical development of mavrilimumab; confirmation of these overall results, precise distinction of efficacy in new-onset and relapsing/refractory disease subgroups, and determination of response durability and glucocorticoid-sparing potential should all be addressable in a larger pivotal clinical trial of longer duration.

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Presented at
Some of the data contained in this manuscript has been previously published in four abstracts: Cid M, Unizony S, Pupim L, *et al* Mavrilimumab (anti GM-CSF Receptor α Monoclonal Antibody) Reduces Time to Flare and Increases Sustained Remission in a Phase 2 Trial of Patients with Giant Cell Arteritis ((abstract)). Arthritis Rheumatol. 2020; 72 (suppl 10); Cid M, Unizony S, Pupim L, *et al*. OP0059 Mavrilimumab (anti GM-CSF Receptor α Monoclonal Antibody) Reduces Risk of Flare and Increases Sustained Remission in a Phase 2 Trial of Patients with Giant Cell Arteritis [(abstract]). Annals of the Rheumatic Diseases. 2021; 80:31-32 (suppl 1); Unizony S, Cid MC, Brouwer E, et al. AB0370 Utility of CRP and ESR in the Diagnosis of Giant Cell Arteritis Relapse in a Phase 2 Trial of Mavrilimumab. Annals of the Rheumatic Diseases 2021;1211-1212; and Unizony S, Cid MC, Blockmans D, et al. Utility of CRP and ESR in the Assessment of Giant Cell Arteritis Relapse in a Phase 2 Trial of Mavrilimumab ((abstract)). Arthritis Rheumatol. 2021; 73 (suppl 10).

Acknowledgements The authors thank the patients for their participation and for making the trial possible. Medical writing assistance was provided by Emily Plummer, Ph.D. of Kiniksa Pharmaceuticals Corp. Editorial and writing assistance was provided by Michelle McDermott, Pharm.D. of Peloton Advantage, an OPEN Health company, and funded by Kiniksa Pharmaceuticals. MCC dedicates her contribution to the Oncology Department, Hospital Clinic, Barcelona, particularly to oncologists Montserrat Muñoz, Meritxell Molla, and Immaculada Alonso for their excellent professional care and encouragement throughout the development of this study. Without their support, her participation would not have been possible.The authors would like to thank all the investigators: Australia—Paul Bird, Catherine Hill, Charles Inderjeeth, Andrew Ostor, Ian Wicks, Robert Will; Belgium—Daniel Blockmans, Yves Boutsen, Michel Malaise, Frédéric Vandergheynst; Croatia—Porin Peric; Estonia—Raili Muller, Andres Pille; Germany—Stephanie Finzel, Bernhard Hellmich, Jörg Henes, Ina Kötter, Peter Oelzner, Hans Jürgen Rech, Elke Riechers, Nils Venhoff; Ireland—Eamonn Molloy; Italy—Lorenzo Dagna, Luca Quartuccio, Carlo Salvarani, Carlo Selmi; Netherlands—Elisabeth Brouwer, Paul van Daele; New Zealand—Nigel Gilchrist, Ketna Parekh; Poland—Bogdan Batko; Serbia—Ksenija Bozic, Nemanja Damjanov, Goran Radunovic; Slovenia – Matija Tomsic; Spain—Francisco Javier Blanco Garcia, Maria C Cid, Federico Diaz-Gonzalez, Eva Galindez Agirregoikoa; United Kingdom—Bhaskar Dasgupta, Alice Lorenzi, Neil McKay, Angela Pakozdi, Hasan Tahir; USA—Yoel Drucker, Joshua June, Lindsay Lally, Yih Chang Lin, Andrew Sulich, Paul Sutej, Sebastian Unizony, Kenneth Warrington. The authors would like to thank all the study site personnel. Special thanks to Georgina Espígol-Frigolé and Roberto Ríos Garcés of Hospital Clínic, University of Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) in Barcelona, Spain; Ana D. Fernandes and Adam Jarvie of Massachusetts General Hospital in Boston, Massachusetts; Ellis Herder-Stok, Janita Bulthuis-Kuiper, and Maria Sandovici, M.D., Ph.D., Department of Rheumatology and Clinical Immunology, University of Groningen, University Medical Centre Groningen, Groningen, The Netherlands; Elena Baldissera, Corrado Campochiaro, Simone Casiraghi, Silvia Sartorelli, and Alessandro Tomelleri of IRCCS San Raffaele Scientific Institute in Milan, Italy; Lorraine O'Neill, M.D., M.R.C.P.I., Phil Gallagher and Lorna Freeman of St Vincent's University Hospital in Dublin, Ireland; Mariagrazia Catanoso, M.D. and Francesco Muratore of Azienda USL-IRCCS di Reggio Emilia and Università di Modena e Reggio Emilia in Reggio Emilia, Italy; Jane M. Jaquith, C.C.R.C. of Mayo Clinic in Rochester, Minnesota.

Collaborators The authors would like to acknowledge the KPL-301-C001 Investigator collaborators: Australia—Paul Bird, Catherine Hill, Charles Inderjeeth, Andrew Ostor, Ian Wicks, Robert Will; Belgium—Daniel Blockmans, Yves Boutsen, Michel Malaise, Frédéric Vandergheynst; Croatia—Porin Peric; Estonia—Raili Muller, Andres Pille; Germany—Stephanie Finzel, Bernhard Hellmich, Jörg Henes, Ina Kötter, Peter Oelzner, Hans Jürgen Rech, Elke Riechers, Nils Venhoff; Ireland—Eamonn Molloy; Italy—Lorenzo Dagna, Luca Quartuccio, Carlo Salvarani, Carlo Selmi; Netherlands—Elisabeth Brouwer, Paul van Daele; New Zealand—Nigel Gilchrist, Ketna Parekh; Poland—Bogdan Batko; Serbia—Ksenija Bozic, Nemanja Damjanov, Goran Radunovic; Slovenia – Matija Tomsic; Spain—Francisco Javier Blanco Garcia, Maria C Cid, Federico Diaz-Gonzalez, Eva Galindez Agirregoikoa; United Kingdom—Bhaskar Dasgupta, Alice Lorenzi, Neil McKay, Angela Pakozdi, Hasan Tahir; USA—Yoel Drucker, Joshua June, Lindsay Lally, Yih Chang Lin, Andrew Sulich, Paul Sutej, Sebastian Unizony, Kenneth Warrington.

Contributors The authors MCC, SHU, and JFP act as guarantors of this work. All authors participated in reviewing and editing the manuscript, data curation and approved the submitted draft. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication. The authors MCC and SHU share first Authorship. The authors MCC, SHU, MS, TZ, LP and JFP accessed and completed formal analysis of all underlying data. The authors MCC, SHU, IPW, LP and JFP participated in conceptualisation. The authors MCC, SHU and JFP supervised the study. The original draft was written by authors MCC and SHU. The sponsor (Kiniksa) participated in trial design and patient recruitment and performed data analysis and interpretation. Kiniksa supported the decision to submit the article for publication.

Funding This study was funded in full by Kiniksa Pharmaceuticals. MCC was supported by Ministerio de Ciencia e Innovación (SAF 2017/88275-R and PID2020-114909RB-I00), co-funded by Fondo Europeo de Desarrollo Regional (FEDER) and CERCA programme. IPW is supported by Practitioner Fellowship 1154325 and Programme Grant 1113577 from the National Health and Medical Research Council of Australia and acknowledges the long-term support of The Reid Charitable Trusts.

Competing interests MCC reports a research grant from Kiniksa; consulting for Janssen, GlaxoSmithKline, and AbbVie; educational support from GlaxoSmithKline, Roche, and Vifor; and meeting attendance support from Roche and Kiniksa. SHU reports research support from Genentech and consulting for Janssen and Kiniksa. DB has nothing to disclose. EB reports receiving, as an employee of the University of Groningen Medical Center, speaker and consulting fees from Roche in 2017 and 2018, paid to the University of Groningen Medical Center. LD reports grants, personal fees, and nonfinancial support from AbbVie, Amgen, Bristol-Myers Squibb, Novartis, Pfizer, Roche, Sanofi-Genzyme, and SOBI; grants and personal fees from Celltrion and Galapagos; grants from Janssen, Kiniksa, and Merck Sharp & Dohme; and personal fees from Biogen and GlaxoSmithKline, outside the submitted work. Bhaskar Dasgupta, M.B.B.S., M.D., FRCP reports receiving grants and personal fees from Roche-Chugai and Sanofi and grants from AbbVie during the conduct of the study. BH has nothing to disclose. EM reports receiving clinical trial expenses from Kiniksa Pharmaceuticals during the conduct of the study; grants and personal fees from AbbVie; and personal fees from Janssen, Gilead, Novartis, Merck, and UCB, outside the submitted work. ES has nothing to disclose. BCT reports receiving personal fees from Kiniksa as a consultant member of DSMB. KJW reports grants from Kiniksa during the conduct of the study; grants from Eli Lilly, Roche/Genentech, and GlaxoSmithKline; and personal fees from Sanofi and Roche/Genentech, outside the submitted work. IW reports receiving scientific consulting fees from CSL and may receive a distribution of royalty income from the Walter & Eliza Hall Institute, which licensed intellectual property related to the alpha chain of the GM-CSF receptor. MS, TZ and LP are employees and stockholders of Kiniksa Pharmaceuticals. JFP is an employee and stockholder of Kiniksa Pharmaceuticals, and is an inventor on patent applications related to mavrilimumab. This study was funded in full by Kiniksa Pharmaceuticals.

Patient consent for publication Not applicable.

Ethics approval The trial was conducted in accordance with the principles of the Declaration of Helsinki, the Good Clinical Practice guidelines of the International Council for Harmonisation, and all required regulations. The protocol was approved by the institutional review boards or independent ethics committees of all participating centres. All patients provided written informed consent. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

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

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CLINICAL SCIENCE

Psoriasis rate is increased by the exposure to TNF inhibition in children with JIA

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221694).

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Received 18 October 2021
Accepted 3 January 2022
Published Online First 27 January 2022

To cite: Zhao Y, Sullivan E, Son MB, *et al*. *Ann Rheum Dis* 2022;**81**:662–665.

ABSTRACT

Objective We aimed to investigate the relationship between tumour necrosis factor inhibitors (TNFi) therapy and the onset of new psoriasis in children with juvenile idiopathic arthritis (JIA) using Childhood Arthritis and Rheumatology Research Alliance (CARRA) Registry data.

Methods De-identified data were obtained from the CARRA Registry. Patients with inflammatory bowel disease or psoriasis documented on or prior to JIA diagnosis date or with incomplete data were excluded. Exposure to TNFi was categorised as: (1) ever use; (2) current use or (3) first use only. Adjusted HRs (aHRs) were calculated between exposed and unexposed groups adjusted for methotrexate exposure, sex, race, family history of psoriasis and initial JIA category.

Results A total of 8225 patients were included with a median follow-up of 3.9 years. Over half of the patients were prescribed TNFi (n=4437, 54%). The aHR of new onset of psoriasis after ever exposure to TNFi was 2.93 (2.15 to 3.98). The incidence rate of psoriasis was the highest in children ever receiving and actively receiving adalimumab. Ever concurrent methotrexate use (HR 0.45, 0.29 to 0.69) was associated with lower risk.

Conclusion In a large prospective JIA patient registry, we observed a nearly threefold increased risk of psoriasis after TNFi exposureCite Now

Key messages

What is already known about this subject?

- Paradoxical psoriasis after exposure to tumour necrosis factor (TNF) inhibitors (TNFi) is described in the paediatric population but the association between TNFi and subsequent psoriasis in juvenile idiopathic arthritis (JIA) has not been systematically assessed using a large prospective clinic-based registry such as Childhood Arthritis and Rheumatology Research Alliance.

What does this study add?

- In a large prospective JIA patient registry, we observed a nearly threefold increased risk of psoriasis after TNFi exposure compared with patients who were never treated with TNFi.
- The risk of new onset TNFi-associated psoriasis was increased in the non-psoriatic arthritis subset with the adjusted HRs (95% CI) of psoriasis of 5.60 (3.47 to 9.05, p<0.01).
- Any concurrent methotrexate use was significantly associated with a lower hazard of psoriasis in patients without the psoriatic subtype of JIA (HR=0.45 (0.29 to 0.69)).

How might this impact on clinical practice or future developments?

- Increasing awareness of this unwanted side effect in JIA patients is important to ensure timely diagnosis and treatment.
- Concurrent methotrexate use could be considered to ameliorate the risk.

INTRODUCTION

Tumour necrosis factor inhibitors (TNFi) have been approved to treat juvenile idiopathic arthritis (JIA) in children. Children with underlying conditions including JIA, inflammatory bowel disease (IBD) and chronic non-bacterial osteomyelitis (CNO) treated with TNFi may develop psoriasis after exposure to TNFi which is sometimes called paradoxical psoriasis, as TNFi also effectively treat psoriasis.[1–6] The most common paediatric presentations included plaque and palmoplantar pustular psoriasis with similar anatomical distribution to psoriatic lesions not associated with TNFi use.[7] In many cases of paradoxical psoriasis, topical treatments lead to sufficient control allowing for continuation of TNFi therapy, whereas TNFi may need to be discontinued in persistent cases.[7 8] In studies to date, paradoxical psoriasis occurred at a median range of 4–20 months after exposure to TNFi.[2 3 5 6 9 10] Demographic characteristics and concomitant therapy were not significantly associated with paradoxical psoriasis from a large paediatric cohort study.[1] A positive family history of psoriasis was noted in 5%–27% of those who developed cutaneous lesions after exposure to TNFi.[2 3 5 6 9 10] A single-centre study that assessed rates of paradoxical psoriasis in JIA, IBD and CNO found that rates of psoriasis in TNFi exposed patients were higher for JIA and IBD, but not for CNO. Data from a large prospective clinic-based registry may help further elucidate the association between TNFi and subsequent psoriasis in JIA. We used data from Childhood Arthritis and Rheumatology Research Alliance (CARRA) Registry to determine the risk from a geographically diverse population.

PATIENTS AND METHODS

Inclusion and exclusion criteria

Data were obtained from the CARRA Registry for patients enrolled with a diagnosis of JIA from 30 June 2015 (Registry inception date) to 1 January 2020 with follow-up data. The CARRA Registry includes patients enrolled from 70 CARRA sites in North America.[11] Enrolment in the CARRA Registry

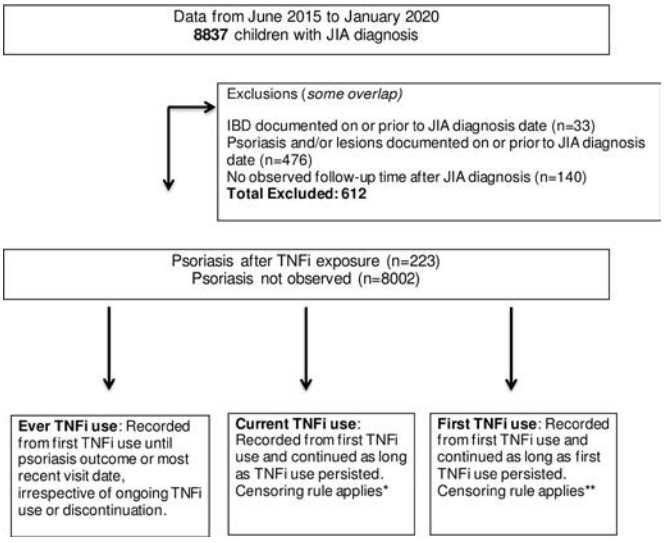

Figure 1 Diagram of data cleaning and censoring rules. *If TNFi was discontinued, then observation time attributed to TNFi was censored 60 days later. Follow-up was resumed for any subsequent restart of TNFi. Follow-up was censored at the first of: psoriasis outcome, most recent visit and most recent TNFi stop date. **If first TNFi was discontinued, then observation time attributed to first TNFi was censored 60 days later. Follow-up was censored at the first of: psoriasis outcome, most recent visit and first TNFi stop date. IBD, inflammatory bowel disease; JIA, juvenile idiopathic arthritis; TNFi, tumour necrosis factor inhibitors.

is not dependent on treatment with TNFi or any other specific medication use (ie, it is not a drug-based registry). The sites are instructed to assess all patients in the CARRA Registry for events of special interest in the same way, irrespective of treatment. It is unlikely that surveillance for psoriasis was increased by treatment with TNFi among Registry patients. New onset skin psoriasis following patient enrolment is considered an 'event of special interest' that Registry sites are explicitly instructed to report. Furthermore, history of psoriasis is obtained at the time of Registry enrolment. International League of Association for Rheumatology ILAR classification is used by site to report the category at baseline and subsequent visits. Psoriatic arthritis is defined as arthritis with psoriasis or meeting at least two of the three criteria (nail pitting, family history of psoriasis, dactylitis) if without psoriasis.[12] Patients with IBD or psoriatic skin lesions documented on or prior to JIA diagnosis date were excluded, as were patients with incomplete data regarding TNFi start and stop dates and patients with no recorded follow-up time after JIA diagnosis (figure 1). New onset psoriasis was defined by first recorded instance of psoriasis following JIA diagnosis. Exposure to TNFi was assessed in multiple ways which were not mutually exclusive as shown in figure 1. We categorised exposure as: (1) Ever TNFi use: follow-up began with first TNFi use and continued until psoriasis outcome or most recent visit date, irrespective of ongoing TNFi use or discontinuation. (2) Current TNFi use: follow-up began with first TNFi use and continued as long as TNFi use persisted. If TNFi was discontinued, then observation time attributed to TNFi was censored 60 days later. Follow-up was resumed for any subsequent restart of TNFi. Follow-up was censored at the first of: psoriasis outcome, most recent visit and most recent TNFi stop date. (3) First TNFi use: follow-up began with first TNFi use and continued as long as first TNFi use persisted. If first TNFi was discontinued, then observation time attributed to first TNFi was censored 60 days later. Follow-up was censored at the first of: psoriasis outcome, most recent visit and first TNFi stop date. The 60-day extension of the exposure risk window was chosen because TNFi may have been discontinued presumptively at the onset of rash before the diagnosis of psoriasis is confirmed. Some TNFi exposure time occurred prior to Registry enrolment. We used the historical medication log (retrospective data at enrolment) and the diagnosis date of comorbid condition at enrolment to make these assessments for some patients/exposures.

Statistics

Baseline characteristics were analysed with descriptive statistics. Incidence of psoriasis was calculated for each TNFi exposure method as number of cases per 1000 person-years, with 95% CIs calculated using mid-p exact methods. Incidence on TNFi was assessed overall (all TNFi) and individually (etanercept, adalimumab and infliximab only). Cox proportional hazard models were used to calculate HRs with 95% Wald CIs comparing the hazard of developing psoriasis among patients ever exposed or first exposed to TNFi versus those never exposed, adjusted for any concurrent methotrexate exposure, sex, race, family history of psoriasis and initial JIA category. Patients with PsA were included in the primary analysis if they had not experienced skin psoriasis prior to TNFi use. Due to concerns about patients with PsA developing skin psoriasis as part of the expression of their disease rather than 'paradoxical' psoriasis, secondary analyses were conducted which stratified by PsA. For modelling, TNFi exposure was treated as a time-varying covariate in Cox regression based on registry recorded start date of first TNFi, after which exposure was treated as a fixed exposure (ever exposed). Exposure to methotrexate was assessed separately within TNFi unexposed and TNFi exposed time and was treated as fixed within the given exposure assessment period. A p value of <0.05

Table 1 Baseline demographics

	Total population N=8225
Gender (%, male)	2371 (28.8%)
Mean (std) age in years at JIA diagnosis	7.5 (4.8)
Initial ILAR category	
Enthesitis related	854 (10.4%)
Oligoarthritis	2937 (35.7%)
Polyarthritis	3177 (38.6%)
Psoriatic	338 (4.1%)
Systemic	711 (8.6%)
Undifferentiated	208 (2.5%)
Race	
White	6253 (76.0%)
Black	341 (4.2%)
Hispanic	896 (10.9%)
Asian	223 (2.7%)
Other	512 (6.2%)
TNFi exposure	
Any use	4437 (53.9%)
Etanercept	2818 (34.3%)
Adalimumab	2516 (30.6%)
Infliximab	590 (7.2%)
First TNFi prescribed for use	
Etanercept	2675 (60.3%)
Adalimumab	1527 (34.4%)
Infliximab	207 (4.7%)

ILAR, International League of Association for Rheumatology; JIA, juvenile idiopathic arthritis; TNFi, tumour necrosis factor inhibitors.

Table 2 Summary of psoriasis incidence

	Incidence rate Cases/1000 person-years (95% CI)	Median follow-up time (months)	Raw totals
Overall (all follow-up time)	5.33 (4.67 to 6.07)	46.9	223 cases/41 831 PY
Never exposed to TNFi	3.27 (2.62 to 4.03)	20.4	84 cases/25 700 PY
Ever exposed to TNFi	8.62 (7.27 to 10.14)	33.3	139 cases/16 132 PY
First exposure to TNFi	8.65 (6.67 to 11.03)	13.5	61 cases/7056 PY
Specific categories of TNFi exposure			
First use of any TNFi			
Etanercept	6.78 (4.69 to 9.50)	15.1	31 cases/4573 PY
Adalimumab	12.32 (8.22 to 17.8)	11.1	26 cases/2110 PY
Infliximab	9.04 (2.30 to 24.59)	12.2	3 cases/332 PY
First ever use of specific TNFi**			
Etanercept	6.97 (4.88 to 9.68)	14.5	33 cases/4731 PY
Adalimumab	11.98 (8.60 to 16.27)	9.4	38 cases/3173 PY
Infliximab	6.44 (2.36 to 14.28)	10.4	5 cases/776 PY
Active use of specific TNFi			
Etanercept	5.46 (3.96 to 7.36)	22.5	40 cases/7324 PY
Adalimumab	13.41 (10.30 to 17.18)	12.2	59 cases/4400 PY
Infliximab	8.77 (4.88 to 14.61)	21.8	13 cases/1483 PY

*With or without other prior TNFi exposure.
CI, confidence interval; TNFi, tumour necrosis factor inhibitors.

was considered statistically significant. Statistics were performed in SAS V.9.4.

RESULTS

A total of 8225 patients with JIA were included with a median follow-up of 3.9 years and analysed as depicted in figure 1. The majority of patients were female and white (table 1). The mean age at the diagnosis of JIA was 7.5 years. Oligoarticular and polyarticular JIA were the most common categories. Over half of the patients were prescribed TNFi (n=4437, 54%) at some point during observation. Etanercept was most commonly used as the first TNFi followed by adalimumab and infliximab. However, the overall usage was similar between etanercept and adalimumab. TNFi prescription patterns did not differ significantly among patients with PsA versus others (online supplemental table 1). The incidence rate of psoriasis was much higher in PsA group (66.34 per 1000 person-years) than in other groups combined (1.17 per 1000 person-years) (online supplemental table 2).

The incidence rate of psoriasis was the highest in children who received adalimumab in ever exposure, current exposure and first exposure only calculations (table 2). The HR of new onset of psoriasis after ever exposure to TNFi was 3.02 (CI 2.26 to 4.02, unadjusted) and 2.93 (2.15 to 3.98, adjusted) (p<0.01) (table 3). The adjusted HR (95% CI) of psoriasis after TNFi exposure was 1.68 (1.11 to 2.54, p=0.01) in psoriatic JIA. The risk of new onset TNFi-associated psoriasis was increased in the non-psoriatic arthritis subset. The adjusted HR (95% CI) of psoriasis after TNFi exposure was 5.60 (3.47 to 9.05, p<0.01) in non-psoriatic arthritis subset. Any concurrent methotrexate use was significantly associated with a lower hazard of psoriasis in patients without the psoriatic subtype of JIA (HR=0.45 (0.29 to 0.69)); similar but non-significant associations were seen in patients with psoriatic JIA (HR for methotrexate: 0.73 (0.49 to 1.08)). Family history of psoriasis and race/ethnicity were not associated with psoriasis development.

Table 3 Association of TNFi exposure with new onset of psoriasis among patients with juvenile idiopathic arthritis

	Unadjusted		Adjusted*	
	HR (95% CI)	P value	HR (95% CI)	P value
TNFi ever use (all JIA)†	3.02 (2.26 to 4.02)	<0.01	2.93 (2.15 to 3.98)	<0.01
TNFi ever use (non-PsA JIA)†	4.57 (2.93 to 7.13)	<0.01	5.60 (3.47 to 9.05)	<0.01
TNFi ever use (only PsA JIA)†	1.62 (1.09 to 2.42)	0.02	1.68 (1.11 to 2.54)	0.01

*Adjusted for any concurrent methotrexate exposure (ever vs never), sex, race, family history of psoriasis and ILAR category at enrolment.
†TNFi 'ever use' defined as any time observed after the initial TNFi prescription, followed until psoriasis or most recent follow-up visit.
CI, confidence interval; HR, hazard ratio; JIA, juvenile idiopathic arthritis; TNFi, tumour necrosis factor inhibitors.

DISCUSSION

This is the largest study in a paediatric population ascertaining the increased risk of paradoxical psoriasis in children with JIA after exposure to TNFi. We found a threefold increase in the odds of psoriasis among patients with JIA treated with TNFi compared with those not treated with TNFi.

We further investigated the risks of new onset of psoriasis in children with JIA during the first TNFi exposure, ever exposure and current exposure and did not find significant differences between exposure ascertainment methods. These results suggest that the risk is not altered by the duration of TNFi treatment and the incidence of psoriasis will continue to occur as patients remain on the treatment. In order to determine the continuing risk after discontinuation of TNFi, a longitudinal cohort of patients with prolonged observation time after complete discontinuation of TNFi is needed.

Among the three most commonly prescribed TNFi, etanercept was most commonly used first, but adalimumab was used in a similar proportion of patients during the entire follow-up. The incidence rate of new onset psoriasis was significantly higher for adalimumab exposure than for etanercept exposure when total exposure time of each TNFi was considered. This result is consistent with published case series in which the majority of psoriasis occurred after exposure to adalimumab or infliximab.[3 4] Type 1 interferon is known to induce psoriasis and suppression of TNF has been associated with upregulated type 1 interferon. Therefore, we hypothesise that monoclonal antibody-based TNFi may be more associated with the development of psoriasis via potentiation of type 1 interferon due to more potent inhibition of TNF.[13 14]

The HR of psoriasis was only 1.68 in children with a diagnosis of psoriatic arthritis without pre-existing psoriasis, which was well below the HR of 5.6 in children with diagnosis of other categories of JIA. This finding suggests that while the overall risk of psoriasis was higher in children with psoriatic arthritis, the exposure to TNFi did not increase the risk in this subset of JIA as much. Both TNF and type 1 interferon may contribute to psoriasis while TNF regulates the production of type 1 interferon. Accordingly, it is possible that patients with psoriatic arthritis have relatively less production of type 1 interferon during the treatment of TNFi compared with other patients with JIA.

Any concurrent methotrexate use was associated with a significantly lower hazard of psoriasis in patients with JIA without psoriatic arthritis, whereas family history of psoriasis and race/ethnicity were not associated with psoriasis development. These results implied that there was potential benefit of combining

methotrexate with TNFi to prevent new onset of psoriasis in this population and demonstrate the importance of educating families about this known side effect of TNFi. Further investigation of this potential association is warranted.

The study has some limitations. As a retrospective cohort study, patients were not randomised to receive TNFi treatment and therefore there is likely some degree of residual confounding in our assessment of the association between TNFi use and development of psoriasis. There may be risk factors for psoriasis development which we did not capture. Additionally, some data collected retrospectively may also have been subject to recall bias. However, appropriate controls, adjustment of covariates and rigorous censoring based on the event and follow-up strengthen the validity of our and others' results.[1]

CONCLUSION

In a large prospective JIA patient registry, we observed a nearly threefold increased risk of psoriasis after TNFi exposure compared with patients who were never treated with TNFi. Increasing awareness of this unwanted side effect in paediatric community is important to ensure timely diagnosis and treatment.

Acknowledgements The authors would like to thank the following colleagues for their contribution during the project: Anne Dennos, Min-Lee Chang and Marc Natter, MD. This work could not have been accomplished without the aid of the following organisations: the NIH's National Institute of Arthritis and Musculoskeletal and Skin Diseases and the Arthritis Foundation. The authors would also like to thank all participants and hospital sites that recruited patients for the Childhood Arthritis and Rheumatology Research Alliance (CARRA) Registry. The authors thank the following CARRA Registry site principal investigators and research coordinators: N. Abel, K. Abulaban, M. Adams, R. Agbayani, J. Aiello, S. Akoghlanian, C. Alejandro, M. Alpizar, E. Anderson, E. Baker, L. Barillas-Arias, K. Baszis, H. Bell-Brunson, E. Beltz, H. Benham, S. Benseler, J. Boland, S. Bowman, H. Brunner, M. Buckley, H. Bukulmez, P. Carper, L. Cerracchio, E. Chalom, J. Chang, V. Chauhan, T. Chinn, K. Chundru, H. Clairman, A. Confair, A. Cooper, S. Cooper, R. Corvalan, D. Costanzo, L. Curiel-Duran, T. Curington, C. Davis, C. Davis, T. Davis, F. De Benedetti, J. Dean, F. Dedeoglu, V. Dempsey, T. Dickson, B. Donaldson, E. Dorsey, S. Dover, J. Dowling, J. Drew, K. Duarte, E. Duverger, M. Eckert, K. Ede, B. Edelheit, Y. Edgerly, B. Ervin, M. Falcon, B. Feldman, I. Ferguson, B. Ferreira, R. Ferrucho, K. Fields, M. Fitzgerald, C. Fleming, O. Flynn, L. Franco, M. Freeman, S. Froese, N. George, E. Giverc, C. Godiwala, I. Goh, H. Goheer, D. Goldsmith, B. Gottlieb, T. Graham, T. Griffin, J. Griswold, M. Guevara, E. Hammelev, M. Hance, A. Hanson, L. Harel, E. Hartigan, K. Hekl, K. Hickey, P. Hill, S. Hillyer, M. Hollander, S. Hong, J. Hsu, A. Huber, C. Hung, J. Huntington, A. Huttenlocher, L. Imundo, C. Inman, H. Jackson, J. Jaquith, S. Jared, J. Jones, J. Jones, S. Jones, R. Joshi, A. Justiniano, N. Karan, A. Kemp, S. Kim, D. Kingsbury, K. Klein, M. Klein-Gitelman, B. Kompelien, A. Kosikowski, J. Kracker, S. Kramer, J. Lam, D. Latham, L. Lentini, L. Lim, M. Lingis, D. Lowman, B. Malla, M. Malloy, M. Mannion, L. Marques, A. Martyniuk, T. Mason, S. Mathus, K. Mcallister, K. McCarthy, K. McConnell, E. McCormick, D. McCurdy, P. McCurdy Stokes, S. McGuire, I. McHale, A. McMonagle, C. McMullen-Jackson, E. Mendoza, R. Mercado, A. Merritt, L. Michalowski, D. Milojevic, E. Mirizio, E. Misajon, N. Mitchell, S. Mohan, K. Moore, L. Moorthy, S. Morgan, C. Moss, E. Muscal, R. Nadler, B. Nahal, N. Nasah, S. Nativ, M. Natter, L. Ng, J. Nicholas, B. Obispo, B. O'Brien, T. O'Brien, J. Olson, K. O'Neil, K. Onel, S. Osei-Onomah, J. Paredes, A. Parsons, J. Patel, K. Pentakota, T. Pfeiffer, K. Phillippi, D. Pires Marafon, L. Ponder, S. Prahalad, S. Pratt, S. Protopapas, B. Puplava, J. Quach, M. Quinlan-Waters, C. Rabinovich, J. Raisian, A. Rakestraw, C. Ramirez, K. Remmel, A. Repp, A. Reyes, S. Ringold, M. Riordan, M. Ritter, A. Robinson, E. Rodela, K. Rojas, T. Ronis, M. Rosenkranz, B. Rosolowski, N. Ruth, A. Sanni, L. Scalzi, S. Scharnhorst, K. Schikler, K. Schollaert-Fitch, T. Seay, A. Shelly, K. Shergill, C. Shivers, J. Sletten, A. Smith, C. Smith, J. Smith, R. Sran, H. Stapp, Y. Sterba Rakovchik, K. Stewart, J. Stokes, J. Sumner, R. Syed, G. Syverson, S. Tapani, L. Tate, A. Taxter, M. Terry, M. Tesher, A. Thatayatikom, K. Tiffany, A. Tipp, M. Toth, V. Tubwell, S. Uriguen, L. Vannoy, C. Varghese, R. Vehe, G. Vilar, N. Volpe, J. Wagner, D. Wahezi, H. Waite, J. Walker, T. Wampler Muskardin, L. Waqar, M. Watson, A. Watts, J. Weiss, P. Weiss, A. Wise, J. Woo, E. Wu, A. Yalcindag, Q. Yu, R. Zapata, A. Zartoshti, A. Zhu and C. Zic.

Contributors Study conception and design and data collection: YZ, MBS and TB. Analysis and interpretation of results, draft manuscript preparation, reviewed the results and approved the final version of the manuscript: all authors. YZ accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish.

Funding This research was funded by a Childhood Arthritis and Rheumatology Research Alliance Registry data analysis grant.

Competing interests YZ receives research funding from Childhood Arthritis and Rheumatology Research Alliance (CARRA), ACR/EULAR, Bristol-Myers Squibb and Washington Research Foundation and consultant fees from Novartis. MBS receives funding from CARRA, the Centers for Disease Control and Disease Prevention, PCORI and the Samara Jan Turkel Clinical Center for Autoimmune Disease. TB receives funding from CARRA and consultant fees from UCB and Novartis.

Patient consent for publication Not applicable.

Ethics approval Institutional review board approval (#2246) was obtained from Seattle Children's Hospital prior to the study. All Registry participants were consented for use of their data in analyses such as this study.

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. Original data request can be made through Childhood Arthritis and Rheumatology Research Alliance via info@carragroup.org and the data analysis protocol can be requested through YZ.

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EPIDEMIOLOGICAL SCIENCE

Osteoarthritis endotype discovery via clustering of biochemical marker data

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221763).

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Received 3 November 2021
Accepted 1 February 2022
Published Online First 4 March 2022

ABSTRACT

Objectives Osteoarthritis (OA) patient stratification is an important challenge to design tailored treatments and drive drug development. Biochemical markers reflecting joint tissue turnover were measured in the IMI-APPROACH cohort at baseline and analysed using a machine learning approach in order to study OA-dominant phenotypes driven by the endotype-related clusters and discover the driving features and their disease-context meaning.

Method Data quality assessment was performed to design appropriate data preprocessing techniques. The k-means clustering algorithm was used to find dominant subgroups of patients based on the biochemical markers data. Classification models were trained to predict cluster membership, and Explainable AI techniques were used to interpret these to reveal the driving factors behind each cluster and identify phenotypes. Statistical analysis was performed to compare differences between clusters with respect to other markers in the IMI-APPROACH cohort and the longitudinal disease progression.

Results Three dominant endotypes were found, associated with three phenotypes: C1) low tissue turnover (low repair and articular cartilage/subchondral bone turnover), C2) structural damage (high bone formation/resorption, cartilage degradation) and C3) systemic inflammation (joint tissue degradation, inflammation, cartilage degradation). The method achieved consistent results in the FNIH/OAI cohort. C1 had the highest proportion of non-progressors. C2 was mostly linked to longitudinal structural progression, and C3 was linked to sustained or progressive pain.

Conclusions This work supports the existence of differential phenotypes in OA. The biomarker approach could potentially drive stratification for OA clinical trials and contribute to precision medicine strategies for OA progression in the future.

Trial registration number NCT03883568.

Key messages

What is already known about this subject?

- There is an unmet need for new therapies that target the underlying pathology in osteoarthritis (OA).
- Computational methods based on unsupervised machine learning have the potential to stratify OA cohorts into subsets that correspond to distinct molecular endotypes.

What does this study add?

- By applying these methods to the IMI-APPROACH cohort, we identified three dominant clusters and characterised them as inflammatory, low-repair and subchondral bone/articular cartilage-driven phenotypes.
- Patients in the discovered clusters had statistically significant differences in clinical characteristics.

How might this impact on clinical practice or future developments?

- The biomarker-based endotype discovery approach could potentially drive stratification for OA clinical trials and contribute in the future to precision medicine strategies for OA care.

To cite: Angelini F, Widera P, Mobasheri A, *et al*. *Ann Rheum Dis* 2022;**81**:666–675.

INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis among older people, affecting more than 500 million people (7% of the global population).[1] It is one of the most frequent causes of physical disability among older individuals and a major contributor to healthcare and societal costs globally.[2] The risk factors for the development of OA include age, sex, obesity, previous joint injuries, repeated stress on the joint, malalignment, genetics, bone shape (including deformities) and certain metabolic diseases.[3] According to studies on the global burden of disease, knee OA represents the greatest burden.[4 5] However, despite the ever-increasing rise in the incidence and burden of OA, there is an unmet need for new therapies that target the underlying pathophysiologies.[6] The currently available pharmacological treatments are only able to target the symptoms of OA, and they have adverse side effects, especially in older adults with common comorbidities.

The development of effective treatments and disease-modifying OA drugs (DMOADs) for this debilitating condition is extremely challenging.[7] Many of the approaches that have been tried thus far have either failed or produced unsatisfactory outcomes. One of the greatest

Table 1 Biochemical markers analysed in the APPROACH cohort sampled from serum (S) and urine (U)

Name	Inter- and intra-CV	Detection range	Description
S_C3M	<15%	1–85 ng/mL	Matrix metalloproteinase (MMP)-mediated type III collagen degradation fragment. Type III collagen is a major collagen of connective tissues, including synovial membrane. C3M has been shown to be released from synovial membranes in the presence of proinflammatory cytokines which activate MMPs.[43]
S_CRPM	<15%	1–110 ng/mL	MMP-mediated C reactive protein (CRP) degradation fragment. CRP is an acute reactant elevated in chronic inflammatory diseases. CRPM is a metabolite of CRP.[44]
S_ARGS	<15%	0.01–0.40 pmol/mL	ADAMTS-mediated aggrecan degradation products. Aggrecan is the major proteoglycan of articular cartilage. Like MMPs, ADAMTS are expressed and activated in the presence of proinflammatory cytokines.[45]
S_C10C	<15%	500–7500 ng/mL	Cathepsin K-mediated type X collagen degradation fragment. Type X collagen is a minor collagen expressed by the cartilage cells called chondrocytes.[46]
S_C2M	<15%	0–10 ng/mL	MMP-mediated type II collagen degradation fragment. Type 2 collagen is the major fibrillar protein of cartilage and C2M is released on activation of MMPs.[47]
S_COLL2_1	<15%	200–2200 nM	Type II collagen degradation fragment similar, but from a different domain compared with C2M.[48]
S_COLL2_1NO2	<15%	150–6000 pg/mL	Inflammation-related (nitrated) type-II collagen degradation fragment. Nitrosylation is a post-translational modification induced by an increase in oxidative stress associated with inflammation.[48]
S_COMP	<15%	1–50 units/L	Cartilage oligomeric matrix protein. COMP is articular cartilage protein, which is released when cartilage is turned over.[49]
S_CTXI	<10%	0–3 ng/mL	Cross-linked, isomerised and cathepsin K-generated fragment of type I collagen C-terminal telopeptide. Type I collagen is the major fibrillar protein of bone and some connective tissues. Cathepsin K is mainly expressed by osteoclast, making CTX-I a marker of bone resorption.[50]
S_HA	<15%	10–800 ng/mL	Hyaluronic acid is a glycosaminoglycan distributed widely across connective, epithelial and neural tissues, including articular cartilage. It is released as part of tissue remodelling and turnover induced by, for example, inflammation.[50]
S_hsCRP	<10%	0–60 mg/L	High-sensitive C reactive protein (hsCRP) is an acute reactant elevated in chronic inflammatory diseases and used as a diagnostic marker in different rheumatic diseases.[51]
S_PRO_C2	<10%	5–1000 ng/mL	Type IIB collagen propeptide (synthesis). When new type II collagen is expressed by cartilage cells, PRO-C2 is released and is a reflection of cartilage formation.[12]
S_NMID	<10%	1–180 ng/mL	Bone gamma-carboxyglutamic acid-containing protein.[52]
S_RE_C1M	<15%	10–500 ng/mL	MMP-mediated type I collagen degradation. See S_C3M and S_CTX-I.[53]
U_CTXI_ALPHA	<15%	0–10 µg/mmol	Cathepsin K-generated fragment of type I collagen C-terminal telopeptide (corrected for creatinine) is a non-isomerised version of S_CTX-I and therefore believed to reflect degradation of young bone in contrast to the isomerised which measures old bone.[54]
U-CTXII	<15%	10–2500 ng/mmol	MMP- and cathepsin K-mediated type II collagen degradation fragment (corrected for creatinine). See CTX-I and C2M as well.[50]

Coefficient of variation (CV) and detection range are shown; for further assay validation, see references.

challenges in OA drug development is the heterogeneity of the disease.[8 9] However, despite being a multifaceted and heterogeneous syndrome, there is an opportunity to target different treatments to patients according to their disease drivers characterised by molecular endotypes (a description of a subset of patients with common molecular characteristics) and clinical phenotypes (an observable characteristic or trait of a disease).[9 10] OA may be amenable to tailored treatments that target specific phenotypes, including inflammatory, low repair, subchondral bone, metabolic or articular cartilage-driven phenotypes.[11–17]

Therefore, development of computational tools that includes objectively measured markers, such as biochemical markers, may facilitate OA drug development through patient subgrouping based on endotypic characteristics.[9 11] An example of an OA endotype could be a group of patients with elevated bone biochemical markers, as compared with the remaining of the OA population. Then, based on the link with clinical data, this subgroup could be annotated as having a bone-driven disease (ie, a OA disease phenotype), and hypothetically, this group of patients should be enriched for in clinical trials testing the efficacy of a bone-modulating drug.[18]

At present, defining the appropriate outcome measures that are needed for OA clinical trials and the objective assessment of new therapies is challenging.[19] Therefore, new computational methods based on machine learning (ML) and big data analytics can help advance this field of research by enabling protocols for patient classification into subtypes, using a combination of clinical, biochemical and/or imaging data.[20–22]

The aim of this study was to develop a methodology based on unsupervised ML (specifically, clustering) to identify/discover OA endotypes in the IMI-APPROACH cohort of patients with knee OA from a panel of 16 biochemical markers related to different joint tissue processes (eg, degradation, formation or inflammation), measured at the baseline of the study. The properties of the discovered clusters were thoroughly analysed using a combination of statistical and ML techniques, and the

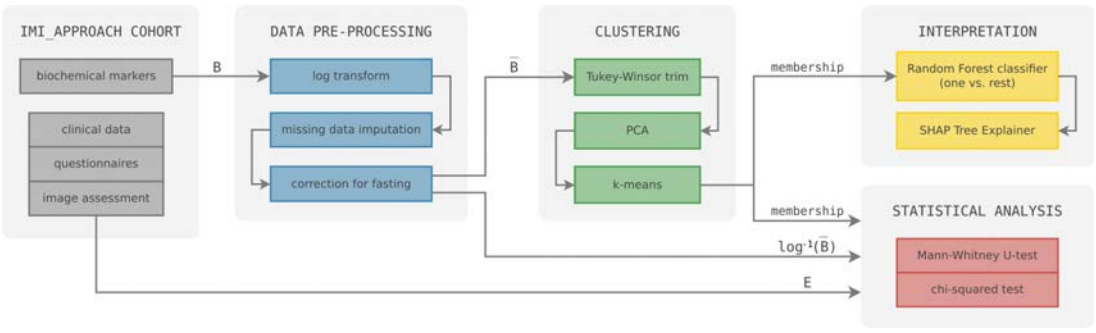

Figure 1 Overview of the data analysis pipeline. PCA, principal component analysis.

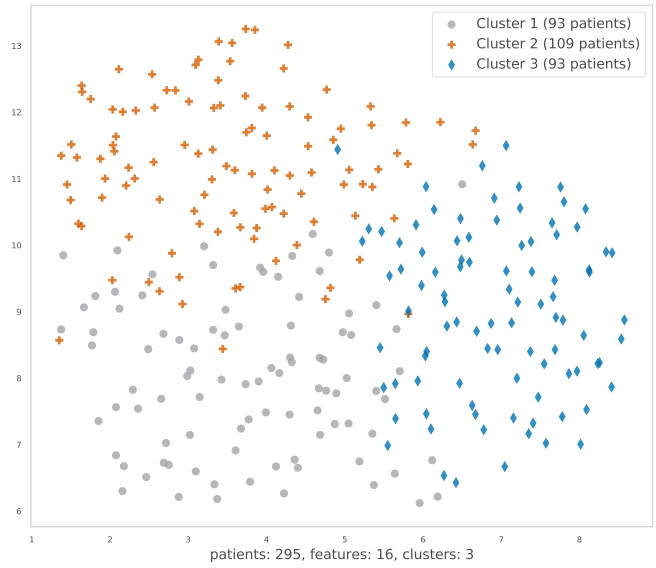

Figure 2 Clustering visualisation (k=3) obtained with UMAP (Uniform Manifold Approximation and Projection).

consistency of the discovered clusters was validated using data from an external cohort.

METHODS

Cohort description and data collection

Applied Public-Private Research enabling OsteoArthritis Clinical Headway funded by the Innovative Medicines Initiative (IMI-APPROACH, trial registration number: NCT03883568) is a prospective cohort study including 297 patients with tibiofemoral OA according to the American College of Rheumatology classification criteria. Patients were (pre)selected from existing cohorts using ML models, developed on data from the CHECK cohort, to display a high likelihood of radiographic joint space width (JSW) loss and/or knee pain progression.[23 24] The ultimate objective of APPROACH is to use real-world data to develop analysis methodologies to define disease subtypes and identify different knee OA clusters/phenotypes, to allow targeted treatment.

The IMI-APPROACH cohort screened 433 patients with OA (at five centres: Utrecht and Leiden, The Netherlands; A Coruña, Spain; Paris, France; Oslo, Norway) and enrolled 297 patients most likely to be pain and/or structural progressors at 2-year follow-up.[24] Enrolled patients were predominantly

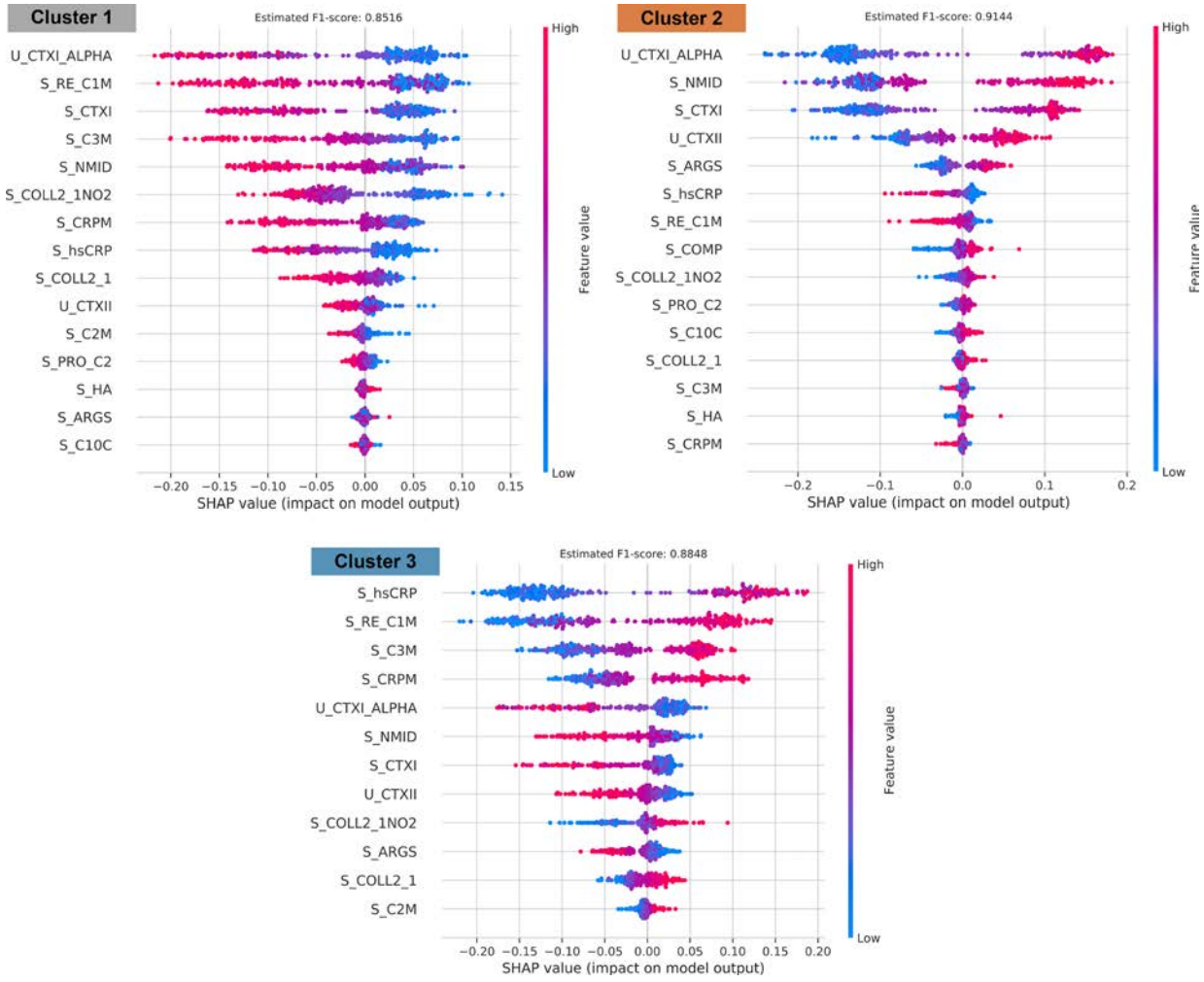

Figure 3 Impact of biomarker values on classification models decisions. Biomarkers are ordered by importance (most important on top). The SHAP values on the x-axis represent strength and direction of impact (positive value indicates increased probability of belonging to the cluster) for each patient. The colour represents the biomarker value (blue if low, red if high).

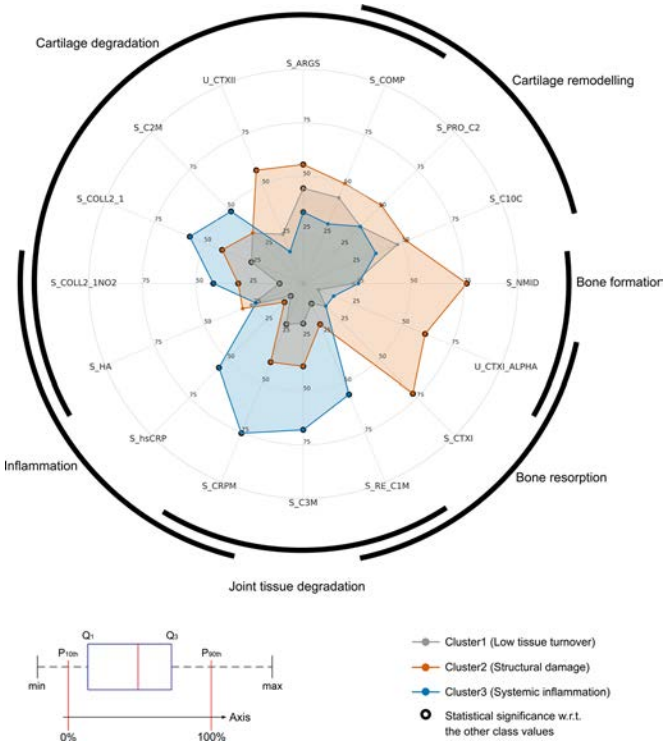

Figure 4 Radar plot showing the median biomarker concentrations for each cluster. When the difference between the medians is statistically different, it is marked with a circle (instead of a dot). The axes show values between the 10% and 90% quantile and are expressed as percentages. The black arcs on the outside show the pathology associated with each biomarker.

women (n=230), predominantly Caucasian/white (n=283), aged 44–82 years (median age: 67.5, IQR 62–71 years) and mostly overweight (median body mass index (BMI): 27 kg/m^2, IQR 24.4–31.6). At baseline, serum (S) and urine (U) samples were collected for analyses of 16 biochemical markers (table 1). The biomarkers were measured in International Organization for Standardization-certified laboratories at Nordic Bioscience (S_RE_C1M, S_C2M, S_C3M, S_C10C, S_CRPM, S_PRO_C2, U_CTXII, S_CTXI, U_CTXI_ALPHA, S_NMID, S_HA, S_COMP and S_hsCRP), Artialis (S_COLL2_1 and S_COLL2_1NO2) and Lund University (S_ARGS). The list of biomarkers was selected based on present knowledge of joint tissue turnover and OA.

In addition to the biochemical markers data (B), extra information (E) was collected as part of the IMI-APPROACH cohort.[23] These included assessment of radiographs of knees and hands, MRIs and CT scans of the knees, and outcomes of physical examinations and questionnaires: Function Index of Hand OA (FIHOA), Hip Disability and Osteoarthritis Outcome Score, Intermittent and Constant Osteoarthritis Pain Score, Knee-Injury and Osteoarthrosis Outcome Score (KOOS) and the 36-Item Short Form Health Survey. See online supplemental table B1. All data used in this paper were collected at the baseline visit of the study, except for the data on progression (Relation of clusters to progression section).

Data preprocessing

The biochemical markers data (B) were log transformed to account for long-tailed distributions. Missing data in B (<0.01% of values) were estimated (imputed) using either random Forest (RF) or k-nearest neighbour (KNN) regression models (see online supplemental appendix A, section 1.1).

As not all patients fasted before the sample collection, the fasting sensitivity of the biomarkers had to be assessed. The Spearman rank correlation with the patient's fasting status was found to be weak, except for U_CTXI (r=0.41). The values for this biomarker were corrected with an imputation approach (see online supplemental appendix A, section 1.2). We opted for a model-agnostic correction (ie, correcting the data rather than altering the analysis model) because it is more suitable for the downstream ML analysis we performed. $\bar{B}$ identifies the processed biomarkers data.

Clustering process

The extremes values of $\bar{B}$ were trimmed with a combination of Tukey and Winsor methods[25] to reduce the effect of outliers. Afterwards, principal component analysis was used to eliminate correlated biomarkers (see online supplemental appendix A, section 1.4). This resulted in 13 principal components which were found to explain 95% of data variance. These components were clustered using the k-means algorithm.[26] The optimum value for k (number of clusters) was identified from the consensus of silhouette score, the j-score and adjusted mutual information score. To obtain a robust estimate of these metrics, for each $k = 1, \ldots, 9$ the k-means algorithm was run 10 times with different random seeds (see online supplemental appendix A, section 1.5). The clustering with the highest quality was found for $k = 2, 3$. We chose $k = 3$ for the rest of the analysis in this paper as we aimed to investigate the highest number of meaningful clusters. The final cluster membership was taken from the algorithm run with the highest silhouette score for $k = 3$.

Cluster interpretation

Using data in $\bar{B}$, we trained three RF classification models predicting membership to each cluster (one cluster vs the rest) and then interpreted the model decisions using the SHAP (SHapley Additive exPlanations) TreeExplainer method,[27] to understand which variables determine the cluster membership. RF hyperparameters were tuned through a nested cross-validation procedure with recursive feature elimination (RFE-CV). See online supplemental appendix A, section 1.6 for more details.

Statistical analysis of cluster differences

To further describe the clusters, statistical tests were conducted for each feature in $\bar{B}$ and E, to assess whether the clusters had statistically different distributions for individual markers. The Mann-Whitney U test was used for continuous and ordinal features, and the $\chi 2$ test for categorical ones. The clusters were compared pairwise, and the null hypothesis was rejected following the Benjamini-Hochberg correction procedure for multiple comparisons applied across features.[28] The features in $\bar{B}$ were inverse log transformed to operate on actual biomarker concentrations (see online supplemental appendix A, section 1.3, for normality tests).

Figure 1 shows an overview of the entire data analysis pipeline described in this section, including data preprocessing, clustering, cluster's interpretation and the statistical analysis.

Validation on an external cohort

The proposed clustering pipeline was also applied to FNIH/OAI. The FNIH/OAI is the largest available OA cohort that was similar to IMI-APPROACH in terms of biomarkers.[29] The two cohorts had 11 biomarkers in common. Incurrent sample remeasurement for the adjustment of technical batch effects could not

be performed, as no samples were left and available from the FNIH/OAI cohort for this purpose. Therefore precise data alignment on the absolute mean concentrations and variance between the two cohorts was not possible to conduct.[30 31] As a result, the only possible type of external validation consisted in replicating the clustering pipeline for the two cohorts restricted to the common set of 11 biomarkers and evaluating the consistency of the identified clusters across cohorts.

Potential age and sex-based bias

To investigate the potential bias of age and gender in the clustering process, we statistically analysed the differences in age and sex across clusters, and we applied our clustering pipeline separately to the male and female subcohorts for both IMI-APPROACH and FNIH/OAI, to assess the consistency across clusters.

Relation of clusters to progression

To verify a relation between the clusters and disease progression, we used 2-year follow-up data to decide for each patient whether and how they have progressed, available only for a subset of 221 IMI-APPROACH participants. We defined one non-progressive category and three progressive categories related to pain, structure, and combined pain and structure.[23 24] Then we analysed the distribution of progressors in each cluster. See online supplemental appendix E for more details.

RESULTS

Cluster interpretation

Our clustering pipeline identified three clusters. These are shown in figure 2 as a two-dimensional projection obtained with UMAP (Uniform Manifold Approximation and Projection).[32] UMAP hyperparameters were optimised via grid search to maximise the two-dimensional silhouette score. The projection preserves the local neighbourhood structure and gives an idea of the strength of the global separation between the clusters in the original multidimensional space.

The classification models trained to predict patient's cluster membership achieved high F1 scores (C1 vs rest: 0.85, C2 vs rest: 0.91, C3 vs rest: 0.88). As a result, the subsequently performed model interpretation was expected to be meaningful. Figure 3 shows which biomarkers were predominantly used by each model to decide the cluster membership. Figure 4 compares the median biomarker concentrations for each cluster in a radar plot. Figure 5 shows the differences in biomarker value distributions across clusters. Bringing all these results together, the three clusters were interpreted as follows:

- *Cluster 1* represents a *low tissue turnover* phenotype: patients have all the inflammation and structural damage related biomarkers in the mid/low ranges.
- Cluster 2 represents a *structural damage* phenotype: patients have high values of the bone and cartilage markers: S_CTXI, U_CTXIALPHA, S_NMID and U_CTXII.
- *Cluster 3* represents a *systemic inflammation* phenotype: patients have high values of the inflammatory and MMP-driven markers: S_hsCRP, S_RE_C1M, S_CRPM and S_C3M. In contrast, these patients show low values of bone and cartilage related markers: U_CTXIALPHA, S_NMID, and S_CTXI.

Clustering stability

The clustering stability was investigated by comparing the results obtained for $k = 3$ with those obtained for $k = 4$ and $k = 5$. We found that clusters and interpretation were reasonably preserved at least until $k = 5$. This demonstrates that the three clusters analysed in this work are well-defined in the data space and robust with respect to finer clustering (see online supplemental appendix A, section 1.7).

Statistical analysis of differences between clusters

Several statistically significant differences in clinical scores were found. Full results are provided in online supplemental appendix B, and here we only present highlights of those findings. All figures cited in this section are provided in online supplemental appendix B.

- Clusters 2 and 3 had a higher percentage of women than cluster 1, and cluster 3 had a higher mean BMI (online supplemental figure B15).
- There was no difference in median age and range, smoking status, comorbidities and use of OA medication (online supplemental figure B14) across the clusters.
- Cluster 3 had statistically more patients experiencing substantial pain when standing (KOOS_P09, online supplemental figure B9), burning sensation (pain detect 09, online supplemental appendix B, B14) and more pain now and on average over the past 4 weeks (pain detect 01 and 03, online supplemental figure B14) than clusters 1 and 2. Patients in cluster 2 also experienced more pain in the past week than those in cluster 1 (pain detect 03, B14). Maximum Numeric Rating Scale (NRS) pain for hands were higher in cluster 3 (online supplemental figure B15), as well as having worse overall health self-assessment (SF36_11d, online supplemental figure B17).
- Cluster 1 has higher knee JSW (mean) than cluster 2 and less severe carpometacarpal Kellgren-Lawrence scores compared with cluster 3 (online supplemental figure B17).

External validation using FNIH/OAI data

We reduced the set of data features to the common subset of 11 biomarkers across the IMI-APPROACH and FNIH/OAI cohorts and applied the same clustering pipeline to both datasets. Figure 6 shows the comparison of obtained clusters. Despite the removal of five biomarkers, the IMI-APPROACH clusters still corresponded to structural damage, inflammatory and low tissue turnover endotypes. The FNIH/OAI clusters were found to consistently exhibit the same patterns, demonstrating cross-cohort robustness of our approach (see online supplemental appendix C).

Analysis of age and gender-based bias

We found no statistical difference between clusters in terms of age, as well as no statistical difference between male and female subcohorts in terms of age (see online supplemental figure D1). However, the male and female subcohorts had statistically different distributions for the following eight biomarkers: S_ARGS, S_C10C, S_COLL2_1, S_COLL2_1NO2, S_CTXI, S_NMID, U_CTXII and U_CTXI_ALPHA. Moreover, the clusters were significantly different in terms of gender, suggesting that it plays an important role in driving the clustering results (online supplemental figure D4). Similar patterns could be found for the FNIH/OAI cohort (see online supplemental appendix D).

Relation of clusters to progression

Table 2 summarises the progression status of the clusters. While we found progressors in all clusters, they were not distributed uniformly by progression type. There was more pain-related

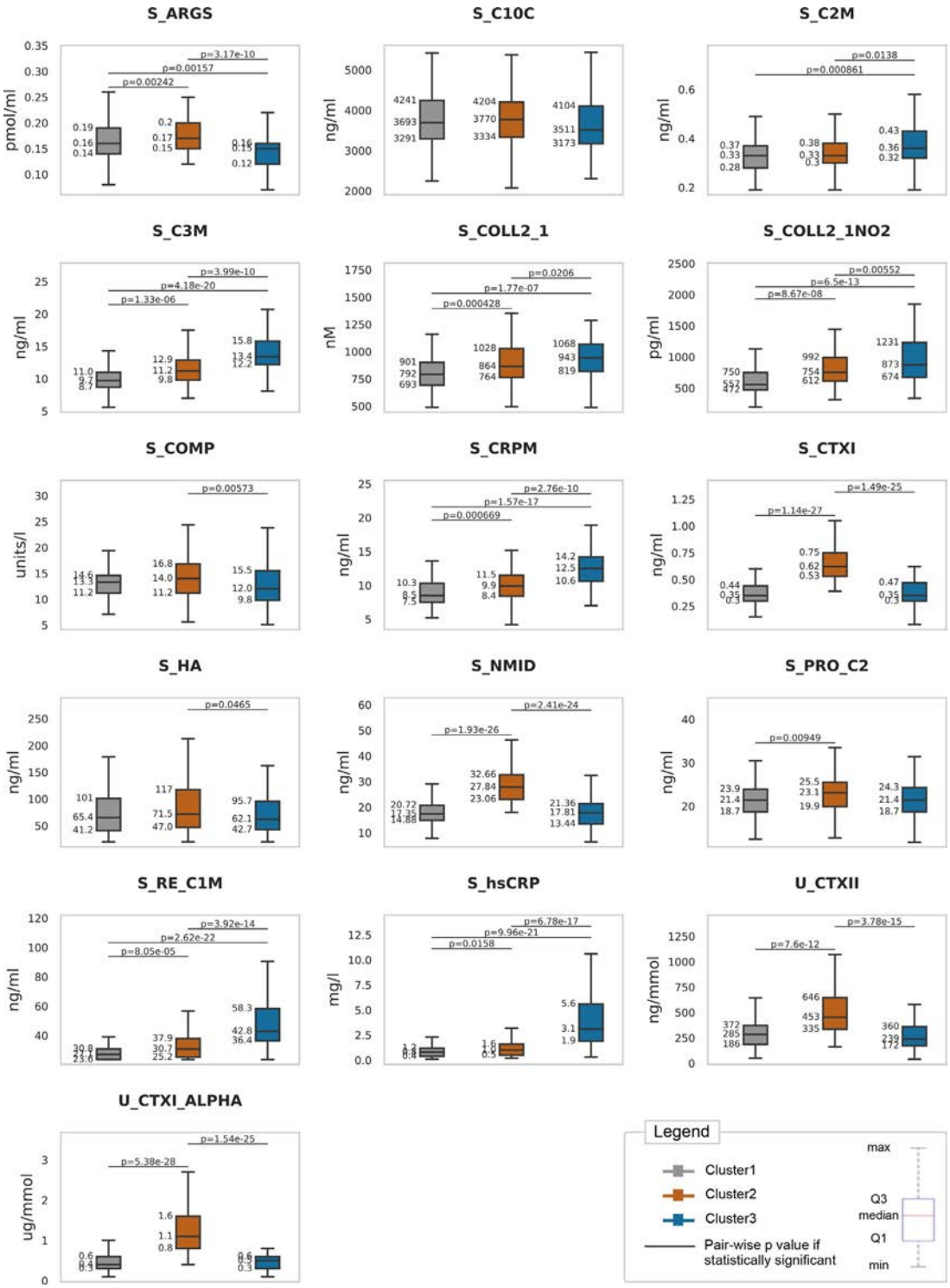

Figure 5 Comparison of biochemical markers' distributions in each cluster, and the statistical relevance of differences between them.

progressors and combined pain and structure progressors in the inflammation cluster (C3). Similarly, there were more structure-related progressors in the structural damage cluster (C2). The highest relative number of non-progressive patients was found in the low tissue turnover cluster (C1) and the lowest in the inflammation cluster (C3).

DISCUSSION

The aim of this work was to test if ML techniques can be used to identify biologically meaningful subgroups of patients with OA in the APPROACH cohort based on selected biochemical markers. By using clustering, that is, an unsupervised ML approach that does not exploit domain knowledge, we were able to identify molecular endotypes from 16 well-defined biochemical markers reflecting different molecular pathways and ongoing pathophysiological processes. We discovered three distinct OA phenotypes associated with the clusters (endotypes): C1—a low tissue turnover phenotype, C2—a structural damage phenotype and C3—systemic inflammation phenotype. The clustering reflects well the current biological and mechanistic understanding of

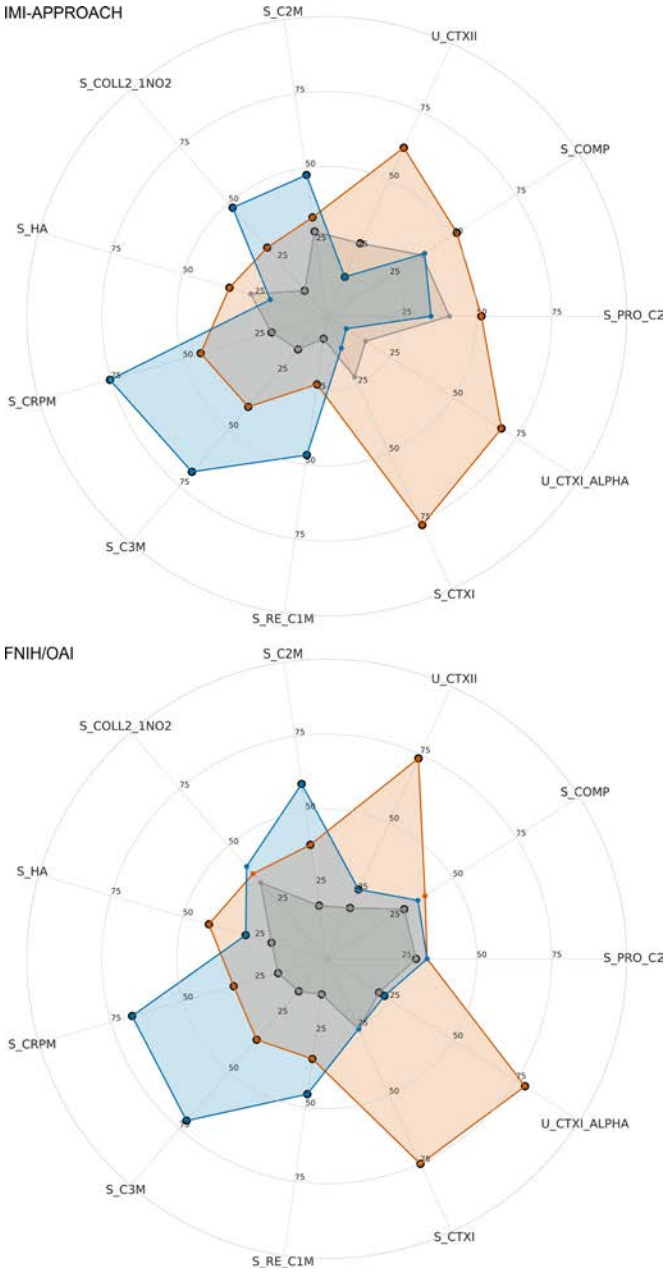

Figure 6 Radar plots comparing clusters found in the IMI-APPROACH and FNIH/OAI cohorts, using common subset of biomarkers. The median biomarker concentration for each cluster is shown. When the difference between the medians is statistically significant, it is marked with a circle (instead of a dot).

the respective biomarkers, in that distinct patterns could be identified for the subtypes. In particular, the combination of different markers describes the underlying biology in the clusters. This result is in line with published results from the FNIH/OAI biomarker initiative,[29 33] and the progression status of the members of each cluster is consistent with the cluster interpretation provided above: C1 has the highest proportion of non-progressors, C2 has the highest proportion of structural progressors and C3 has the highest proportion of pain-related progressors, and those progressing both in pain and in structure. However, although the proportions varied (ranging from 43% to 56%) progressive patients were found in all clusters. This means that the clusters represent different disease subtypes, within which the progression may occur.

Table 2 Distribution of progressive IMI-APPROACH patients across clusters.

Cluster (members)	No progression	Only pain	Only structure	Both
C1 (69)	39 (57%)	20 (29%)	7 (10%)	3 (4%)
C2 (84)	45 (54%)	21 (25%)	16 (19%)	2 (2%)
C3 (68)	30 (44%)	25 (37%)	7 (10%)	6 (9%)

Putting this in context of the work conducted on markers in clinical interventional trials, a few things can be learnt. Oral salmon calcitonin was tested as an antiresorptive treatment for OA. The phase III clinical trials failed to meet their clinical endpoints. Interestingly, calcitonin did significantly modulate CTX-I and CTX-II.[34] There are likely several reasons why this study failed, however, it begs to wonder what would the outcome have been if the study was enriched for C2 patients? Another failed trial was testing the efficacy of the IL-1 monoclonal antibody in OA and found markers from C3 modulated by treatment.[35] Would it still fail if it was enriched for C3 patients?

Despite a large and growing disease burden in OA, many pharmaceutical companies have de-emphasized or even abandoned OA drug development due to perceived hurdles. Crucial in this is the lack of appropriate predictive and outcome measures that can robustly identify patients early in the disease, which may benefit from a specific therapy. The lack of specific and sensitive baseline characteristics and subsequent endpoints to differentiate between responders and non-responders, both at the level of pain and tissue structure modification (ie, DMOAD), has led to trials that included hundreds of patients in each arm with at least 3-year follow-up. Despite these enormous trials, European Medicines Agency and Food and Drug Administration have not approved any DMOAD yet.[36] There is a general lack of understanding of OA pathogenesis which appears rather variable and likely reflects different phenotypes with fundamental differences in disease aetiology, tissue alterations, clinical manifestations (pain/mobility) and disease progression. Although the current mindset for drug treatment in the field is moving to a more personalised medicine and patient stratification approach, there are no accepted methods or guidelines to classify patients with OA, for example, to predict the underlying pathophysiology, to select patients according to their prognosis or to differentiate between patients in terms of diagnosis methodology and treatment plan. However, several initiatives have been initiated to generate more focus on the development of projects for identifying endotypes. For example, a framework for conducting and reporting phenotyping research was provided[37]—this may very well be the first step toward integrating the concept of phenotyping in research.

A better understanding of disease stratification and acceptance of a guideline to classify patients with OA will provide clear phenotype-directed protocols for DMOAD trials that enable us to target subgroups with OA that have uniform disease characteristics, thereby increasing the chances of success. We propose that the biomarker clustering analysis performed herein can be used to stratify patients with OA into groups with distinct molecular endotypes. This approach could potentially drive OA clinical trials stratification and serve as the basis for precision medicine strategies for OA progression in the future. Although there are limited data publicly available, there have been a few attempts to identify multimarker endotypes in OA. Sonh *et al* showed that several cytokines were elevated in synovial fluid and serum of patients with OA compared with normal samples when looking at an average level; however, it was also obvious

that the pattern was very heterogeneous.[38] Werdyani *et al* identified three distinct endotypes using metabolomics.[39] One of those clusters showed some association with muscle weakness. These data suggest that a subset of patients could belong to an inflammatory endotype.

Moreover, we focused on biochemical markers measured at the baseline of the study, and not their longitudinal changes, as this analysis would be more useful to inform future clinical trials. Longitudinal monitoring of biomarkers can give insight in the pharmacodynamic effects or provide early proof of effectiveness of a compound in interventional clinical trials, however often fail to predict progression in the study population in these trials.[34 40–42] Therefore, although longitudinal monitoring of individual biomarkers are only modestly predictive (if at all) of knee OA progression, they might have some utility as patient stratification like described herein for enriching OA trials for progressors.[29]

As more longitudinal data of the IMI-APPROACH cohort becomes available (currently an ongoing process), future investigations could explore the longitudinal data on biomarkers, imaging and other markers in IMI-APPROACH to further refine the description of the phenotypes and possibly explore more detailed stratifications. This analysis could take many different directions, for example, analyse cluster membership differences between visits or on comparison of the entire patient trajectories over 2 years of the study.

The main limitations of this work were the small numbers of patients in the IMI-APPROACH cohort and being able to perform only a partial validation with an external cohort, limited to a common subset of biomarkers. It would be beneficial for the field if future biomarker studies use a superset of the FNIH/OAI and IMI-APPROACH biomarkers, to allow for a complete validation of the discovered clusters. The use of predefined set of biochemical markers limits the discovery potential to certain molecular mechanisms. This could be avoided if clustering was performed on data generated by an untargeted platform (eg, RNA-seq); however, the analysis of such high-dimensional data is often much less robust, especially on small sample sizes. Finally, more research should be conducted on more abundant cohorts to fully evaluate the gender bias in clustering analysis of OA-related biochemical markers. From our analysis in the IMI-APPROACH and FNIH/OAI cohorts, we believe it is advisable for future studies to consider male and female patients separately and possibly draw conclusions that are gender based, if sample sizes are large enough.

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Acknowledgements We thank Agnes Lalande (Institut de Recherches Internationales Servier, Suresnes, France) for clinical study coordination, Thomas Lohr (GSK) for sample handling logistics and Janneke Boere and colleagues at Lygature for coordinating the research activity.

Contributors FA, PW, JBa contributed to the conception and design of the study, AS, MU, YH, ACM, MK, FJB, IKH, FB and ACBJ contributed to the acquisition of data. AM, JBl, CL, JL and ACB-J contributed to the analysis and interpretation of data. JBa is the paper's guarantor. All authors contributed to drafting the article or revising it critically for important intellectual content. All authors gave final approval of the version to be submitted.

Funding The research leading to these results has received support from the Innovative Medicines Initiative Joint Undertaking under Grant Agreement no 115770, resources of which are composed of financial contributions from the European Union's Seventh Framework Programme (FP7/2007–2013) and EFPIA companies' in-kind contribution. See http://www.imi.europa.eu/ and http://wwwapproachprojecteu/.

Competing interests ACB-J is a full-time employee and shareholder of Nordic Bioscience, a privately owned company involved in the development and commercialisation of biomarkers for fibroinflammatory disorders. YH is the founder and president of Artialis, and MU is a full-time employéé of Artialis, a spin-off company of the University of Liège. YH has also received fees from Tilman, Genequine, Seikagaku, Expanscience, Nestlé, Immubio, Biose and Labhra. CL was an employee of Merck at project start. IKH consults for Abbvie and Novartis and has received funding from Pfizer. JL is employed by and shareholder in GlaxoSmithKline. FB reports personal fees from AstraZeneca, Boehringer, Bone Therapeutics, CellProthera, Expanscience, Galapagos, Gilead, Grunenthal, GSK, Eli Lilly, Merck Sereno, MSD, Nordic, Nordic Bioscience, Novartis, Pfizer, Roche, Sandoz, Sanofi, Servier, UCB, Peptinov, 4P Pharma, 4Moving Biotech and grants from TRB Chemedica, outside the submitted work. FJB reports funding from Gedeon Richter, Bristol-Myers Squibb, Sun Pharma Global FZE, Celgene, Janssen Cilag, Janssen Research & Development, Viela Bio, Astrazeneca, UCB BIOSCIENCES, UCB BIOPHARMA SPRL, AbbVie Deutschland, Merck, Amgen, Novartis Farmacéutica, Boehringer Ingelheim España, CSL Behring, Glaxosmithkline Research & Development, Pfizer, Lilly, Corbus Pharmaceuticals, Biohope Scientific Solutions for Human Health, Centrexion Therapeutics, Sanofi, TEDEC-MEIJI FARMA, Kiniksa Pharmaceuticals, Fundación para la Investigación Biomédica Del Hospital Clínico San Carlos, Grünenthal and Galapagos. MK receives consulting fees from Abbvie, Pfizer, Levicept, GlaxoSmithKline, Merck-Serono, Kiniksa, Flexion, Galapagos, Jansen, CHDR, Novartis, UCB. AM receives fees/funding from Merck, Kolon TissueGene, Pfizer, Galapagos-Servier, Image Analysis Group (IAG), Artialis, Aché Laboratórios Farmacêuticos, AbbVie, Guidepoint Global, Alphasights, Science Branding Communications, GSK, Flexion Therapeutics, Pacira Biosciences, Sterifarma, Bioiberica, SANOFI, Genacol, Kolon Life Science, BRASIT/BRASOS, GEOS, MCI Group, Alcimed, Abbot, Laboratoires Expansciences, SPRIM Communications, Frontiers Media and University Health Network Toronto.

Patient consent for publication Not required.

Ethics approval This study involves human participants. The study is being conducted in compliance with the protocol, Good Clinical Practice (GCP), the Declaration of Helsinki, and applicable ethical and legal regulatory requirements (for all countries involved), and is registered under ClinicalTrials.gov: NCT03883568. All participants have received oral and written information and provided written informed consent. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. Data are available on request to the APPROACH Steering Committee.

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TRANSLATIONAL SCIENCE

Mechanical overloading promotes chondrocyte senescence and osteoarthritis development through downregulating FBXW7

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221513).

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Received 14 September 2021
Accepted 27 December 2021
Published Online First 20 January 2022

ABSTRACT

Objectives To investigate the role of mechanical stress in cartilage ageing and identify the mechanistic association during osteoarthritis (OA) progression.

Methods F-box and WD repeat domain containing 7 (FBXW7) ubiquitin ligase expression and chondrocyte senescence were examined in vitro, in experimental OA mice and in human OA cartilage. Mice with *Fbxw7* knockout in chondrocytes were generated and adenovirus-expressing *Fbxw7* (AAV-Fbxw7) was injected intra-articularly in mice. Destabilised medial meniscus surgery was performed to induce OA. Cartilage damage was measured using the Osteoarthritis Research Society International score and the changes in chondrocyte senescence were determined. mRNA sequencing was performed in articular cartilage from *Fbxw7* knockout and control mice.

Results Mechanical overloading accelerated senescence in cultured chondrocytes and in mice articular cartilage. FBXW7 was downregulated by mechanical overloading in primary chondrocytes and mice cartilage, and decreased in the cartilage of patients with OA, aged mice and OA mice. FBXW7 deletion in chondrocytes induced chondrocyte senescence and accelerated cartilage catabolism in mice, as manifested by an upregulation of $p16^{INK4A}$, p21 and Colx and downregulation of Col2a1 and ACAN, which resulted in the exacerbation of OA. By contrast, intra-articular injection of adenovirus expressing *Fbxw7* alleviated OA in mice. Mechanistically, mechanical overloading decreased *Fbxw7* mRNA transcription and FBXW7-mediated MKK7 degradation, which consequently stimulated JNK signalling. In particular, inhibition of JNK activity by DTP3, a MKK7 inhibitor, ameliorated chondrocyte senescence and cartilage degeneration

Conclusions FBXW7 is a key factor in the association between mechanical overloading and chondrocyte senescence and cartilage ageing in the pathology of OA.

Key messages

What is already known about this subject?

- Mechanical overloading and chondrocyte senescence play essential roles in osteoarthritis (OA) development.
- *Fbxw7* deletion leads to $p16^{INK4a}$ and p19 elevation to facilitate the cell cycle and promote cell senescence.

What does this study add?

- F-box and WD repeat domain containing 7 (FBXW7), a ubiquitin ligase, is a key factor in the association between mechanical stress and chondrocyte senescence in OA pathology.
- Excessive mechanical loading downregulates FBXW7 to activate MKK7–JNK signalling, which stimulates chondrocyte senescence and consequently initiates and accelerates OA development.
- Inhibition of JNK activity ameliorated chondrocyte senescence and cartilage degeneration.

How might this impact on clinical practice or future developments?

- This study suggests that targeting FBXW7-MKK7–JNK signalling may be a novel therapeutic approach for OA treatment.

INTRODUCTION

Osteoarthritis (OA) is the most common global age-related and post-traumatic degenerative joint disorder, which will become the disease with the highest disability rate globally by 2030.[1 2] Although mechanical overloading and advancing age have been recognised as the two most important risk factors for developing OA, much of the aetiology remains unkown.[3–5]

Proper mechanical loading is essential for joint health, while mechanical overloading can result in articular cartilage being prone to degenerative lesions that lead to OA onset and progression. Due to the progressive loss of articular cartilage that mainly occurs in load-bearing joints, OA was previously, for many decades, considered to be a mechanical issue.[6] In the clinic, the knee axis of most patients with OA is misaligned, resulting in various deformities leading to further exacerbation of wear and accelerating OA progress.[7 8] Previous studies have shown that a variety of signalling pathways are activated during OA progression. However, the specific mechanism through which mechanical overloading induces OA has not been fully elucidated.[9–11]

Advancing age has been identified as the prominent biological mechanism for OA development

To cite: Zhang H, Shao Y, Yao Z, *et al*. *Ann Rheum Dis* 2022;**81**:676–686.

and progression.[12–14] Because chondrocytes are the only cell type in articular cartilage, when they display dysfunctional metabolism, this leads to cartilage damage, which has been widely studied. Accumulated evidence shows that senescent chondrocytes are increased in human aged and OA cartilage lesions compared with that of young and healthy cartilage, suggesting a strong correlation between chondrocyte senescence and OA severity.[15] Senescent chondrocytes exhibit a senescence-associated secretory phenotype (SASP) and secrete enzymes capable of digesting cartilage extracellular matrix, resulting in cartilage degeneration and disruption.[16 17] Interestingly, senescent cell clearance in the mouse joint not only prevents disease progression but also maintains tissue structure.[18 19] Together, chondrocyte senescence and cartilage ageing play essential roles in OA development; however, the factors that stimulate chondrocyte senescence and the underlying mechanisms remain to be identified.

As mechanosensitive cells, chondrocytes perceive and respond to mechanical stress throughout life.[20 21] Indeed, different types of exercise will lead to different stress intensities on cartilage, resting (0%–10%), walking and exercise (20%–40%), and joint trauma and injurious loading (60%–90%).[22 23] Accumulating evidence indicates that high intensity mechanical stress accelerates chondrocyte catabolism, while moderate intensity stimulates the chondrocyte to secrete collagen. Moreover, high intensity stress can induce the chondrocyte to express SASPs, and shear stress alone can induce chondrocyte senescence.[24–27] Therefore, chondrocyte senescence was identified as a turning point regarding chondrocyte phenotype in OA and its SASP activity is essential in cartilage erosion. However, the role of mechanical stress in chondrocyte senescence and cartilage ageing is unclear and their mechanistic association has not been reported.

In this study, we found that mechanical overloading stimulated senescence in cultured chondrocytes and in mice articular cartilage, and identified that F-box and WD repeat domain containing 7 (FBXW7), a ubiquitin ligase, to be a key factor in the association between mechanical stress and chondrocyte senescence in OA pathology. FBXW7, as a ubiquitin ligase, is emerging as having a key role in controlling cell growth, differentiation and tumorigenesis, but its role in OA progression has not previously been investigated. Excessive mechanical loading downregulates FBXW7 to activate MKK7–JNK signalling, which stimulates chondrocyte senescence and consequently initiates and accelerates OA development. Targeting FBXW7-MKK7–JNK signalling represents a novel therapeutic approach for OA treatment.

RESULTS

Excessive mechanical loading induces chondrocyte senescence in vitro and in mice

To examine the effect of mechanical loading on chondrocyte senescence, mouse primary chondrocytes were treated with 0.5 Hz and 5%, 10% and 20% cyclic tensile strain loading for 0–24 hours. Consistent with previous results, 0.5 Hz with 5% and 10% cyclic tensile strain loading upregulated *Col2a1* but downregulated *Mmp13* mRNA levels, indicating the chondrogenic effect of low mechanical loading in chondrocytes.[28 29] However, excessive mechanical loading by 20% cyclic tensile strain not only promoted catabolic effects but also stimulated chondrocyte senescence (figure 1A,B). The number of cells with senescence-associated β-galactosidase (SA-βGal) staining, a classical indicator of senescence, and γH2AX, a marker of DNA damage, were increased in a time-dependent manner after excessive mechanical loading (figure 1C,D, and online supplemental figure S1A). In addition, 20% cyclic tensile strain loading increased the mRNA levels of $p16^{ink4a}$, *p21*, *Gadd45* and *Il-6* but decreased *LaminB1*, while 5% and 10% loading had a protective effect against cell senescence (online supplemental figure S1B–D). Furthermore, human primary chondrocytes were used to confirm the effect of mechanical loading on chondrocyte metabolism in both cyclic tensile strain loading model and compression loading model (online supplemental figure S2A–D). Interestingly, examination of chondrocyte monolayer features in response to several stretch amplitudes revealed gradual, time-dependent reorientation of filamentous actin (F-actin) to the stretch direction. Monolayer alignment was initiated at 6 hour and completed at 24 hours of continuous 20% cyclic tensile strain. In contrast, 5% and 10% stretch were insufficient to trigger alignment, even at longer time scales (figure 1E). In addition, cyclic 20% strain resulted in an enlarged nuclear area, a feature of senescent cells (figure 1E,F). These findings demonstrate that excessive mechanical loading induces senescence in primary cultured chondrocytes.

We further assessed the effect of mechanical overloading on chondrocyte senescence in mouse articular cartilage. As expected, the application of multiple loading episodes at a peak load of 13.5 N for 14 days induced proteoglycan loss with significant fibrillation of the articular surface on mouse knee joints, whereas no significant changes were detected on low mechanical loading (9 N). The number of articular chondrocytes stained for $p16^{INK4a}$ and p21 increased markedly, accompanied by the loss of cartilage structure by 13.5 N peak loads (figure 1G,H). Together, these findings demonstrate that excessive mechanical loading accelerates chondrocyte senescence in vitro and in articular cartilage, suggesting a potential mechanism in OA pathogenesis and development.

Chondrocyte FBXW7 is reduced by mechanical overloading and is decreased in articular cartilage of patients with OA, aged mice and OA mice

We subsequently investigated the mechanism through which mechanical overloading stimulates chondrocyte senescence. Isobaric tags for relative and absolute quantitation proteomic analysis was performed to quantitatively analyse and map proteins in mouse primary chondrocytes subjected to 20% elongation strain loading for 24 hours. Among the 813 differentially expressed proteins, FBXW7 was the most highly downregulated by mechanical stress (online supplemental table 1). FBXW7, a ubiquitin ligase and a member of the F-box family proteins, was of particular interest. It contributes to the degradation of proteins that positively regulate the cell cycle, but its role in chondrocyte and OA development is unknown. Immunohistochemical (IHC) staining of cartilage confirmed a marked decrease of chondrocyte FBXW7 by mechanical stress (figure 2A). Consistent with this, the *Fbxw7* mRNA and protein levels were decreased by 20% cyclic tensile strain loading in human and mouse primary chondrocyte culture (online supplemental figure S3A–C). Furthermore, human primary chondrocytes were used to confirm the effect of mechanical loading on Fbxw7 expression in a compression loading model (online supplemental figure S3D). To further understand whether chondrocytes are responding to mechanical overload by producing less FBXW7 or are degrading FBXW7 protein more quickly, mouse primary chondrocytes were treated with MG132 (10 μM) to inhibit proteolysis or with

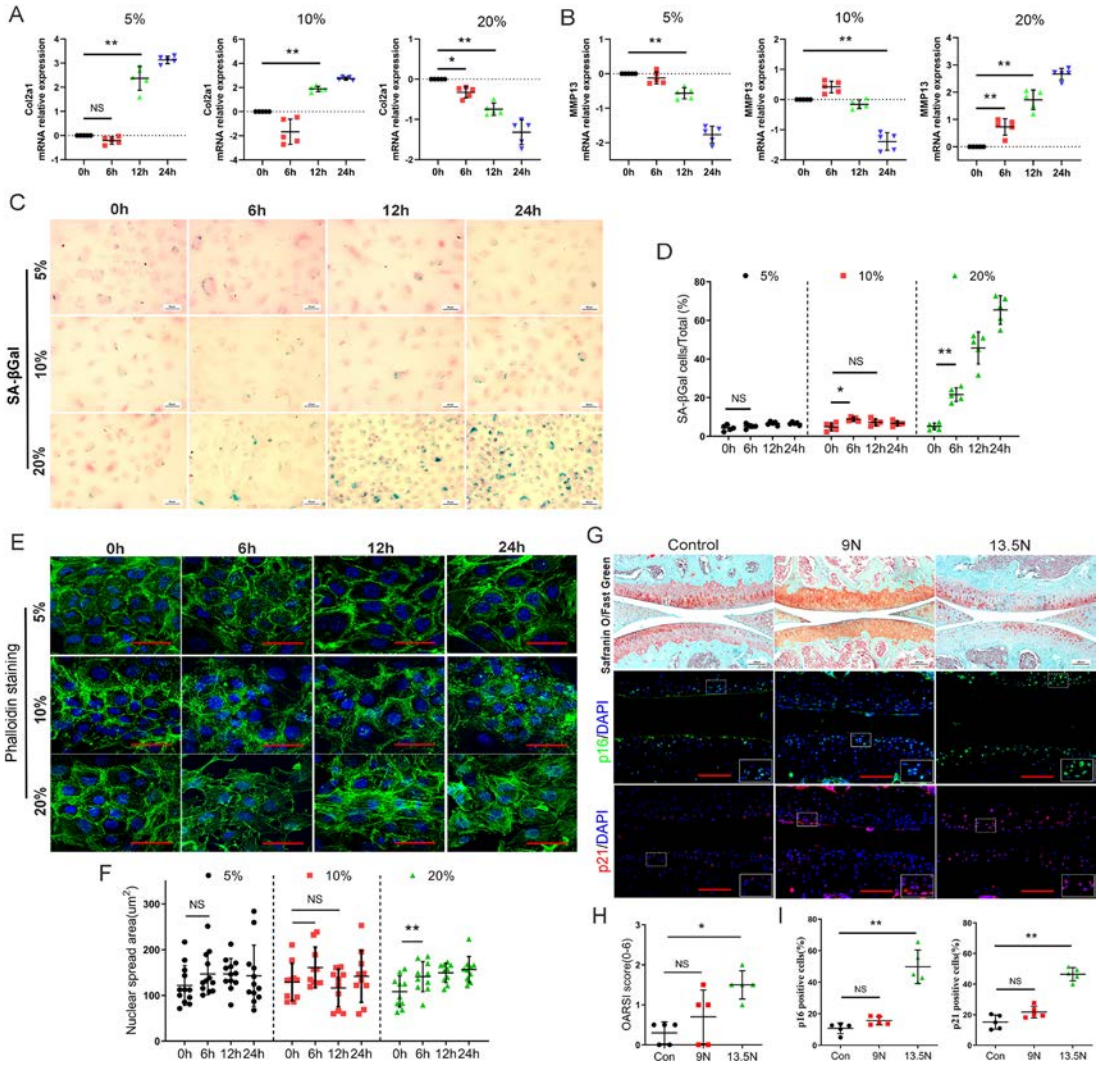

Figure 1 Excessive mechanical loading induces chondrocyte senescence in vitro and in mice. (A,B) Quantitative PCR analysis of *Col2a1* and *Mmp13* in primary chondrocytes treated with different elongation strain loading (5%,10% or 20%) for 0, 6, 12 and 24 hours. n=5 per time point. (C,D) Representative images and quantification of SA-βGal staining in primary chondrocytes treated with different elongation strain loading (5%, 10% or 20%) for 0, 6, 12 and 24 hours. n=5 per time point. Scale bar: 50 µm. (E,F) Representative images and quantification of F-actin (phalloidin) staining in chondrocytes described in (C) . n=10 per time point. Scale bar: 50 µm. (G) Representative images of safranin O/Fast green staining (top) and immunofluorescence of $p16^{INK4a}$ (middle) and p21 (bottom) in articular cartilage of mice treated with multiple loading episodes at peak loads of 9.0 and 13.5 N. Scale bars: 100 µm (first row) and 50 µm (second and third rows). (H) Quantitative analysis of the OARSI scale. n=5 per group. (I) Quantification of $p16^{INK4a}$-positive and p21-positive chondrocytes as a proportion of the total chondrocytes in mice cartilage described in (G). n=5 per group. Data are shown as mean±SD. Statistical analyses were conducted using one-way analysis of variance followed by Dunnett's multiple comparison test (A,B,I), two-way analysis of variance followed by Dunnett's multiple comparisons test (D,F) or Kruskal-Wallis test followed by Dunn's multiple comparisons test (OARSI score) (H). Boxed area is enlarged in the bottom right corner. *P<0.05, **P<0.01. DAPI, 4',6-diamidino-2-phenylindole; NS, not significant; OARSI, Osteoarthritis Research Society International.

cycloheximide (CHX) (50 µM) to block new protein synthesis under 20% elongation strain loading for 24 hours. When treated with CHX alone, the expression level of FBXW7 was reduced significantly. However, there was no significant difference in the levels of FBXW7 protein under mechanical overloading whether treated with CHX or not (online supplemental figure S3E). Furthermore, inhibition of proteolysis by MG132 increased FBXW7 protein level in control cells but could not prevent the marked decrease of FBXW7 induced by 20% elongation strain loading (online supplemental figure S3F). The results suggested that the loss of FBXW7 in response to mechanical overloading is mainly caused by a decrease in FBXW7 mRNA synthesis. In addition, when protein synthesis was blocked by CHX, we found that the level of FBXW7 decreased time-dependently as previously reported.[30] These results indicate that the loss of FBXW7 protein at 24 hours under 20% elongation strain loading might be caused by the initial drop in the mRNA level at 6 hours (online supplemental figure S3G–J).

Moreover, IF staining showed that FBXW7 was decreased in association with cartilage damage in patients with OA, which was further confirmed by western blotting analysis (figure 2B–E and online supplemental figure S4A–C). Interestingly, a loss of FBXW7-expressing chondrocytes was observed in aged (24-month-old) mice compared with young (4-month-old) mice (figure 2F–H and online supplemental figure S4D,E). Additionally, FBXW7-positive articular chondrocytes were progressively reduced in a mechanical load-induced (destabilisation of the medial meniscus (DMM))

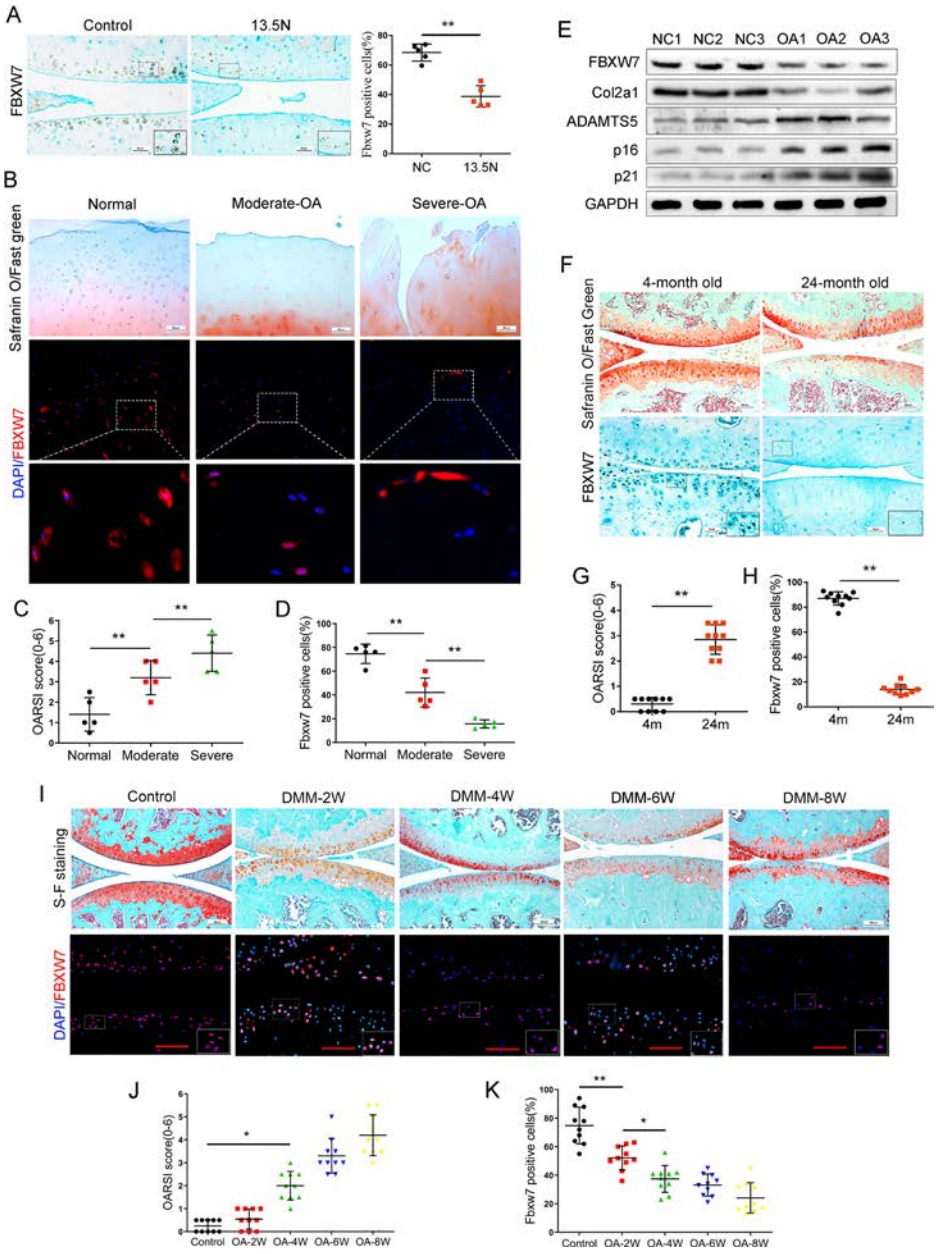

Figure 2 Chondrocyte FBXW7 is reduced by mechanical overloading and decreased in OA articular cartilage. (A) IHC staining of FBXW7 in chondrocytes of mice treated with multiple loading episodes at peak loads of 13.5 N and controls, and quantitative analysis of FBXW7-positive chondrocytes as a proportion of the total chondrocytes. n=5 per group. Scale bar: 50 µm. P<0.001, 95% CI −39.30 to −20.16. (B) Representative images of safranin O/fast green staining (top) and immunofluorescence of FBXW7 (middle and bottom) in normal, moderate OA and severe OA cartilage. Scale bars: 200 µm (first row) and 100 µm (second row). (C,D) Quantitative analysis of OARSI scale and FBXW7-positive chondrocytes as a proportion of the total chondrocytes. n=5 per group. (E) Western blotting analysis of FBXW7 COL2A1, ADAMTS5, $p16^{INK4a}$ and p21 in normal and severe OA cartilage. (F) Representative images of safranin O/fast green and IHC staining of FBXW7 in chondrocytes of mice aged 4 and 24 months. Scale bars: 100 µm (first row) and 50 µm (second row). (G) Quantification of the OARSI scale based on staining results in (F). n=10 per group. (H) Quantification of FBXW7-positive chondrocytes based on staining results in (F). n=10 per group. P<0.001, 95% CI −77.58 to −68.62. (I) Representative images of safranin O/fast green staining and immunofluorescence of FBXW7 in chondrocytes of controls and mice at 2, 4, 6 and 8 weeks after DMM surgery. Sale bars: 100 µm (first row) and 50 µm (second row). (J) Quantitative analysis of the OARSI scale of controls and DMM mice. n=10 per group. (K) Quantification of FBXW7-positive chondrocytes as a proportion of the total chondrocytes of control and DMM mice. n=10 per group. Data are shown as mean±SD. Statistical analyses were conducted using Student's t-test (A,H), one-way analysis of variance followed by Tukey's multiple comparison test (D,K), Kruskal-Wallis test followed by Dunn's multiple comparisons test (OARSI score (C,J), or non-parametric Mann-Whitney U tests (OARSI score (G). Boxed area is enlarged in the bottom right corner. *P<0.05, **P<0.01. DMM, destabilisation of the medial meniscus; FBXW7, F-box and WD repeat domain containing 7; GAPDH, glyceraldehyde-3phosphate dehydrogenase; IHC, immunohistochemical; NC, control; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International.

OA mouse model, which exhibited a senescence phenotype, indicating that both mRNA and protein levels of FBXW7 were reduced with the progression of articular cartilage degeneration (figure 2I–K and online supplemental figure S4F–H). Taken together, these observations show that chondrocyte FBXW7 is reduced by mechanical overloading and is decreased in the articular cartilage of patients with OA, aged mice and OA mice, implicating a potential role for FBXW7 in

the association between excessive mechanical load and chondrocyte senescence during OA development.

FBXW7 deletion in chondrocytes accelerates cartilage ageing and exacerbates OA development in mice

To determine the casual relationship between the loss of chondrocyte FBXW7 and OA progression, we generated mice with a conditional deletion (knockout) of the *Fbxw7* gene in chondrocytes (*Fbxw7*KO) by crossing *Fbxw7*$^{flox/flox}$ mice with *Col2a1*-cre mice, and genotypes were determined by PCR (online supplemental figure S5A,B). We found a slight decrease in the body length in *Fbxw7*KO mice aged 4 weeks, and delayed formation of secondary ossification centre in *Fbxw7*KO mice at postnatal day 7 (P7), compared with controls. However, no significant differences either in gross appearance or organisation of the articular cartilage and the growth plates were found between *Fbxw7*KO mice and littermate controls at the ages of 4 and 12 weeks, together with no significant increased expression of COLX in growth plate. These results indicated that the retarded growth of *Fbxw7*KO mice may cause by delayed formation of secondary ossification centre around P7 (online supplemental figures S5C–G and S6A–D). In addition, ablation of FBXW7 in articular chondrocytes of *Fbxw7*KO mice was further confirmed by IHC staining (figure 3A).

At the age of 3 months, no significant changes in chondrocyte senescence markers were observed between control and *Fbxw7*KO mice (online supplemental figure S6E). However, by the age of 18 months, *Fbxw7*KO mice exhibited significant cartilage erosion and loss of both proteoglycans and cellularity in the articular cartilage compared with control mice, which was further validated by Osteoarthritis Research Society International (OARSI) scale analysis (figure 3B–D). The number of p16^{INK4a}- and p21-positive articular chondrocytes was markedly increased in aged *Fbxw7*KO mice compared with their littermate controls. FBXW7 deletion also promoted chondrocyte senescence in vitro because enhanced SA-β-galactosidase staining was observed in FBXW7-deficient primary chondrocyte culture at passage 6, indicating that the loss of FBXW7 induced chondrocyte senescence and contributed to OA development (figure 3B–D and online supplemental figure S7A). In addition, FBXW7 deletion accelerated experimental OA in a DMM model. Colx and TUNEL-positive chondrocytes were increased, whereas Col2a1 and aggrecan were significantly decreased in *Fbxw7*KO mice compared with control mice (figure 3E,F and online supplemental figure S7B,C). In addition, we also detected synovial inflammation and bone changes in *Fbxw7*KO mice and their littermate controls. In both aged and DMM model mice, *Fbxw7*KO mice developed larger periarticular osteophytes and more synovitis inflammation compared with littermate controls but exhibited no statistically significant difference in bone density (online supplemental figure S8A–D). These results indicate that the aggravated cartilage damage also accelerates synovial inflammation and osteophyte formation in *Fbxw7*KO mice. Collectively, these results reveal that FBXW7 deletion in chondrocytes accelerates cell senescence and cartilage ageing, and leads to grossly observable cartilage destruction in aged and traumatic OA mice.

FBXW7 overexpression in cartilage alleviates OA development

We then performed an experiment with human or mouse primary chondrocytes treated with or without adenovirus containing FBXW7 (Ad-*Fbxw7*) under 20% elongation strain loading for 24 hours. Increased expression of Col2a1 and decreased MMP13 were found in Ad-Fbxw7-treated chondrocytes, demonstrating that addition of FBXW7 can rescue the promotion of the catabolic effect caused by excessive mechanical loading (figure 4A,B). Subsequently, adenovirus expressing FBXW7 (AAV-*Fbxw7*) and comparable amounts of AAV-negative control were injected intra-articularly once a week from 3 days after DMM surgery. GFP distribution indicated that intra-articular injection of adenovirus mainly affected articular cartilage, accompanied by significantly elevated FBXW7 expression in the chondrocytes of the middle and deep zones in AAV-*Fbxw7*-treated mice, demonstrating successful AAV-delivered overexpression of FBXW7 (figure 4C). As expected, AAV-*Fbxw7* effectively alleviated OA development in mice, as manifested by reduced chondrocyte hypertrophic differentiation and attenuated cartilage destruction and proteoglycan loss, together with increased Col2a1 expression and decreased MMP13 and Colx expression in the tibial cartilage of AAV-*Fbxw7*-treated mice. Importantly, p16^{INK4a}-positive, p21-positive and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)-positive articular chondrocytes were also significantly decreased by AAV-*Fbxw7* (figure 4D, E and online supplemental figure S9A). Together, these data suggest that FBXW7 protects against mechanically induced chondrocyte senescence and OA development.

FBXW7 loss by excessive mechanical loading activates MKK7–JNK signalling to promote chondrocyte senescence

To explore the mechanisms through which FBXW7 regulates chondrocyte senescence, the mRNA expression profile of articular cartilage from *Fbxw7*KO mice and their littermate controls was analysed (SRA accession codes PRJNA78345).[31] By performing Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis, we found that genes related to the MAPK signalling pathway were abundantly expressed in *Fbxw7*KO mouse cartilage, and MKK7 (Map2k7) was the most highly upregulated gene among those relating to the MAPK signalling pathway (figure 5A). It has been established that JNK activation can promote the ageing process. Phosphorylation of JNK did not significantly change in the articular cartilage of *Fbxw7*KO mice at 3 months old but was markedly enhanced together with the protein level of MKK7, a MAPK kinase responsible for JNK activation, under stimulation of mechanical overload (figure 5B–D). By contrast, MKK4, another JNK signalling activator, remained unchanged in *Fbxw7*KO cartilage (figure 5C). In addition, we further deciphered the mechanism underlying the effects of FBXW7 on MKK7 and cartilage homeostasis using ATDC5 cells incubated with either *Fbxw7*-overexpression adenovirus or siRNA. Results showed that FBXW7 associated with MKK7, and mediated MKK7 ubiquitination and degradation by proteasomes. Both the proteasome inhibitor MG132 and FBXW7 deficiency attenuated MKK7 ubiquitination and degradation (figure 5E,F).

We subsequently investigated whether the loss of FBXW7 promoted chondrocyte senescence via activation of MKK7. DTP3, a MKK7 inhibitor, inhibited JNK phosphorylation and rescued SA-βGal staining enhanced by FBXW7 deletion in primary chondrocytes (figure 6A). Additionally, mechanical stress-stimulated γH2AX, *p16^{ink4a}*, *p21*, *Gadd45*, *Laminab1*, *Mmp13* and *Il-6* in chondrocytes from *Fbxw7*KO mice could also be partially rescued by DTP3 (online supplemental figure S9B,C). Taken together, these results indicate that FBXW7 loss by excessive mechanical loading activates MKK7–JNK signalling to promote chondrocyte senescence.

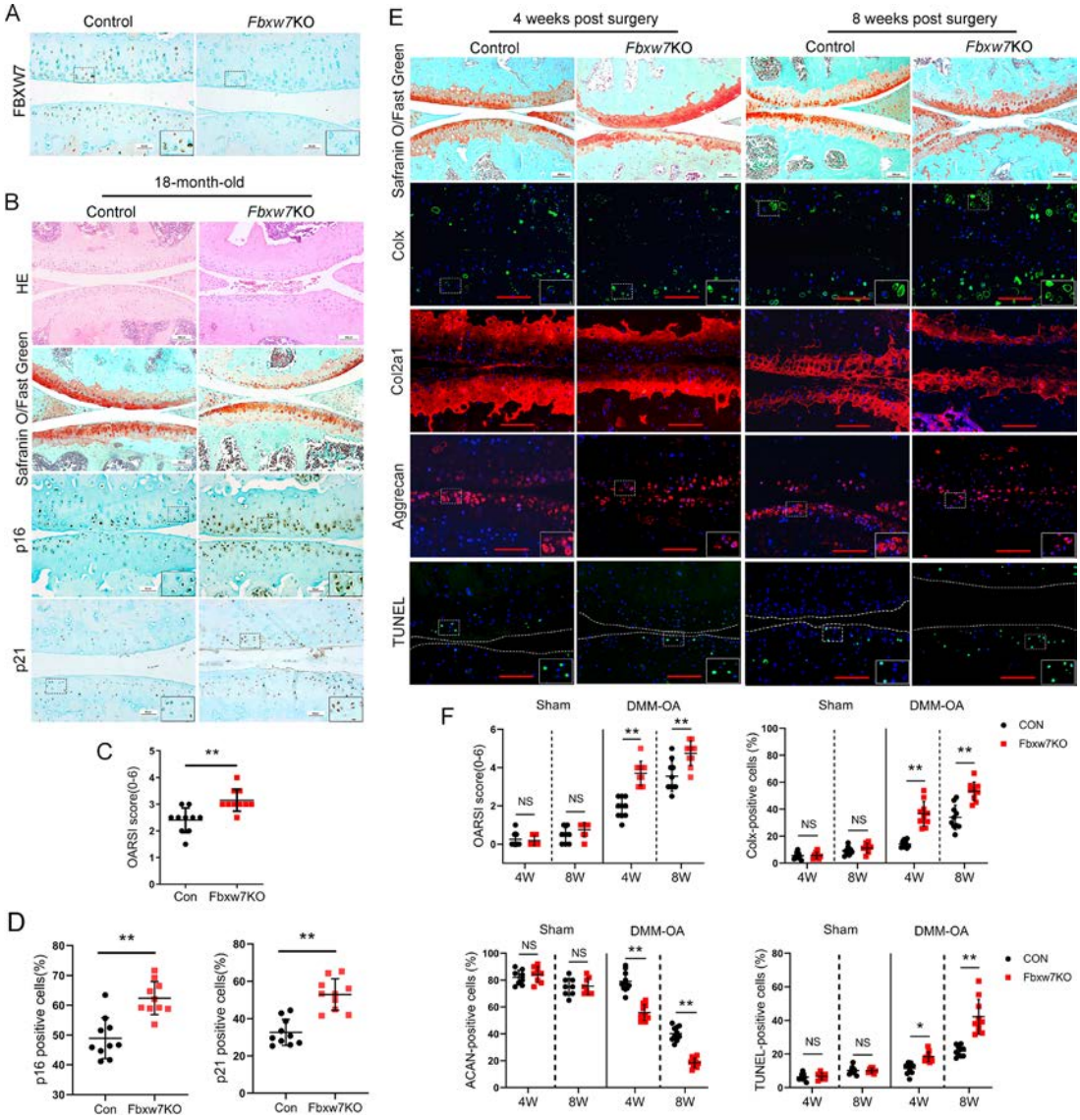

Figure 3 Fbxw7 loss in chondrocyte accelerates mouse OA development. (A) Representative images of IHC staining of FBXW7 in articular cartilage of *Fbxw7*KO and Control mice aged 3 months. Scale bar: 50 µm. (B) Representative images of H&E staining (first row), safranin O/fast green staining (second row) and IHC staining of $p16^{INK4a}$ (third row) and p21 (fourth row) in articular cartilage of *Fbxw7*KO and Control mice aged 18 months. Scale bars: 100 µm (first and second rows) and 50 µm (third and fourth rows). (C,D) Quantitative analysis of the OARSI scale and $p16^{INK4a}$-positive and p21-positive chondrocytes in *Fbxw7*KO mice and Controls. n=10 per group. (E) Representative images of safranin O/fast green staining and immunofluorescence staining of Colx, COL2A1, aggrecan and TUNEL in chondrocytes of *Fbxw7*KO and Controls at 4 and 8 weeks after DMM surgery. Scale bars: 100 µm (first row) and 50 µm (rest rows). (F) OARSI scale and quantification of Colx, ACAN and TUNEL-positive chondrocytes based on staining results in (E). n=10 per group. Data are shown as mean±SD. Statistical analyses were conducted using Student's t-test (D), three-way analysis of variance followed by Tukey's multiple comparison test (F) or non-parametric Mann-Whitney U tests (OARSI score) (C). Boxed area is enlarged in the bottom right corner. *P<0.05, **P<0.01. Con, control; DMM, destabilisation of the medial meniscus; FBXW7, F-box and WD repeat domain containing 7; IHC, immunohistochemical; NS, not significant; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

Inhibition of MKK7–JNK signalling delays chondrocyte senescence and OA development

To further verify the role of MKK7 in OA development induced by mechanical overloading in vivo, DTP3 was injected intraperitoneally to inhibit MKK7 after DMM surgery. The results showed a reduction in p-JNK-positive cells in articular cartilage by DTP3 injection and significant alleviation of the cartilage destruction and the OARSI score compared with the vehicle-treated mice. Catabolic factor expression and chondrocyte senescence and apoptosis were markedly reduced by DTP3 treatment (figure 6B and online supplemental figure S10A,B). These protective effects of DTP3 on OA were further verified in *Fbxw7*KO mouse in which MKK7 was highly expressed in chondrocytes (figure 6C and online supplemental figure S10C,D). Taken together, these data suggest that FBXW7 loss promotes chondrocyte senescence and OA development partially through MKK7–JNK activation.

DISCUSSION

Ageing and mechanical overload play important roles in OA development. This study for the first time established a mechanistic association between these two critical risk factors for

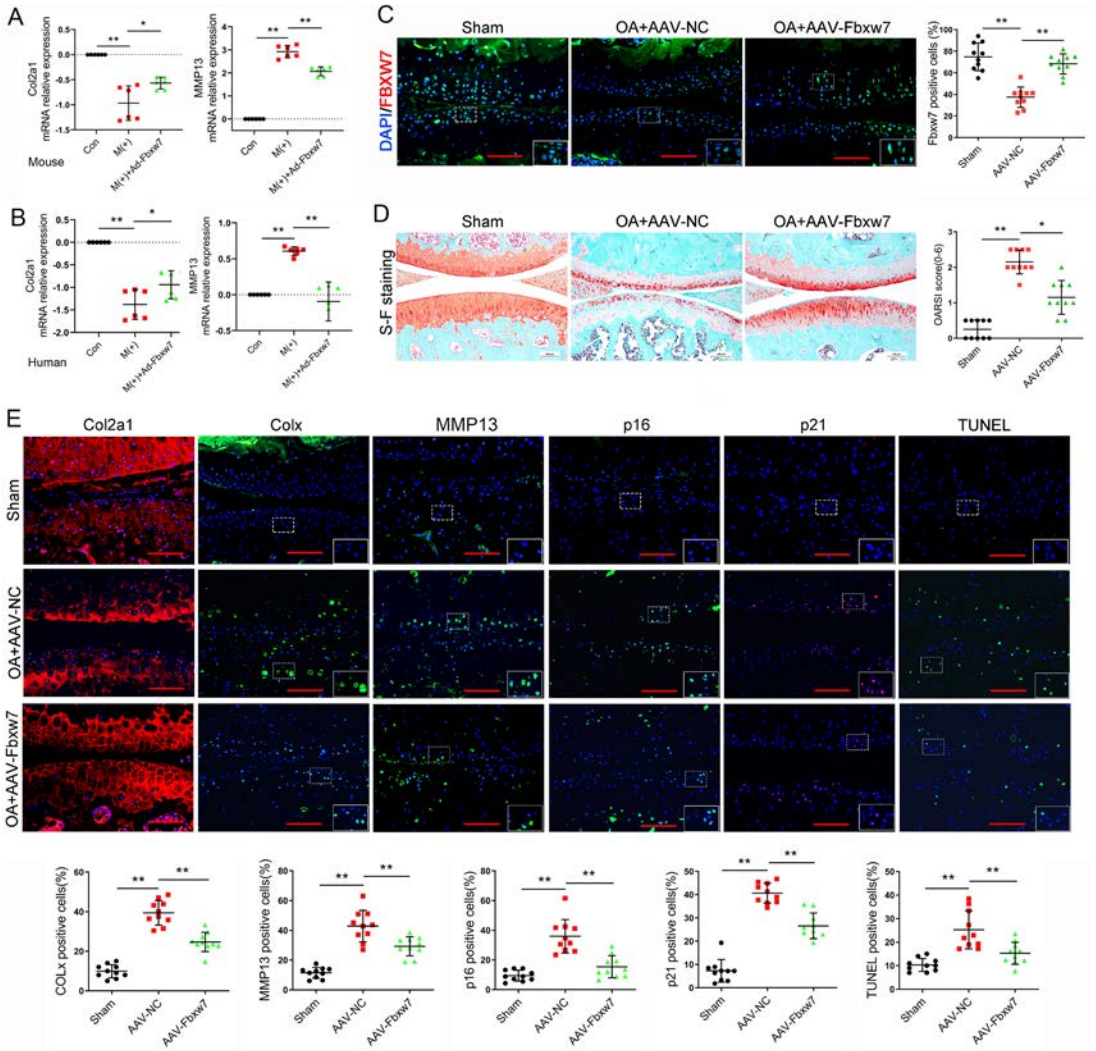

Figure 4 FBXW7 overexpression in chondrocytes alleviates experimental OA in mice. (A) Quantitative PCR analysis of COL2A1 and MMP13 in mouse primary chondrocytes treated with or without adenovirus containing FBXW7 (Ad-*Fbxw7*) under 20% elongation strain loading for 24 hours. n=6 per time point. (B) Quantitative PCR analysis of COL2A1 and MMP13 in human primary chondrocytes treated with or without Ad-*Fbxw7* under 20% elongation strain loading for 24 hours. n=6 per time point. (C) Representative images and quantitative analysis of FBXW7-positive chondrocytes in the cartilage of mice intra-articularly injected with AAV-NC or AAV- *Fbxw7* after DMM surgery. n=10 per group. Scale bar: 50 µm. (D) Safranin O/fast green staining of joints from AAV- *Fbxw7* and Control mice at 8 weeks after DMM surgery and quantitative analysis of the OARSI scale. n=10 per group. Scale bar: 100 µm. AAV-NC versus AAV-Fbxw7: p<0.002, 95% CI 0.5397 to 1.460. (E) Immunofluorescence staining and quantification of COL2A1, Colx, MMP13, p16^{INK4a}, p21 and TUNEL in chondrocytes of AAV- *Fbxw7* and Control mice at 8 weeks after DMM surgery. n=10 per group. Scale bar: 50 µm. Data are shown as mean±SD. Statistical analyses were conducted using one-way analysis of variance followed by Tukey's multiple comparison test (A–C,E), or Kruskal-Wallis test followed by Dunn's multiple comparisons test (OARSI score (D). Boxed area is enlarged in the bottom right corner. *P<0.05, **P<0.01. AAV- *Fbxw7*, adenovirus expressing FBXW7; AAV-NC, negative control; Con, control; DMM, destabilisation of the medial meniscus; FBXW7, F-box and WD repeat domain containing 7; NS, not significant, OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

OA. We showed that mechanical overloading stimulated chondrocyte senescence in vitro and in mice articular cartilage, and identified FBXW7 as a key factor in the association between biomechanics and chondrocyte senescence in OA pathology. Excessive mechanical loading downregulated FBXW7 and thereby reduced FBXW7-mediated MKK7 ubiquitination and degradation. MKK7 accumulation subsequently activated JNK signalling, which promoted chondrocyte senescence and consequently accelerated cartilage degeneration and OA development. Supplementation of FBXW7 or inhibition of MKK7–JNK is thus a potential therapeutic target for OA treatment (figure 6D).

Increasing age is strongly correlated with cartilage degeneration and the presence of senescent cells in cartilage isolated from patients undergoing total knee artificial implants has been noted,[32] but the direct relationship between the ageing process and OA development is not completely understood.[33] In the current study, we found senescent chondrocytes accumulated with age and in human OA cartilage. The presence of senescent chondrocytes near the osteoarthritic lesions, but not in intact tissue, further suggests an association between chondrocyte senescence and OA development. Additionally, mechanical overload stimulated both primary chondrocytes and cartilage ageing in mice, indicating the interaction between biomechanics and the biological context during OA.

FBXW7 has emerged as one of the substrate-recognition subunits of an SKP1-Cullin1-F-box protein (SCF)-type ubiquitin

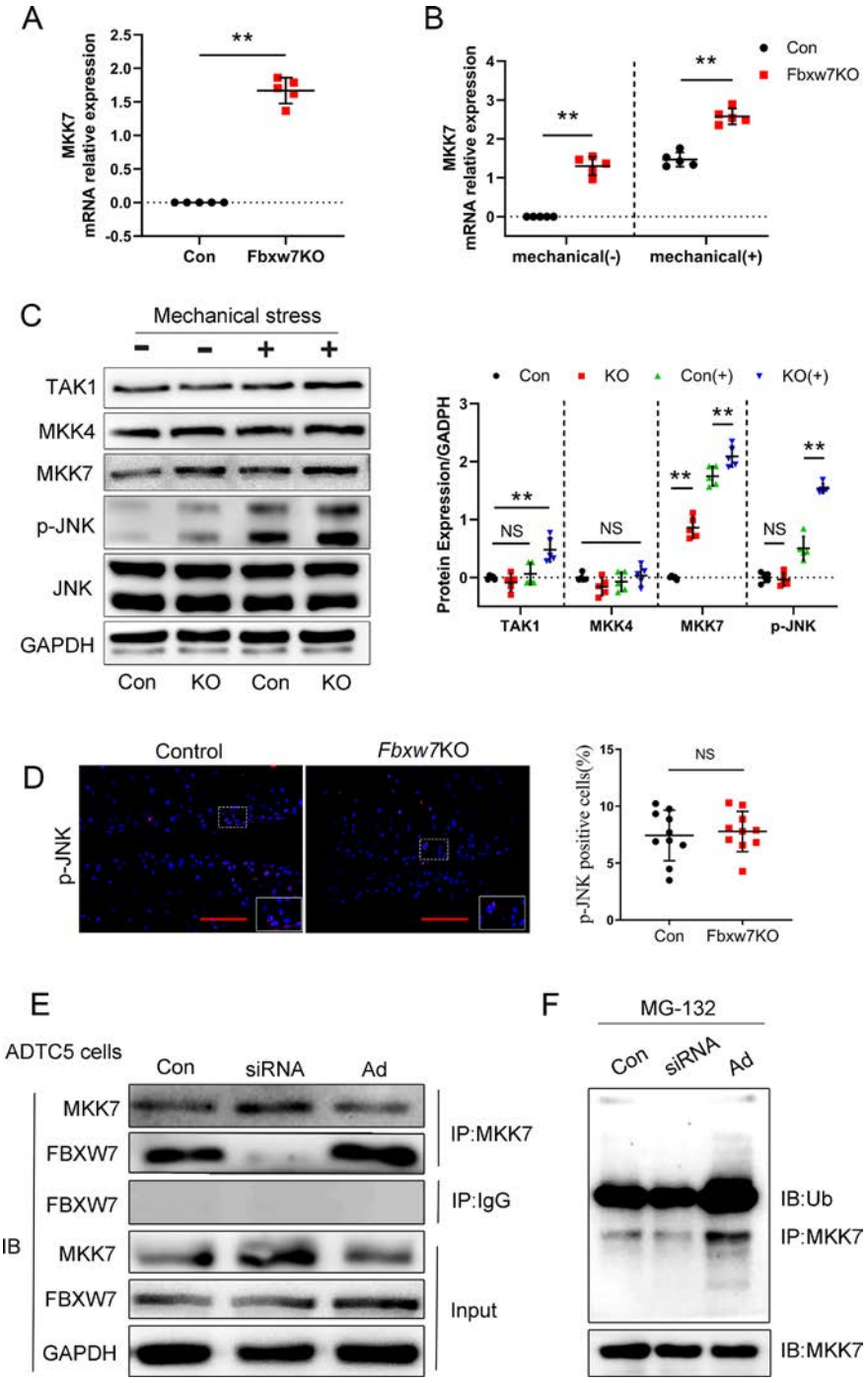

Figure 5 FBXW7 regulates MKK7 ubiquitination and degradation. (A) Quantitative PCR analysis of *Mkk7* in articular cartilage from *Fbxw7*KO mice and littermate Controls. n=5 per group. P<0.002, 95% CI 1.471 to 1.867. (B) Quantitative PCR analysis of *Mkk7* in primary chondrocytes from *Fbxw7*KO mice and littermate Controls treated with or without tensile strain loading. n=5 per group. (C) Western blot and quantification of TAK1, MKK4, MKK7, p-JNK and JNK in mechanical stress-treated primary chondrocytes from *Fbxw7*KO mice and Controls. (D) Representative images of immunofluorescence of p-JNK in cartilage from *Fbxw7*KO mice and Controls, and quantitative analysis of p-JNK-positive chondrocytes compared with total chondrocytes. n=10 per group. Scale bar: 100 µm. (E) MKK7 was immunoprecipitated from ATDC5 cells after transfection with either adenovirus containing *Fbxw7* (Ad-*Fbxw7*) or *Fbxw7*-siRNA. The presence of MKK7 and FBXW7 in the immunoprecipitates was evaluated by immunoblotting. (F) MKK7 was immunoprecipitated from ATDC5 cells after stimulation with MG-132 and transfection with either Ad-*Fbxw7* or *Fbxw7*-siRNA. Western blotting detected the ubiquitination level of MKK7. Data are shown as mean±SD. Statistical analyses were conducted using Student's t-test (A,D), two-way analysis of variance followed by Sidak's multiple comparison test (B,C). Boxed area is enlarged in the bottom right corner. **P<0.01. Con, control; FBXW7, F-box and WD repeat domain containing 7; IB, immunoblotting; IP, immunoprecipitate; KO, knockout; NS, not significant.

(E3) ligase complex and targets several pathways for the degradation of various mammalian oncoproteins that control cell growth, differentiation, and tumorigenesis.[34–37] It has been reported that FBXW7 deletion leads to $p16^{INK4a}$ and p19 elevation to facilitate the cell cycle and promote cell senescence.[38 39] Additionally, FBXW7 has been shown to negatively regulate mTOR signalling, which plays a vital role in OA development, as reported in our previous studies.[40–42] Although FBXW7 has been widely studied, its role in chondrocyte senescence and OA development has not been reported. An interesting finding of

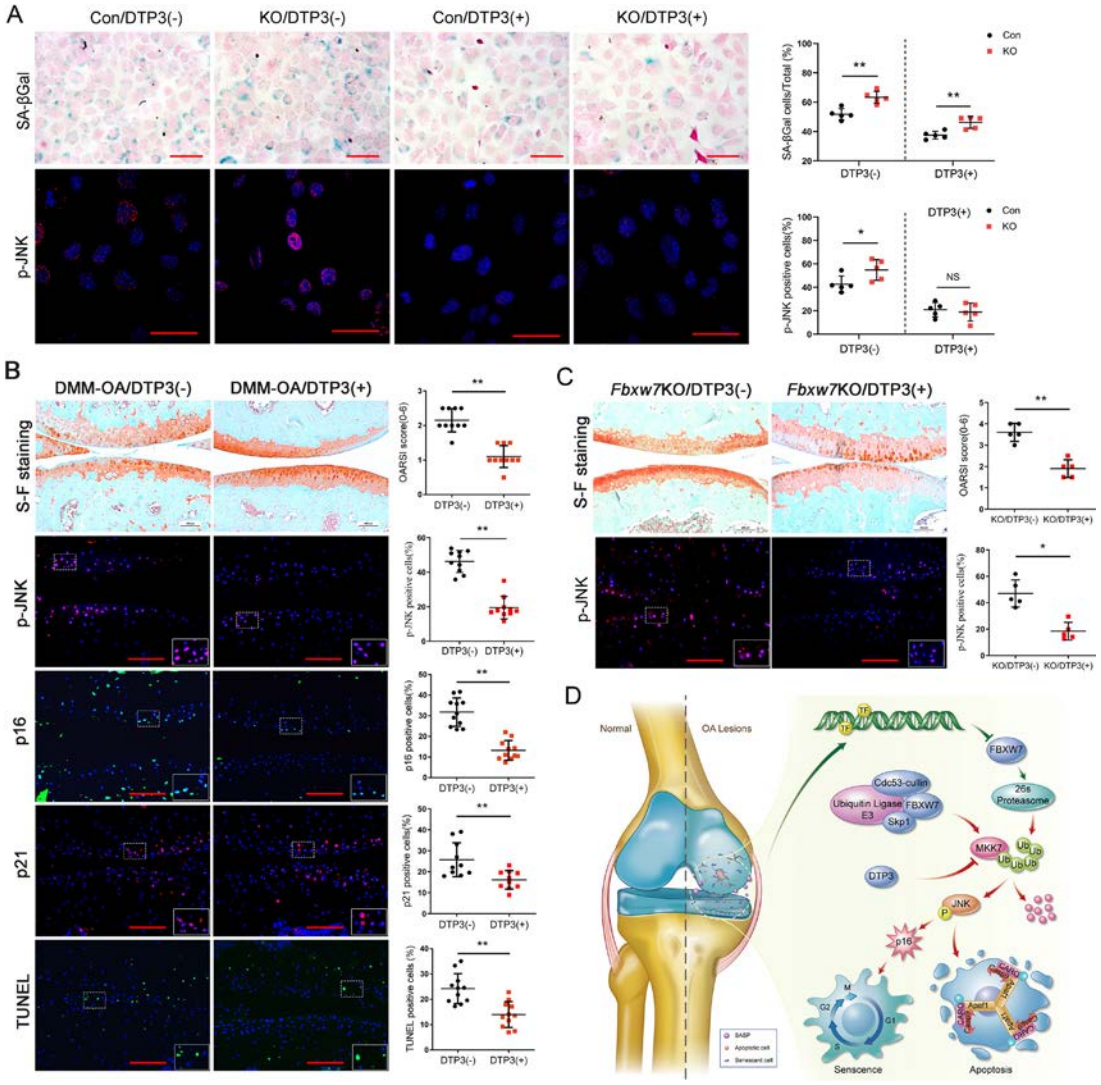

Figure 6 Inhibition of MKK7–JNK signalling delays chondrocyte senescence and OA development. (A) Representative images and quantification of SA-βGal staining and immunofluorescence staining of p-JNK in primary chondrocytes from *Fbxw7*KO mice and controls after elongation strain loading for 24 hours with or without DTP3 treatment. n=5 per group. Scale bar: 50 µm. (B) Representative images and quantification of safranin O/fast green staining and immunofluorescence staining of p-JNK, $p16^{INK4a}$, p21 and TUNEL in chondrocytes of DTP3-treated mice (intraperitoneal injection, 10 mg/kg, every other day) at 4 weeks post-DMM surgery. n=10 per group. Scale bars: 100 µm (first row) and 50 µm (the rest of the rows). (C) Safranin O/fast green staining and immunofluorescence staining of p-JNK of joints from DTP3-treated *Fbxw7*KO mice at 4 weeks post-DMM surgery. Quantitative analysis of the OARSI score and p-JNK-positive chondrocytes are shown on the right. n=5 per group. Scale bars: 100 µm (first row) and 50 µm (second row). (D) Schematic diagram representing molecular pathways in which excessive mechanical loading induces OA development through FBXW7. Data are shown as mean±SD. Statistical analyses were conducted using two-way analysis of variance followed by Sidak's multiple comparison test (A), Student's t-test (B and C), or non-parametric Mann-Whitney U tests (OARSI score) (B,C). Boxed area is enlarged in the bottom right corner. *P<0.05, **P<0.01. DMM, destabilisation of the medial meniscus; FBXW7, F-box and WD repeat domain containing 7; OA, osteoarthritis; OARSI, Osteoarthritis Research Society International; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

the present study was that FBXW7 was markedly downregulated in chondrocytes by excessive mechanical loading both in vitro and in vivo. FBXW7 deletion in chondrocytes resulted in the senescence and degeneration of articular cartilage and exacerbation of OA. In addition, we detected senescent chondrocytes in articular cartilage of patients with OA and DMM OA mice, which were enhanced by FBXW7 deletion and reversed by FBXW7 overexpression. However, no significant changes in chondrocyte senescence were observed between *Fbxw7*KO mice and controls at a young age, indicating that FBXW7 deficiency alone was not sufficient to induce cell senescence, unless under the context of mechanical loading or old age. Therefore, we propose that mechanical overloading reduces FBXW7 in cartilage chondrocytes, which represents a novel mechanism for chondrocyte senescence during OA.

To identify FBXW7 downstream signalling during chondrocyte senescence and OA development, we screened out MKK7, and one MAPK kinase was shown to activate JNK.[43] MAPKs, including the JNK and p38MAPK signalling pathways, have been suggested to be extensively involved in OA.[44] JNK signalling can be phosphorylated by activating MKK4 or MKK7, while MKK4 can activate p38MAPK and JNK, whereas MKK7 is specifically involved in only JNK activation.[45–47] We found that MKK7, but not MKK4, participated in mechanical overloading-induced JNK activation. Although JNK signalling plays a key role in cell proliferation, differentiation and apoptosis in response to stress,

the contributions of MKK7–JNK in cartilage ageing and OA progression in response to mechanical stress are not known.[48 49] In the present study, we found that FBXW7 inhibition by excessive mechanical loading strongly elevated MKK7 expression and subsequently induced an increase in the JNK signal in chondrocytes, resulting in enhanced cell senescence. Additionally, FBXW7 loss-induced chondrocyte senescence and cartilage degeneration were ameliorated by MKK7 inhibitor DTP3. These results indicate that the MKK7–JNK pathway plays an important role in mechanical stress-induced chondrocyte senescence and cartilage degeneration.

To conclude, our study found an association between mechanical loading and cell senescence in OA development. FBXW7 loss and activation of MKK7–JNK signalling play a crucial role in biomechanically induced chondrocyte senescence. Overexpression of FBXW7 by targeting its transcriptional regulators or upstream lncRNAs, or targeting MKK7 by DTP3 might represent novel therapeutic approaches for OA treatment.

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Contributors HyZ and XB conceived the ideas for experimental designs, analysed data and wrote the manuscript. HyZ and YS conducted the majority of the experiments and helped with manuscript preparation. ZY conducted the majority of the experiments and analysed data during the revision of the article. CZ, HbZ and LL performed immunohistochemistry and immunofluorescence and confocal imaging. HbZ and KL conducted cell cultures and western blot experiments. JY and ZY collected human tissue samples. CZ, XB and DC developed the concept, supervised the project and conceived the experiments. All authors approved the final version of the manuscript. XB accepted full responsibility for the finished work, had access to the data and controlled the decision to publish.

Funding This work was supported by grants from the National Natural Science Foundation of China (grant numbers 81974341, 81991510 and 81991511) and the Natural Science Foundation of Guangdong Province (2020A1515011062).

Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s)

Ethics approval This study involves human participants, and patient consent and the approval of the ethics committee of the Third Affiliated Hospital of Southern Medical University (Guangzhou, China) were obtained before the human tissue samples were harvested (201711001). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Not applicable.

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CLINICAL SCIENCE

Additional heterologous versus homologous booster vaccination in immunosuppressed patients without SARS-CoV-2 antibody seroconversion after primary mRNA vaccination: a randomised controlled trial

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Handling editor Gerd-Rüdiger R Burmester

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221558).

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Received 22 September 2021
Accepted 10 December 2021
Published Online First 13 January 2022

To cite: Bonelli M, Mrak D, Tobudic S, *et al*. *Ann Rheum Dis* 2022;**81**:687–694.

ABSTRACT

Objectives SARS-CoV-2-induced COVID-19 has led to exponentially rising mortality, particularly in immunosuppressed patients, who inadequately respond to conventional COVID-19 vaccination.

Methods In this blinded randomised clinical trial, we compare the efficacy and safety of an additional booster vaccination with a vector versus mRNA vaccine in non-seroconverted patients. We assigned 60 patients under rituximab treatment, who did not seroconvert after their primary mRNA vaccination with either BNT162b2 (Pfizer–BioNTech) or mRNA-1273 (Moderna), to receive a third dose, either using the same mRNA or the vector vaccine ChAdOx1 nCoV-19 (Oxford–AstraZeneca). Patients were stratified according to the presence of peripheral B cells. The primary efficacy endpoint was the difference in the SARS-CoV-2 antibody seroconversion rate between vector (heterologous) and mRNA (homologous) vaccinated patients by week 4. Key secondary endpoints included the overall seroconversion and cellular immune response; safety was assessed at week 1 and week 4.

Results Seroconversion rates at week 4 were comparable between vector (6/27 patients, 22%) and mRNA (9/28, 32%) vaccines (p=0.6). Overall, 27% of patients seroconverted; specific T cell responses were observed in 20/20 (100%) vector versus 13/16 (81%) mRNA vaccinated patients. Newly induced humoral and/or cellular responses occurred in 9/11 (82%) patients. 3/37 (8%) of patients without and 12/18 (67%) of the patients with detectable peripheral B cells seroconverted. No serious adverse events, related to immunisation, were observed.

Conclusions This enhanced humoral and/or cellular immune response supports an additional booster vaccination in non-seroconverted patients irrespective of a heterologous or homologous vaccination regimen.

Key messages

What is already known about this subject?
- A third COVID-19 vaccination has been recommended by the US Food and Drug Administration for certain immunocompromised individuals.
- First clinical trial data have now reported on efficacy of a third vaccination in patients under immunosuppressive therapy.
- No clinical trial data exist which compare efficacy and safety of a heterologous versus homologous vaccination strategy in non-seroconverted patients under rituximab therapy.

What does this study add?
- The results from our study support efficacy and safety of an additional heterologous or homologous booster vaccination in immunosuppressed patients.
- Cellular and humoral immune response can be induced in B cell depleted patients undergoing rituximab treatment.

How might this impact on clinical practice or future developments?
- Based on these data, COVID-19 booster vaccination is recommended for non-seroconverted rituximab-treated patients.

INTRODUCTION

The current pandemic caused by SARS-CoV-2 has led to exponentially rising morbidity and mortality worldwide. Apart from aggressive quarantine and infection control hygiene measures, the most effective way to combat SARS-CoV-2 spread is a population-wide vaccination strategy, foremost in those at high risk to develop severe COVID-19.[1 2] Two types of vaccines have been currently approved by the European Medicines Agency: vector vaccines, such as ChAdOx1 nCoV-19 (Oxford–AstraZeneca) and Ad26.COV2-S (Johnson & Johnson), and mRNA vaccines, such as BNT162b2 (Pfizer–BioNTech) and mRNA-1273 (Moderna). However, immune responses to these vaccines vary between individuals, and antibody levels wane over time.[3–6]

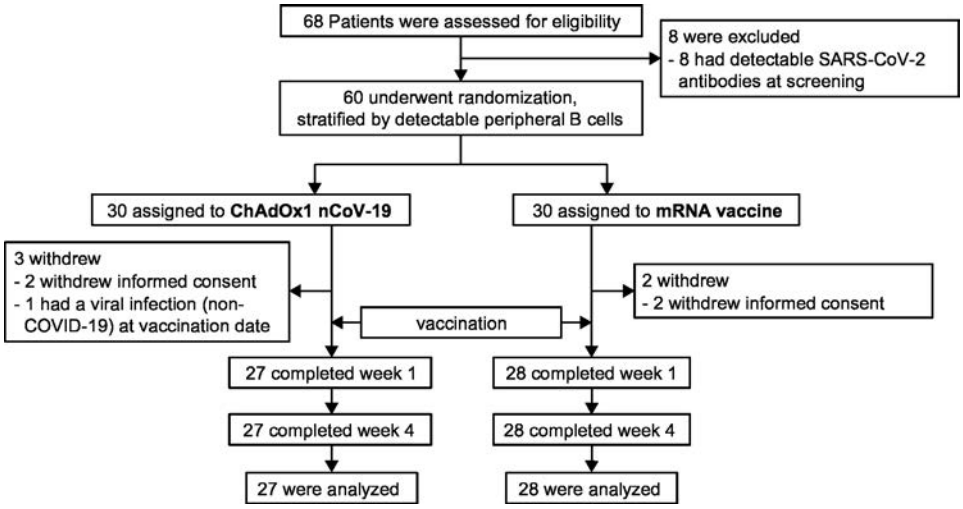

Figure 1 Screening, randomisation and follow-up of patients.

Application of an additional booster dose has most recently been investigated.[7–15] Several countries already started a third vaccination, especially in patients at high risk. Most recently, the US Food and Drug Administration authorised an additional vaccine dose for certain immunocompromised patients.[16]

Patients under immunosuppressive therapy with rituximab, a B cell depleting monoclonal antibody against the CD20 surface antigen, are at a high risk for severe COVID-19 requiring hospitalisation and ICU admission.[17 18] At the same time, B cell depletion reduces immune responses to vaccination.[19–21] This combination poses a dilemma and, therefore, a highly unmet clinical need for this group of patients. Those lacking B lymphocytes in the periphery at the time of vaccination and thus did not yet start reconstituting their B cell pool often fail to seroconvert.[22 23] Although B cell depleted patients can develop a T cell response, to date, it is unclear to what extent cellular and humoral responses contribute to protection against SARS-CoV-2 infection.

The development of a humoral immune response currently constitutes a good surrogate of protection and its absence is, therefore, often considered an alarm signal for an insufficient vaccination response. In order to stimulate the humoral immune response of rituximab-treated patients who do not respond to the conventional scheme of COVID-19 vaccination, an additional booster vaccination may be an obvious clinical strategy. Recent studies also evaluated the safety and efficacy of homologous versus heterologous schemes for primary and secondary vaccination in healthy individuals.[24–26] However, it is unknown whether a heterologous approach could benefit those who completely lack a humoral immune response after basic immunisation. Furthermore, no data exist on responses to an additional booster vaccination in patients who had completely failed to mount a specific antibody response after the primary two-vaccination schedule.

In this blinded, randomised, controlled trial, we addressed this question and the general inducibility of a humoral or T cell response in rituximab-treated autoimmune disease patients without anti-SARS-CoV-2 antibodies after their basic mRNA vaccination.

METHODS

In this prospective patient and efficacy (laboratory), blinded randomised controlled trial adults (≥18 years) with chronic inflammatory rheumatic or neurologic diseases under current rituximab therapy and without detectable SARS-CoV-2 spike (S) protein antibodies at least 4 weeks after their second standard vaccination with an mRNA vaccine (BNT162b2 or mRNA-1273) were included. Key exclusion criteria were previous infection with SARS-CoV-2 or known allergies to study compounds. The detailed inclusion/exclusion criteria can be found in the trial protocol (online supplemental file 1). The trial was registered

Table 1 Baseline characteristics of patients vaccinated with a third dose

	Vector	mRNA
n	27	28
Age (years)	60.9 (15.0)	58.9 (18.4)
Gender: female	18 (66.7%)	23 (82.1%)
Diagnosis		
Arthritis	11 (40.7%)	10 (35.7%)
Connective tissue diseases	7 (25.9%)	9 (32.1%)
Vasculitis	4 (14.8%)	4 (14.3%)
Multiple sclerosis	3 (11.1%)	3 (10.7%)
IgG4-related disease	2 (7.4%)	2 (7.1%)
Months between RTX and screening	7.0 (6.2)	6.0 (3.6)
Weeks between second vaccination and screening	8.2 (3.7)	6.6 (2.3)
Patients with detectable B cells	8 (29.6%)	10 (35.7%)
Concomitant medication		
Any csDMARD	10 (37.0%)	16 (57.1%)
Methotrexate	3 (11.1%)	7 (25.0%)
Mycophenolate mofetil	2 (7.4%)	4 (14.3%)
Azathioprine	2 (7.4%)	3 (10.7%)
Leflunomide	3 (11.1%)	1 (3.6%)
Hydroxychloroquine	0 (0.0%)	4 (14.3%)
Immunoglobulin therapy	1 (3.7%)	1 (3.6%)
Prednisone	7 (25.9%)	8 (28.6%)
Prednisone dose (mg)	5.7 (2.3)	4.6 (2.7)
Primary vaccination with BNT162b2	21 (78%)	21 (75%)
Primary vaccination with mRNA-1273	6 (22%)	7 (25%)

Data are n (%) or mean (SD).
csDMARD defined here as concomitant treatment with at least one of the following: methotrexate, mycophenolate mofetil, azathioprine, leflunomide and hydroxychloroquine.
csDMARD, conventional synthetic disease-modifying antirheumatic drug; IgG4, immunoglobulin G4; RTX, rituximab.

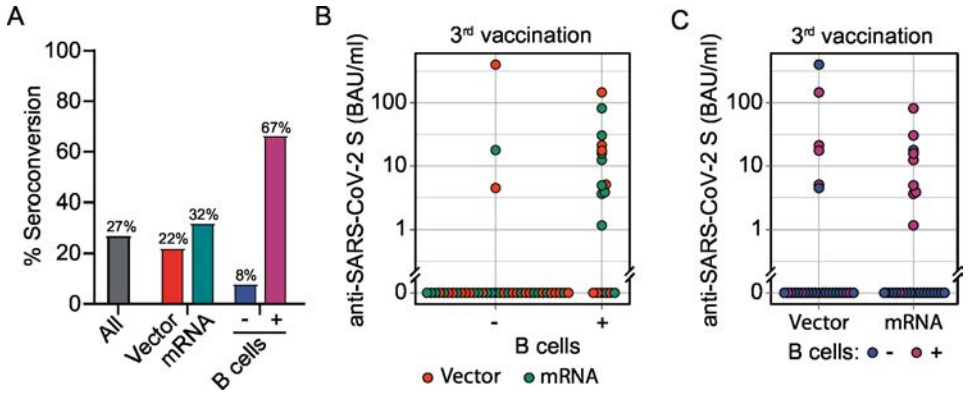

Figure 2 Antibody seroconversion rate 4 weeks after vector vs mRNA booster vaccination. Antibodies to the RBD of the viral Sprotein were determined using an anti-SARS-CoV-2 immunoassay. (A) Seroconversion rate was calculated based on the presence of anti-RBD antibodies in patients stratified by booster vaccination with vector vaccine or mRNA vaccine, in all patients and in patients with and without detectable peripheral B cells. (B)Anti-RBD antibody levels in patients with (n=18) and without (n=37) peripheral B cells, with colour of the circles indicating the type of vaccine. (C) Anti-RBD antibody levels in patients 4 weeks after booster vaccination with vector vaccine (n=27) or mRNA vaccine (n=28), with colour of the circles indicating the presence or absence of detectable peripheral CD19$^+$ B-cells. RBD, receptor-binding domain; S,spike.

with EudraCT (2021-002348-57) on 10 May before inclusion of the first patient.

Randomisation

Patients were block-randomised in a 1:1 ratio based on the presence or absence of peripheral B lymphocytes by a computerised randomisation algorithm to receive either a third dose of an mRNA vaccine (BNT162b2 or mRNA-1273, respective of their initial vaccination compound) or a third vaccination with a vector COVID-19 vaccine (ChAdOx1 nCoV-19).

Interventions

During the screening visit (visit one), data on demographics, concomitant medication, possible hypersensitivity reactions to the previous SARS-CoV-2 vaccination and medical history regarding SARS-CoV-2 infections were collected. The absence of detectable SARS-CoV-2 antibodies against nucleocapsid and S protein was verified before enrolment and the level of peripheral B lymphocytes was assessed. The vaccination was applied during a baseline visit (visit 2, within 28 days after screening) followed by visits 3 and 4 (1 week and 4 weeks after vaccination, respectively) to determine the efficacy and safety of the third COVID-19 vaccination. Serum samples obtained during visits 1, 3 and 4 were stored below −70°C at the Biobank of the Medical University of Vienna, a centralised facility for the preparation and storage of biomaterial with certified quality management (International Organization for Standardization (ISO) 9001:2015).[27] Peripheral blood mononuclear cells were isolated at screening and week 1 by density gradient centrifugation and stored in the vapour phase of liquid nitrogen until further use.

All patients were blinded throughout visit 4, mainly to allow objectivity in safety assessment of the two strategies; blinding of vaccines was ensured by using pre-arranged dose aliquots in syringes without reference to the type used by the Central Pharmacy of the Vienna General Hospital. The City of Vienna provided the vaccines for this study free of charge. The study was conducted in following Good Clinical Practice guidelines and the Declaration of Helsinki. All trial visits were conducted in a tertiary hospital (Vienna General Hospital). The first patient was included on 25 May 2021 and the last patient finalised the 4 week follow-up on 5 August 2021.

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.

Assessment, outcomes and sample size

The quantification of CD19$^+$ peripheral B cells, the anti-SARS-CoV-2 antibody testing and T cell assays is detailed in the methods section of the online supplemental file 2. Laboratory assessors were blinded to randomisation.

The primary study endpoint was defined as difference in antibody seroconversion rates between the vector and mRNA vaccinated groups.

Secondary endpoints included seroconversion rate and SARS-CoV-2 antibody levels at week 4 overall and stratified for patients with and without detectable peripheral B cells as well as cellular immune response defined by T lymphocyte restimulation potential before and 1 week after vaccination. Safety was reported and evaluated for incidence and severity of adverse events as well as potential effects on the underlying disease activity over a period of 28 days. Additionally, a paper-based patient diary was used. The study sample size was pragmatically targeted at 60 individuals, based on the number of rituximab-treated patients potentially eligible during the tight recruitment period, including estimates of non-responders to a standard protocol of mRNA vaccination, and expected participation rates. Based on a χ^2 test comparing vector versus mRNA vaccine, this number of patients would allow to achieve at least 80% power at a minimal detectable difference of 28% (5% of responders in one group vs 33% in the other).

Statistical analysis

All subjects vaccinated with a third dose were included in the analysis. Primary outcome was assessed using χ^2 test. Secondary outcomes and safety data are presented in a descriptive manner. Post-hoc exploratory analyses were performed to evaluate factors associated with seroconversion rates by univariate logistic regression analyses. Variable selection was based on previous data in rituximab-treated patients, and included age, concomitant medication, type of booster vaccination and the presence or absence of detectable peripheral B cells.[22] GraphPad Prism (V.9.1.0) was used for the graphical presentation of the data. 'R' V.4.0.3 (R Development Core Team. Vienna, Austria) was used for the entire statistical analysis. Following

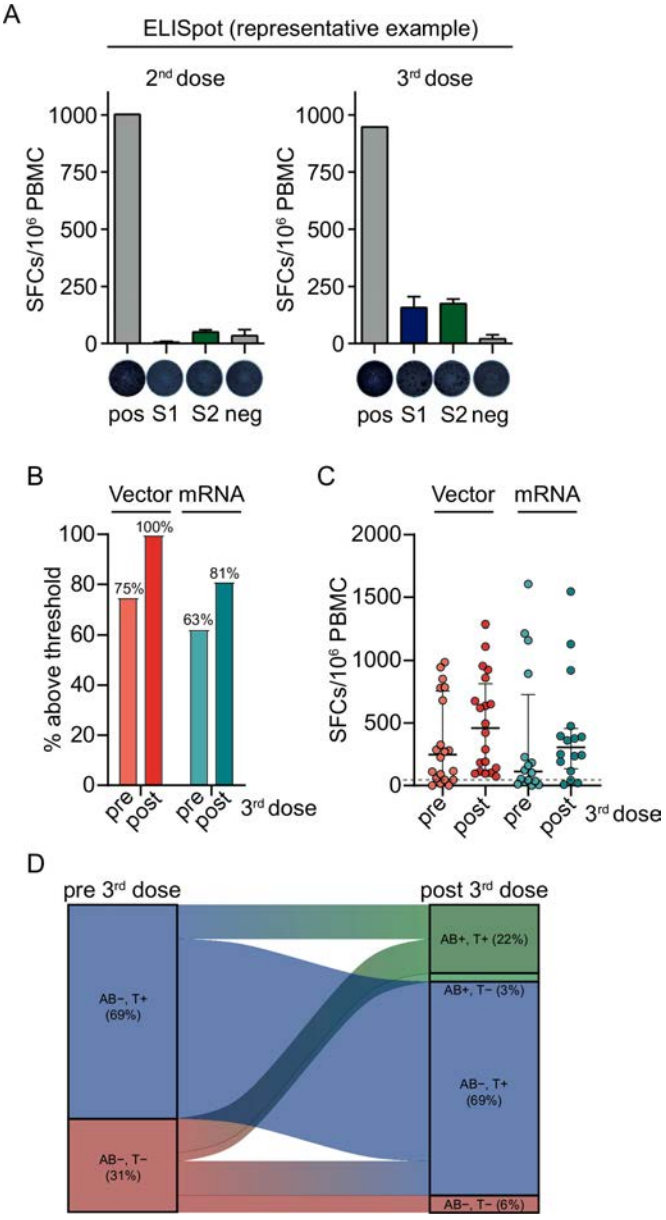

Figure 3 SARS-CoV-2-specific T after additional vector or mRNA booster vaccination in rituximab-treated patients. (A) One representative ex vivo IFN-γ enzyme-linked immune absorbent spot (ELISpot) result from PBMCs stimulated with S subunit, S1 and S2, peptide pools shown for one patient before and after booster vaccination. Y axis indicates the number of SFCs per 10^6 PBMCs. (B) Per cent of patients without T before and after third vaccination with vector and mRNA vaccine. (C) Composite ELISpot results from 36 patients before and after the third vaccination with mRNA (n=16) and vector vaccine (n=20). Circles show sum of total response from S1 and S2 peptide pools. Vertical line shows median, whiskers IQR. Dotted lines represent the cut-off as defined by the mean SFC count plus three times the SD from pre-pandemic controls. (D) Humoral and cellular immune responses before and after the third vaccination. AB, antibody; IFN, interferon; PBMCs, peripheral blood mononuclear cells; SFCs, spot-forming cells; T, T cell response.

packages were used: 'ggplot2', 'ggbeeswarm' and 'sjPlot' for creating plots and 'tableone' to create baseline tables.

RESULTS

Sixty-eight patients under rituximab treatment who had been immunised with two doses of mRNA vaccine were screened

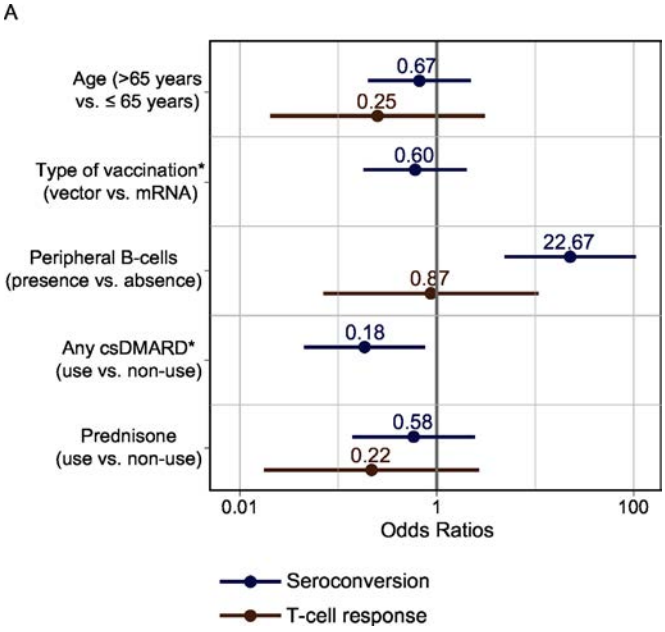

Figure 4 ORs of logistic regression assessing humoral and cellular immune responses. *All patients treated with vector vaccine developed a T cell response and all patients without T cell response were co-treated with csDMARDs, so consequently no OR could be calculated due to non-convergence of the respective models. csDMARDs, conventional synthetic disease-modifying antirheumatic drugs.

for eligibility. Eight patients were excluded due to the presence of detectable SARS-CoV-2-specific antibodies. Sixty non-seroconverted patients were randomised, of whom 30 were assigned to receive vector vaccine and 30 to receive mRNA vaccine as the third dose; 5 patients withdrew consent between screening and baseline visit (figure 1). A total of 27/30 patients were vaccinated with a vector vaccine and 28/30 received an mRNA vaccine. All patients subsequently presented at follow-up visits and completed the trial at week 4 after vaccination. Patient characteristics were similar between the two randomised groups (table 1).

Seroconversion rates at week 4 were numerically lower in the vector group than in the mRNA group (6/27, 22% of patients compared with 9/28, 32% of patients) (figure 2A). Despite the numerical difference in favour of the homologous vaccination group, disadvantage of the heterologous group cannot be supported statistically (p=0.6).

Even though the primary endpoint was not met, 27% of all vaccinated patients seroconverted independent of the vaccine used with a median SARS-CoV-2 S antibody level of 15.7 BAU/mL (IQR: 4.7, 25.8 BAU/mL). Neutralising antibodies (titre : ≥10) against SARS-CoV-2 were observed in 4/15 (27%) of all seroconverted patients. Seroconversion rate was higher in patients with detectable peripheral $CD19^+$ B cells versus those without (figure 2A). Among patients with no detectable peripheral B cells (37/55, 67%), antibodies to the receptor-binding domain (RBD) of the viral S protein (anti-RBD antibodies) were detectable in 3/37 (8%) patients; in patients with detectable peripheral B cells, seroconversion rate was 67% (12/18) at week 4 (figure 2A–C). Median levels of anti-RBD antibodies were 19.4 (IQR: 8.2, 114.8) and 12.4 (IQR: 3.8, 17.8), respectively, in seroconverted vector and mRNA vaccinated patients (figure 2B;online supplemental table 1).

SARS-CoV-2-specific T cell responses were determined by enzyme-linked immune absorbent spot (ELISpot) assay in all

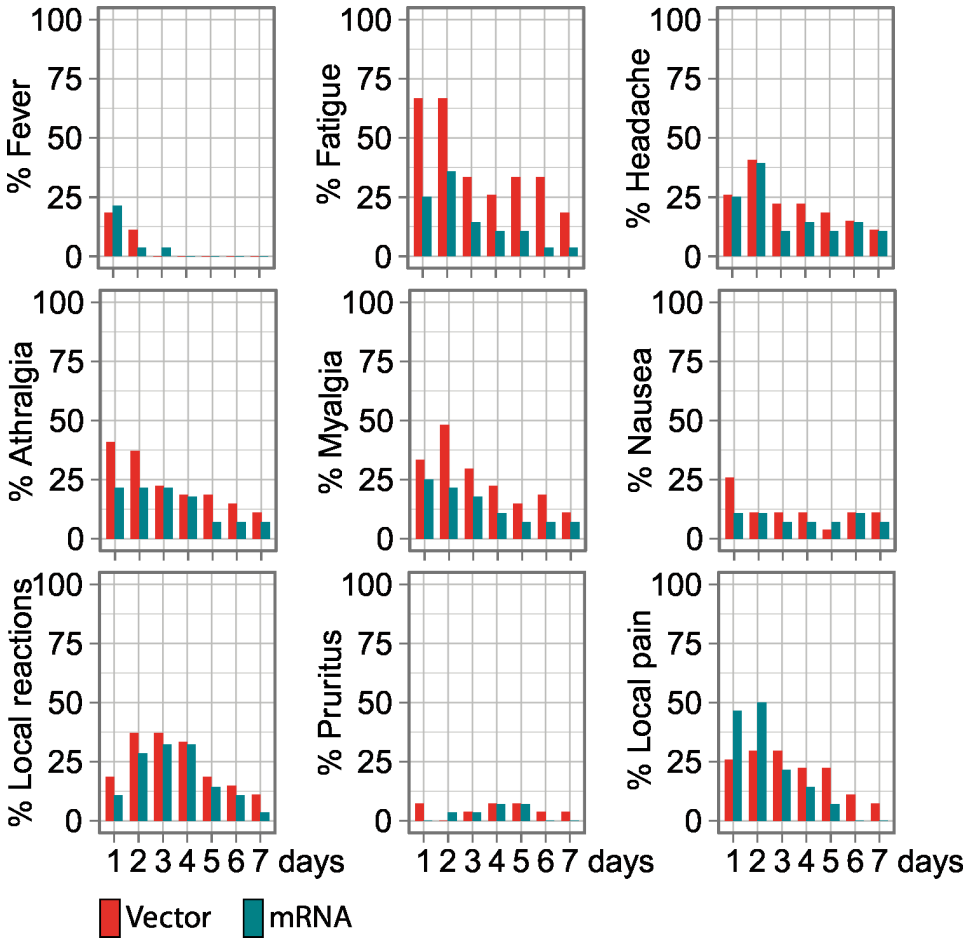

Figure 5 Safety. Systemic reactogenicity was evaluated daily during the first week after vaccination.

patients before and after booster vaccination. Matched samples before and after the third vaccination were available from 36 patients. Patient characteristics for this group stratified by third vaccination are presented as online supplemental table 2. At screening, 15/20 (75%) of patients assigned to the vector group and 10/16 (63%) assigned to the mRNA group had detectable S-specific T cells. Administration of a third vaccine dose led to an increase to 20/20 (100%) in the vector group and 13/16 (81%) in the mRNA group (figure 3A–B). The number of spot-forming cells to the S peptide pools (S1/S2) was slightly higher after boosting with vector vaccine (median: 459, IQR: 133, 722) as compared with mRNA vaccine (median: 305, IQR: 171, 416) (figure 3C).

Integrative analysis of humoral and T cell responses for 36 patients with matched samples before and after the third vaccination was performed: before third vaccination, 11/36 patients (31%) had neither anti-RBD antibodies nor T cell response (AB−, T−), and 25/36 patients (69%) did not have a humoral but exhibited a cellular immune response (AB−, T+). After the third vaccination, 8/36 (22%) showed a humoral and T cell response (AB+, T+), 1/36 (3%) had a humoral but no detectable cellular immune response (AB+, T−), in 25/36 (69%) a cellular but no humoral immune response (AB−, T+) was observed and 2/36 (6%) developed neither a humoral nor a cellular immune response. Overall, a cellular and/or humoral immune response could be achieved through an additional booster vaccination in 9/11 (82%) of those patients who did not respond to conventional vaccination strategy with two doses of mRNA vaccine (figure 3D, online supplemental tables 2 and 3).

Exploratory post-hoc univariate logistic regression models revealed that detectable peripheral B cells strongly favoured the likelihood of seroconversion (OR: 22.67, 95% CI 5.46 to 125.10), while co-medication with any conventional synthetic disease-modifying antirheumatic drug (csDMARD) favoured non-seroconversion. Compared with mRNA booster vaccination, the vector vaccine showed a lower likelihood of inducing humoral response though not statistically significant. With respect to T cell response, no association with age, use of prednisone or the presence of peripheral B cells could be observed (figure 4). All patients vaccinated with the vector regimen developed a T cell response, while all patients without T cell response were co-treated with a csDMARD, resulting in non-convergence of the respective regression models (online supplemental table 4).

Systemic reactogenicity was evaluated by the patients using a paper-based diary daily during the first week after vaccination. Adverse events, in general, were monitored until 28 days after vaccination. One serious adverse event was reported after the screening visit prior to vaccination. Most side effects were similar between vector and mRNA booster vaccine groups. Numerically, a higher prevalence of systemic reactogenicity after the booster dose was reported by patients in the heterologous vaccine group compared with homologous vaccine schemes for fatigue, arthralgia and myalgias. Thirteen out of 27 (48%) of vector-vaccinated patients developed arthralgias as compared with 8/28 (29%) of patients with mRNA booster vaccination. Myalgia was reported in 15/27 (56%) of vector-vaccinated patients compared with 9/28 (32%) of mRNA-vaccinated patients. Fatigue was

present in 21/27 (78%) of vector-vaccinated patients, while only 13/28 (46%) of mRNA-boosted patients experienced fatigue. Local pain at the injection site was more frequent during the first 2 days in mRNA-vaccinated patients (16/28, 57%) than vector-vaccinated patients (8/27, 30%). The local and systemic reactogenicity for the first week after vaccination is displayed in figure 5.

No thrombocytopenia or antibodies against platelet factor 4 were observed after additional booster vaccination 1 week and 4 weeks after vaccination. Mean thrombocyte counts were 285 G/L±85 before and 296 G/L±79 one week after vaccination. None of the patients experienced any anaphylactoid reaction or neurological complication. Seven patients (13%) reported an alteration or worsening in their underlying disease 1 week after vaccination, but no disease flare that required glucocorticoid treatment or change in immunosuppressive medication was reported within the study period.

DISCUSSION

In this randomised, controlled clinical trial, we enrolled patients treated with rituximab for an underlying autoimmune disease, who had not seroconverted on vaccination with two doses of an mRNA vaccine, and thus continued to be at a high risk for a severe disease course of SARS-CoV-2 infection. The additional SARS-CoV-2 booster vaccination evaluated in this trial resulted in the development of a humoral immune response in 27% of this initially vaccination-refractory patient population. Moreover, the additional booster vaccination reduced the proportion of patients lacking both a humoral and cellular immune response to primary vaccination from 31% to 6%.

Currently approved vector and mRNA vaccination strategies against SARS-CoV-2 consider only homologous vaccination. However, recent studies indicate a better humoral and cellular immune response after heterologous prime-boost vaccination in healthy individuals.[24 26 28–30] In our study, no significant advantage for either the homologous or heterologous vaccination strategy was found: the primary outcome showed a 10% higher seroconversion rate for mRNA (homologous) versus vector (heterologous) vaccination. Conversely, the inducibility of a T cell response was numerically higher for the vector vaccine. However, while unlikely, these findings cannot rule out a higher efficacy of an additional heterologous versus homologous booster vaccination. Larger patient cohorts are needed to sufficiently address this question.

To date, limited data exist that report on the efficacy and safety of a third vaccine in immunosuppressed patients to guide the vaccination strategy on non-seroconverted patients, particularly those at a high risk for severe COVID-19 infections. Data published so far report on increased immunogenicity of a third vaccine in patients under immunosuppression or healthy individuals.[7–15] However, most of the patients included in these trials had already shown some humoral response, as evidenced by the inclusion criteria, which allowed for the presence of low antibody levels against SARS-CoV-2 after two vaccinations. In contrast, none of the patients in our study had detectable anti-SARS-CoV-2 antibodies at baseline.

Detectable peripheral B cells serve as a key factor for seroconversion in rituximab-treated patients[22] and randomisation was, therefore, stratified by the presence or absence of peripheral B cells. As described after conventional vaccination with two mRNA vaccine doses, the presence of detectable peripheral B cells was the strongest determinant for seroconversion also in patients receiving an additional booster vaccination. These data support the critical consideration of the timing of rituximab treatment, potentially suggesting postponing its application until after vaccination, or that vaccination should be timed after peripheral B cells have repopulated. Which strategy may be preferable will be guided by the perceived severity of underlying disease as well as the risk from a severe COVID-19 infection. Although it did not reach statistical significance, co-medication with any csDMARD favoured lack of seroconversion. These data are in line with recent data, which indicate a role of csDMARDs on seroconversion on primary vaccination.[19 31–33] Larger cohorts are certainly needed to sufficiently address the impact of co-medication on humoral as well as cellular immune responses.

The concern with such booster vaccination, also among candidate patients, may mostly relate to the risk of adverse reactions. Although no serious adverse events after booster vaccination were reported in both groups, our data show a numerically higher incidence of adverse events in patients boosted with the heterologous vector vaccine than with the homologous mRNA vaccine. These data are in line with recently published reports, which describe an increase in systemic reactogenicity in participants receiving heterologous schedules as compared with homologous schedules.[25] Reactogenicity was similar on third vaccination as reported previously.[24] In our trial, typical general systemic reactions (like fever, myalgias and similar) were observed that were within the scope of the reports from the large approval studies,[34–36] with some numerical differences seen between the two treatment groups.

One limitation of the trial is the absence of a placebo control, which was considered unethical in this high-risk population. While the small sample size precluded delivering ultimate statistical evidence concerning the clinical question of differences in immune responses to booster vaccination with homologous vs heterologous products, the important result of our study is that a third booster vaccination is effective in inducing an immune response in these refractory patients. Since we cannot generalise these data to the wider population of non-responders to COVID-19 vaccines, that is, beyond immunosuppressed patients, broader population-based programmes are needed to evaluate the impact of an additional booster vaccination in non-responding healthy individuals. It is important to note, that it still needs to be determined how humoral and cellular immune responses (or their absence) relate to protection against clinical infection with SARS-CoV-2.

Our data show that a cellular and/or humoral immune response can be achieved on a third COVID-19 vaccination in most of the patients who initially developed neither a humoral nor a cellular immune response. The efficacy data together with the safety data seen in our trial provide a favourable risk/benefit ratio and support the implementation of a third vaccination for non-seroconverted high-risk autoimmune disease patients treated with B cell depleting agents. This might be a viable way to protect this group of patients from more dire consequences of an acquired SARS-CoV-2 infection.

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Acknowledgements We thank all the patients who participated. We thank Martina Durechova, Daffodil Dioso and Michael Zauner for their support. We thank Brigitte Meyer, Birgit Niederreiter, Carl-Walter Steiner, Ursula Sinzinger, Amelie Popovitsch, Jutta Hutecek, Sebastian Weiss, Patrick Mucher, Astrid Radakovics and Manuela Repl for their technical assistance. We thank Sylvia Taxer, Zoltan Vass, Eva Rath, Nikolaus Hommer, Lisa Göschl and Jochen Zwerina for their support. We thank Franz X Heinz for critically reading the manuscript.

Contributors All authors revised the manuscript and were involved in editing or quality control. MB, DA, DS, DM, MZ, ST and SW contributed to the study design. DM, HR, LH and DS contributed to data analysis. TP, HH and KS performed antibody measurements. JHA, MK, MM and PH contributed to cellular assays. MB, DA, JSS, DM, DS, LXH and MZ contributed to the first manuscript draft. ST, DM, MB, PM, BK, ES, RF-S, KR and EH-M contributed to patient recruitment. RT determined leucocyte subsets. MZ performed randomisation. MB and DM had access to all the data, accept full responsibility for the work and conduct of the study and controlled the decision to publish.

Funding Provision of vaccines and laboratory testing was provided free of charge by the City of Vienna and the Medical University of Vienna via the Vienna General Hospital. Laboratory testing was supported by the Medical-Scientific fund of the Mayor of the federal capital Vienna to JHA (grant number: Covid003). Otherwise, there was no specific funding or grant for this research from any funding agency in the public, commercial or not-for-profit sectors. The funders had no role in considering the study design or in the collection, analysis or interpretation of data, the writing of the report or the decision to submit the article for publication.

Competing interests BK has received honoraria for lecturing/consulting from Biogen, BMS Celgene, Johnson & Johnson, Merck, Novartis, Roche, Sanofi-Genzyme and Teva. PM reports speaker fees from AbbVie, Janssen and Novartis and research grants from AbbVie, BMS, Novartis, Janssen, MSD and UCB. MB reports about personal fees from Eli-Lilly. DA received grants and consulting fees from AbbVie, Amgen, Lilly, Merck, Novartis, Pfizer, Roche and Sandoz. JSS reports about grants, consulting and personal fees from AbbVie, Astra-Zeneca, Lilly, Novartis, Amgen, Astro, Bristol-Myers Squibb, Celgene, Celltrion, Chugai, Gilead, ILTOO, Janssen, Merck Sharp & Dohme, Novartis-Sandoz, Pfizer, Roche, Samsung and UCB. KS received a research grant from Pfizer. MZ received grants and consulting fees from Nabriva, AntibioTxApS, Shionogi, NovoNordisk, Merck, Infectopharm and Pfizer. HH received grants from Glock Health, BlueSky Immunotherapies and Neutrolis. All other authors declare no competing interests.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants. The study protocol and all relevant documents were approved by the competent authorities and the ethics committee of the Medical University of Vienna in May 2021 (EK#: 1481/2021). All participants gave informed written consent.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. Anonymous patient data is available under specific conditions. Proposals will be reviewed and approved by the sponsor, scientific committee and staff on the basis of scientific merit and the absence of competing interests. Once the proposal has been approved, data can be transferred through a secure online platform after the signing of a data access agreement and a confidentiality agreement.

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EPIDEMIOLOGICAL SCIENCE

Safety of vaccination against SARS-CoV-2 in people with rheumatic and musculoskeletal diseases: results from the EULAR Coronavirus Vaccine (COVAX) physician-reported registry

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221490).

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Received 10 September 2021
Accepted 8 December 2021
Published Online First 31 December 2021

To cite: Machado PM, Lawson-Tovey S, Strangfeld A, *et al*. *Ann Rheum Dis* 2022;**81**:695–709.

ABSTRACT

Objectives To describe the safety of vaccines against SARS-CoV-2 in people with inflammatory/autoimmune rheumatic and musculoskeletal disease (I-RMD).

Methods Physician-reported registry of I-RMD and non-inflammatory RMD (NI-RMDs) patients vaccinated against SARS-CoV-2. From 5 February 2021 to 27 July 2021, we collected data on demographics, vaccination, RMD diagnosis, disease activity, immunomodulatory/immunosuppressive treatments, flares, adverse events (AEs) and SARS-CoV-2 breakthrough infections. Data were analysed descriptively.

Results The study included 5121 participants from 30 countries, 90% with I-RMDs (n=4604, 68% female, mean age 60.5 years) and 10% with NI-RMDs (n=517, 77% female, mean age 71.4). Inflammatory joint diseases (58%), connective tissue diseases (18%) and vasculitis (12%) were the most frequent diagnostic groups; 54% received conventional synthetic disease-modifying antirheumatic drugs (DMARDs), 42% biological DMARDs and 35% immunosuppressants. Most patients received the Pfizer/BioNTech vaccine (70%), 17% AstraZeneca/Oxford and 8% Moderna. In fully vaccinated cases, breakthrough infections were reported in 0.7% of I-RMD patients and 1.1% of NI-RMD patients. I-RMD flares were reported in 4.4% of cases (0.6% severe), 1.5% resulting in medication changes. AEs were reported in 37% of cases (37% I-RMD, 40% NI-RMD), serious AEs in 0.5% (0.4% I-RMD, 1.9% NI-RMD).

Conclusion The safety profiles of SARS-CoV-2 vaccines in patients with I-RMD was reassuring and comparable with patients with NI-RMDs. The majority of patients tolerated their vaccination well with rare reports of I-RMD flare and very rare reports of serious AEs. These findings should provide reassurance to rheumatologists and vaccine recipients and promote confidence in SARS-CoV-2 vaccine safety in I-RMD patients.

Key messages

What is already known about this subject?

- People with inflammatory/autoimmune rheumatic and musculoskeletal diseases (I-RMDs) were excluded from SARS-CoV-2 vaccine clinical development programmes; therefore, concerns regarding the safety and effectiveness of SARS-CoV-2 vaccines in this population still exist.
- Previous studies in people with I-RMDs were small albeit reassuring in terms of the incidence of I-RMD flares and adverse events.

INTRODUCTION

The WHO declared the SARS-CoV-2 outbreak a Public Health Emergency of International Concern on 30 January 2020 and a pandemic on 11 March 2020. The COVID-19 pandemic has led to a dramatic loss of human life and an unprecedented challenge to public health and healthcare systems worldwide.[1]

Since the publication of the genome sequence of SARS-CoV-2 on 11 January 2020, the development of vaccines against SARS-CoV-2 accelerated at an extraordinary pace; in December 2020, two vaccines using mRNA technology (Pfizer/BioNTech and Moderna) and one vaccine using a non-replicating adenoviral vector expressing the spike protein (AstraZeneca/Oxford) were authorised for use by several national and international drug regulatory bodies.[1] According to the WHO, on 17 August 2021, there were 112 candidate vaccines in human clinical trial phases and 183 candidates in preclinical development worldwide.[2]

Vaccines are a key pillar of public health and the WHO estimates that vaccine immunisation currently prevents 4–5 million deaths every year.[3]

Key messages

What does this study add?

- In this large international registry of patients with I-RMDs vaccinated against SARS-CoV-2, the overwhelming majority of patients tolerated their vaccination well with rare reports of I-RMD flare (4.4%, 0.6% severe, 1.5% requiring medication changes) and very rare reports of serious adverse events (AEs) (0.4%) and breakthrough infections, namely in fully vaccinated patients (0.7%).
- The AE profile was similar to the one observed in patients with non-inflammatory RMDs (and the general population). They were mainly non-serious transient local and systemic reactions.

How might this impact on clinical practice or future developments?

- These findings will support discussions with patients regarding the safety profile and benefit/risk ratio of vaccination against SARS-CoV-2 and the development of recommendations by competent organisations.
- These findings should provide reassurance to rheumatologists, other health professionals and vaccine recipients and promote confidence in SARS-CoV-2 vaccine safety in I-RMD patients.

Many more lives are expected to be saved with immunisation against SARS-CoV-2, which has been shown to be highly effective.[4–8] However, vaccination also raises questions, especially for patients with inflammatory/autoimmune rheumatic and musculoskeletal diseases (I-RMDs) and/or treated with drugs that may influence the functional competence of their immune system.

Patients with immune-mediated inflammatory diseases (including I-RMDs) were excluded from SARS-CoV-2 vaccine clinical development programmes; therefore, questions regarding the safety, effectiveness and potential measures that may increase the safety and effectiveness of vaccination against SARS-CoV-2 are unanswered.[9 10] Lack of data has led to some contradictory advice from rheumatology organisations and healthcare professionals regarding some of these vaccination aspects.[11 12] Further data will contribute to more informed decisions by patients and healthcare professionals and more robust and homogeneous evidence-based recommendations from relevant organisations. Our aim was therefore to describe the safety of vaccines against SARS-CoV-2 in people with I-RMDs.

Of note, adverse events reported in these manuscript should be considered adverse events following immunisation (AEFI), as defined by the WHO that is, 'any untoward medical occurrence which follows immunization and which does not necessarily have a causal relationship with the usage of the vaccine'. Investigating causality of AEFIs, particularly those that are more serious, is a much more challenging and complex process that should take the consistence, strength, specificity, temporal relation and biological plausibility of the association into account.

METHODS

Data source

The European Alliance of Associations for Rheumatology (EULAR) Coronavirus Vaccine (COVAX) physician-reported registry was launched on 5 February 2021. Data are entered voluntarily by rheumatologists or other members of the clinical rheumatology team; patients are eligible for inclusion if they have a pre-existing I-RMD or non-inflammatory rheumatic and musculoskeletal disease (NI-RMD) and have received one or more doses of any vaccine against SARS-CoV-2. Data are entered directly into an online data entry system or transferred from national registries (for Portugal). Patients with NI-RMDs are included as a control group.

Providers were asked to report as many cases as possible of patients with rheumatic and musculoskeletal disease (RMDs) vaccinated against SARS-CoV-2, with or without adverse events. Cases could be collected in outpatient, day care or inpatient settings, with the number of reported cases per session varying depending on feasibility. When reporting only a subset of patients from, for example, a full clinic list, providers were asked to select cases randomly, in order to avoid selection bias. Furthermore, the time from vaccination to the reporting of the case/outcome was allowed to vary between individuals, and providers were also asked not to report adverse events that, in the opinion of the reporter, were definitely not related with the vaccine administration (eg, death as a consequence of road traffic accident).

Data are collected using REDCap, a secure web application for building and managing online surveys and databases.[13 14] The survey (available at https://www.eular.org/eular_covax_registry.cfm) was developed by a EULAR COVID-19 Task Force of representatives of its constituents, patients and health professionals in rheumatology and rheumatologists. Input and support was also received from the European Reference Network (ERN) on Rare and Complex Connective Tissue and Musculoskeletal Diseases (ERN ReCONNET) and the European Reference Network on Rare Immunodeficiency, Autoinflammatory and Autoimmune Diseases Network (ERN RITA), two virtual networks involving healthcare providers across Europe, part of the EU-supported ERN initiative.

Given the registry collects anonymous non-interventional data, the UK Health Research Authority (HRA) does not class the registry as a research study (in line with the HRA decision tool), and patient consent is not required. By submitting cases, providers accept the privacy notice available on the data collection website.

Data collected

The following information is collected: patients' age (years), sex at birth, country of residence, COVID-19 vaccine received, number of doses and dates, diagnosis of COVID-19 before or after vaccination, primary (and secondary) RMD diagnoses, physician global assessment of disease activity (only applicable to I-RMDs and categorised as remission/inactive disease, low, moderate or severe/high disease activity), exposure to immunomodulatory/immunosuppressive treatments at the time of vaccination,[9] I-RMD flare following vaccination and other probably/possibly vaccine-related adverse events (AEs), including AEs of special interest. SARS-CoV-2 infections stratified by vaccination status were defined as per US Centers for Disease Control and Prevention definitions[15]: (1) infection in 'partially vaccinated' cases if occurring ≥14 days after dose one to <14 days after dose two, and (2) infection in 'fully vaccinated' cases if occurring ≥14 days after dose two or after a single-dose vaccine.

Immunomodulatory/immunosuppressive treatments

Exposure to the following immunomodulatory/immunosuppressive treatments[16] at the time of COVID-19 vaccination is collected:

1. Conventional synthetic (cs) disease-modifying antirheumatic drugs (DMARDs), namely antimalarials

(hydroxychloroquine and chloroquine), leflunomide, methotrexate and sulfasalazine.
2. Biological (b) DMARDs, namely abatacept, belimumab, rituximab, interleukin (IL)-1 inhibitors (including anakinra, canakinumab and rilonacept), IL-6 inhibitors (including tocilizumab, sarilumab), IL-12/23 inhibitors (ustekinumab), IL-23 inhibitors (including guselkumab, risankizumab and tildrakizumab), IL-17 inhibitors (including secukinumab, ixekizumab and brodalumab) and tumour necrosis factor (TNF) inhibitors (including adalimumab, certolizumab, etanercept, golimumab, infliximab and biosimilars).
3. Targeted synthetic (ts) DMARDs, namely apremilast and JAK inhibitors (including tofacitinib, baricitinib and upadacitinib).
4. Immunosuppressants: glucocorticoids (GCs), azathioprine/6-mercaptopurine, cyclophosphamide, ciclosporin, mycophenolate mofetil and tacrolimus.
5. Intravenous immunoglobulin.

For each medication, information about changes in the original therapeutic regimen before or after COVID-19 vaccination (including stopping/holding/reducing the medication) is also collected.

Flares

For patients with I-RMDs, information about flares is collected, namely: (1) type of flare (fever, weight loss, increase in fatigue, increase in dryness, enlarged lymph nodes, arthralgia, arthritis flare, cutaneous, pulmonary, renal, neurological, muscular, cardiac, gastrointestinal or haematological flare or other type of flare); (2) severity of flare (mild/minor, moderate, severe/major without hospitalisation and severe/major with hospitalisation); (3) information about changes in medication (including dosage increase) due to the flare; and (4) period of time between vaccination and the flare.

Adverse events

Two main types of AEs are collected:
1. Early AEs within 7 days from vaccination (reactogenicity): pain, redness or swelling at the site of injection, generalised muscle or joint pain, headache, fever, chills, fatigue, vomiting and diarrhoea.
2. AEs of special interest: collected based on organ/system affected, with the possibility to add free-text descriptors.

Information about the period of time between vaccination and the AE, degree of confidence in the relationship between the AE and the COVID-19 vaccine, outcome (ongoing/continuing, recovered/resolved without sequelae, recovered/resolved with sequelae, death and unknown) and if the AE was serious or not is also collected.

Serious AEs (SAEs) are further categorised into six possible groups: resulting in an important medical event, resulting in hospitalisation or prolongation of existing hospitalisation (hospitalisation being defined as at least 24 hours in a hospital or an overnight stay), life-threatening event, resulting in persistent or significant disability/incapacity, resulting in death or resulting in congenital anomaly/birth defect.

Statistical analysis

Descriptive statistics, including means and SD, frequencies and proportions, are used to describe the data. Data are presented separately for patients with I-RMD and NI-RMD. Crystal arthropathies were included in the NI-RMD group as these patients are not usually treated with immunomodulatory/immunosuppressive drugs. Missing data were treated as missing.

RESULTS

Demographics

Between 5 February 2021 and 27 July 2021, 5121 cases were submitted to the EULAR COVAX registry (table 1). Seventy per cent of these cases were female, the mean age was 61.6 (SD 15.2), and over half of the cases were over the age of 60 years (56%). Cases were submitted from 30 countries, the majority from France (40%), Italy (16%) and Portugal (14%). Providers were from diverse rheumatology practices, including academic and non-academic centres, and a minority of private practices. The I-RMD group made up 90% of all cases (n=4604), with a mean age of 60.5 (SD 15.1) and 68% of this group were female. The NI-RMD group (10%, n=517) had a higher percentage of female cases (77%) and a higher mean age (71.4, SD 12.5), with 80% of the group having an age over 60 years. Mean time between first vaccine dose and case reporting was 66 days (SD 40), 66 days (SD 40) in the I-RMD group and 64 days (SD 40) in the NI-RMD group.

RMD data

Over half of the cohort had an inflammatory joint disease as their primary RMD diagnosis (58%), 18% had a connective tissue disease, 12% vasculitis and 2% another I-RMD (table 2). The most common I-RMDs were rheumatoid arthritis (33%), axial spondyloarthritis (11%) and psoriatic arthritis (10%). Osteoarthritis (5%) and osteoporosis (2%) were the most frequent NI-RMDs.

The majority of the I-RMD group had minimal (41%) or low (28%) disease activity, although these data were missing in 17% of cases.

Fifty-four per cent of the I-RMD group received csDMARDs, 42% bDMARDs and 35% immunosuppressants. The most common individual medications were methotrexate (MTX; 34%), GCs (30%) and TNF-inhibitors (25%). Overall, there were few medication changes either before or after vaccination; however, changes were more prevalent in some drugs than others. Seven per cent of patients taking rituximab and IL-6 inhibitors held their medication before vaccination, 6% of TNF-inhibitor patients held the drug prior to vaccination and 6% and 4% of MTX cases held the medication before and after vaccination, respectively (table 2).

Vaccine information

Most patients received the Pfizer/BioNTech vaccine (70%), 17% had the AstraZeneca/Oxford and 8% the Moderna vaccine (table 3). One quarter of cases had one vaccine dose, whereas almost three quarters (74%) had two and 1% had three. Mean time between the first and second dose of the vaccine (if applicable) was 34 days (SD 62), 33 days (SD 18) in the I-RMD group and 43 days (SD 189) in the NI-RMD group. Mean time between the first and second vaccine doses in the Pfizer group was 28 days (SD 12), 30 days (SD 8) in the Moderna group and 78 days (SD 14) in the AstraZeneca/Oxford group.

The split of vaccine types, doses and postvaccination SARS-CoV-2 infection was similar between the I-RMD and NI-RMD groups (table 3), although 12 I-RMD cases received a combination of vaccines (Pfizer/BioNTech and either AstraZeneca/Oxford or CoronaVac/Sinovac).

SARS-CoV-2 infection after vaccination occurred in 46 cases (0.9%), with 42 cases occurring in the I-RMD (0.9%) and 4

Table 1 Patient demographics

		Inflammatory RMDs	Non-inflammatory RMDs	All patients
Total number		**4604**	**517**	**5121**
Gender	Female	3152 (68)	398 (77)	3550 (70)
	Male	1410 (31)	117 (23)	1527 (30)
	Other/unknown	42 (1)	2 (<1)	44 (1)
Age (years)	Mean (SD)	60.5 (15.1)	71.4 (12.5)	61.6 (15.2)
	Range (min to max)	15 to 96	22 to 98	15 to 98
Age categories (years)	<18	6 (<1)	–	6 (<1)
	18–40	526 (11)	11 (2)	537 (10)
	41–60	1640 (36)	94 (18)	1734 (34)
	61+	2432 (53)	412 (80)	2844 (56)
Country	Belgium	197 (4)	3 (1)	200 (4)
	France	1838 (40)	232 (45)	2070 (40)
	Italy	615 (13)	194 (38)	809 (16)
	Latvia	107 (2)	19 (4)	126 (2)
	Monaco	296 (6)	36 (7)	332 (6)
	Portugal	737 (16)	–	737 (14)
	Ireland	76 (2)	7 (1)	83 (2)
	Romania	61 (1)	4 (1)	65 (1)
	Slovak Republic	204 (4)	11 (2)	215 (4)
	Spain	164 (4)	5 (1)	169 (3)
	Turkey	78 (2)	1 (<1)	79 (2)
	UK	72 (2)	1 (<1)	73 (1)
	Other countries*	159 (3)	4 (1)	163 (3)

All values are n (%) unless stated otherwise.
*Other countries classified as those who submitted <50 cases: Albania, Australia, Austria, Croatia, Czechia, Estonia, Germany, Greece, Hungary, Lithuania, Luxembourg, Netherlands, Republic of Moldova, Russian Federation, Slovenia, Switzerland, Ukraine and USA.
RMDs, rheumatic and musculoskeletal diseases.

cases occurring in the NI-RMD group (0.8%); however, only 21 cases (0.7%) occurred in fully vaccinated patients (n=18, 0.7%; n=3, 1.1%; in the I-RMD and NI-RMD group, respectively).

When stratified by vaccine type, the percentage of cases with postvaccination SARS-CoV-2 infection was equal across vaccine types in the I-RMD group (online supplemental table 1) but only reported following the Pfizer/BioNTech vaccine or other vaccine types in the NI-RMD group (online supplemental table 2), though this is explained by the low number of cases vaccinated with Oxford/AstraZeneca and Moderna in the NI-RMD group.

Flares

Flare following vaccination was reported in 4.4% (n=204) of I-RMD cases, though these data were missing in 15% of cases. Mean time between the most recent vaccine dose (prior to flare) and the flare was 6 days (SD 8). The most common flares were arthritis flare, polyarthralgia and increase in fatigue (2.1%, 1.8%, and 0.7% of the I-RMD cohort, respectively). Most flares were mild (1.5%) or moderate (2.1%), with 29 cases (0.6%) being severe and 68 cases (1.5%) having started a new medication or increased existing medication dosage as a result of the flare (table 3).

The percentage of cases reporting a flare, flare severity and medication changes due to the flare were consistent among different vaccines (online supplemental table 1). The percentage of flares was slightly higher in patients with moderate/high disease activity (5.2%) compared with patients in remission/low disease disease activity (4.8%), with similar results observed for severe flares (1.0% vs 0.7%), though disease activity information was missing in 17% of cases. These findings raise the possibility of an association between higher disease activity and higher flare rate.

When stratified by I-RMD group (table 4), patients with inflammatory joint diseases experienced a slightly higher percentage of flares compared with the connective tissue disease and vasculitis groups (5.1% vs 3.1% vs 3.2%, respectively). Flare prevalence was similar across most medication groups in I-RMD cases (table 5), although patients on monotherapy or combination therapies of TNF-inhibitors (5.5%), other biologicals (5.3%), other csDMARDs (excluding methotrexate) (4.7%) and tsDMARDs (4.6%) reported a slightly higher percentage of flares than other medication groups (2.7%–3.6%). The lower flare rate was observed for rituximab and immunosuppressants (both 2.7%).

Adverse events

There were possible/probable vaccine-related AEs in 37% of all cases, 37% in the I-RMD group and 40% in the NI-RMD group. The majority were early AEs, mostly pain at injection site (19%), fatigue (12%), generalised muscle pain (7%) and fever (7%). Overall, the pattern and proportion of early AEs was similar between I-RMD and NI-RMD cases (table 3).

When I-RMD cases were stratified by vaccine type (online supplemental table 1), the percentage of AEs was similar across the group (32%–37%), except for Moderna, where a slightly higher percentage was observed (42%). The percentages of most individual types of early AEs were also similar across vaccines; however, a larger proportion of Moderna (26%) and a lower proportion of AstraZeneca/Oxford cases (12%) had pain at the injection site, and higher percentages of AstraZeneca/Oxford

Table 2 Rheumatic and musculoskeletal disease information

Primary RMD diagnosis	**Inflammatory RMDs**	**4604 (90)**
	Inflammatory joint diseases	**2979 (58)**
	Rheumatoid arthritis	1686 (33)
	Axial spondyloarthritis (including ankylosing spondylitis)	573 (11)
	Psoriatic arthritis	505 (10)
	Other peripheral spondyloarthritis (including reactive arthritis)	114 (2)
	Juvenile idiopathic arthritis, not systemic	23 (<1)
	Systemic juvenile idiopathic arthritis	7 (<1)
	Other inflammatory arthritis	70 (1)
	Connective tissue diseases	**928 (18)**
	Systemic lupus erythematosus	367 (7)
	Primary anti-phospholipid syndrome	26 (1)
	Sjogren's syndrome	223 (4)
	Systemic sclerosis	162 (3)
	Idiopathic inflammatory myopathy (myositis)	69 (1)
	Mixed connective tissue disease	37 (1)
	Undifferentiated connective tissue disease	43 (1)
	Ehlers-Danlos syndromes	1 (<1)
	Vasculitis	**593 (12)**
	Large vessel vasculitis – Takayasu arteritis	14 (<1)
	Large vessel vasculitis – giant cell arteritis	141 (3)
	Polymyalgia rheumatica	239 (5)
	Medium-vessel vasculitis (polyarteritis nodosa, Kawasaki disease)	11 (<1)
	ANCA-associated vasculitis (MP, GPA, EGPA)	127 (2)
	Immune complex small vessel vasculitis	7 (<1)
	Behcet's syndrome	33 (1)
	Other vasculitis	21 (<1)
	Other immune-mediated inflammatory diseases	**106 (2)**
	Monogenic autoinflammatory syndrome	13 (<1)
	Non-monogenic autoinflammatory syndrome	12 (<1)
	IgG4-related disease	16 (<1)
	Sarcoidosis	56 (1)
	Relapsing polychondritis	7 (<1)
	Chronic recurrent multifocal osteomyelitis	2 (<1)
	Non-inflammatory RMDs	**517 (10)**
	Gout or other crystal arthritis	62 (1)
	Osteoporosis	112 (2)
	Osteoarthritis	240 (5)
	Fibromyalgia	36 (1)
	Chronic mechanical back pain	16 (<1)
	Radiculopathy or regional pain	7 (<1)
	Other mechanical RMD (eg, tendinitis and bursitis)	44 (1)
Rheumatic disease activity (only applicable to patients with inflammatory RMD; n=4604)	Remission or inactive disease	1867 (41)
	Minimal or low disease activity	1276 (28)
	Moderate disease activity	610 (13)
	Severe or high disease activity	76 (2)
	Missing/unknown	775 (17)
Medication exposure at the time of vaccination (only applicable to patients with inflammatory RMD; n=4604)	**csDMARDS**	**2497 (54)**
	Antimalarials (including hydroxychloroquine and chloroquine)	568 (12)
	Held before vaccination	*2*
	Reduced before vaccination	*1*
	Held after vaccination	*3*
	Reduced after vaccination	*2*
	Leflunomide	211 (5)
	Held before vaccination	*7*
	Reduced before vaccination	*1*
	Held after vaccination	*2*
	Methotrexate	1557
	Held before vaccination	*58*
	Reduced before vaccination	*3*
	Held after vaccination	*90*
	Reduced after vaccination	*1*
	Sulfasalazine	161 (4)
	Held before vaccination	*1*
	Held after vaccination	*1*
	Reduced after vaccination	*1*
	bDMARDS	**1944 (42)**
	Abatacept	103 (2)
	Held before vaccination	*5*
	Held after vaccination	*3*
	Belimumab	32 (1)
	Held before vaccination	*2*
	Rituximab	260 (6)
	Held before vaccination	*18*
	Reduced before vaccination	*2*
	Held after vaccination	*1*
	Reduced after vaccination	*1*
	IL-1 inhibitors (including anakinra, canakinumab, rilonacept)	19 (<1)
	IL-6 inhibitors (including tocilizumab and sarilumab)	222 (5)
	Held before vaccination	*16*
	Held after vaccination	*4*
	Reduced after vaccination	*1*
	IL-12/23 inhibitors (including ustekinumab)	34 (1)
	Held before vaccination	*2*
	IL-23 inhibitors (guselkumab, risankizumab and tildrakizumab)	2 (<1)
	IL-17 inhibitors (including secukinumab, ixekizumab and brodalumab)	99 (2)
	Held before vaccination	*4*
	Held after vaccination	*4*
	Reduced after vaccination	*1*
	TNF-inhibitors (including adalimumab, certolizumab, etanercept, golimumab, infliximab and biosimilars)	1173 (25)
	Held before vaccination	*67*
	Reduced before vaccination	*5*
	Held after vaccination	*29*
	Reduced after vaccination	*2*
	tsDMARDS	**175 (4)**
	Apremilast	13 (<1)
	JAK inhibitors (including tofacitinib, baricitinib and upadacitinib)	162 (4)
	Held before vaccination	*5*
	Reduced before vaccination	*1*
	Held after vaccination	*10*
	Immunosuppressants	**1621 (35)**
	Glucocorticoids (systemic)	1385 (30)
	Held before vaccination	*6*
	Reduced before vaccination	*6*
	Held after vaccination	*6*
	Reduced after vaccination	*9*
	Azathioprine/6-mercaptopurine	88 (2)
	Held before vaccination	*2*
	Cyclosporine	15 (<1)
	Held before vaccination	*2*
	Reduced before vaccination	*1*
	Cyclophosphamide	8 (<1)
	Mycophenolate mofetil/mycophenolic acid	123 (3)
	Held before vaccination	*6*
	Held after vaccination	*2*
	Reduced after vaccination	*1*
	Tacrolimus	2 (<1)
	Other	**78 (2)**
	Intravenous immunoglobulin	15 (<1)
	Held after vaccination	*1*
	Antifibrotics (pirfenidone and nintedanib)	5 (<1)
	Thalidomide/lenalidomide	2 (<1)
	Colchicine	24 (<1)
	Denosumab	26 (1)
	Mepolizumab	4 (<1)
	Pembrolizumab	1 (<1)
	Vedolizumab	1 (<1)
	Unknown/missing	**43 (1)**
	None	**393 (9)**

All values are n (%) unless stated otherwise.
ANCA, anti-neutrophil cytoplasmic antibody; bDMARDs, biological disease-modifying antirheumatic drugs; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; EGPA, eosinophilic granulomatosis with polyangiitis; GPA, granulomatosis with polyangiitis; IL, interleukin; JAK, Janus kinase; MP, microscopic polyangiitis; TNF, tumour necrosis factor; tsDMARDs, targeted synthetic disease-modifying antirheumatic drug.

Table 3 COVID-19 vaccines, SARS-CoV-2 infections after vaccination, flares and adverse events in patients with inflammatory and non-inflammatory RMDs

		Inflammatory RMDs	Non-inflammatory RMDs	All patients
Vaccine	mRNA/nucleic acid (Pfizer/BioNTech)	3218 (70)	382 (74)	3600 (70)
	mRNA/nucleic acid (Moderna)	398 (7)	30 (6)	428 (8)
	Viral vector (AstraZeneca/Oxford)	759 (16)	96 (19)	855 (17)
	Viral vector (Janssen/Johnson & Johnson)	45 (1)	5 (1)	50 (1)
	Viral vector (Sputnik V)	5 (<1)	1 (<1)	6 (<1)
	Inactivated vaccine (CoronaVac/Sinovac)	53 (1)	1 (<1)	54 (1)
	Other	4 (<1)		4 (<1)
	Unknown/missing	110 (2)	2 (<1)	112 (2)
	Vaccine combination (Pfizer/BioNTech and AstraZeneca/Oxford)	11 (<1)		11 (<1)
	Vaccine combination (Pfizer/BioNTech and CoronaVac/Sinovac)	1 (<1)		1 (<1)
Vaccine doses	One	1149 (25)	132 (26)	1281 (25)
	Two	3406 (74)	384 (74)	3790 (74)
	Three	46 (1)	1 (<1)	47 (1)
	Unknown/missing	3 (<1)		3 (<1)
SARS-CoV-2 infection after vaccination	Yes	42 (1)	4 (1)	46 (1)
	No	4380 (95)	490 (95)	4870 (95)
	Unknown/missing	182 (4)	23 (4)	205 (4)
Vaccination status	Fully vaccinated cases	2622 (57)	270 (52)	2892 (56)
	Partially vaccinated cases	1982 (43)	247 (48)	2229 (44)
SARS-CoV-2 infection after vaccination, according to vaccination status (vaccination status is the denominator)	Fully vaccinated cases	18/2622 (1)	3/270 (1)	21/2892 (1)
	Partially vaccinated cases	24/1982 (1)	1/247 (<1)	25/2229 (1)
Flare following vaccination (only applicable to patients with inflammatory RMD; n=4604)	Yes	204 (4)	–	–
	No	3706 (81)	–	–
	Unknown/missing	694 (15)	–	–
Type of flare (data presented as percentage of total number of inflammatory RMD cases (n=4604))	Fever	18 (<1)	–	–
	Weight loss	1 (<1)	–	–
	Increase in fatigue	30 (1)	–	–
	Increase in dryness	4 (<1)	–	–
	Enlarged lymph nodes	4 (<1)	–	–
	Polyarthralgia	83 (2)	–	–
	Arthritis flare	95 (2)	–	–
	Cutaneous flare	16 (<1)	–	–
	Pulmonary flare	3 (<1)	–	–
	Renal flare	1 (<1)	–	–
	Neurological flare	2 (<1)	–	–
	Muscular flare	15 (<1)	–	–
	Cardiac flare	3 (<1)	–	–
	Gastro-intestinal flare	1 (<1)	–	–
	Haematological flare	3 (<1)	–	–
	Other	17 (<1)	–	–
	Unknown/missing	7 (<1)	–	–
Severity of flare (data presented as percentage of total number of inflammatory RMD cases (n=4604))	Mild/minor	69 (2)	–	–
	Moderate	98 (2)	–	–
	Severe/major without hospitalisation	20 (<1)	–	–
	Severe/major with hospitalisation	9 (<1)	–	–
	Unknown/missing	8 (<1)	–	–
	New medication or dosage increase due to flare	68 (1)	–	–
Vaccine-related AEs	Yes	1688 (37)	206 (40)	1894 (37)
	No	2916 (63)	311 (60)	3227 (63)

Continued

Table 3 Continued

		Inflammatory RMDs	Non-inflammatory RMDs	All patients
Early AEs	Pain at injection site	881 (19)	75 (15)	956 (19)
	Redness at injection site	70 (2)	4 (1)	74 (1)
	Swelling at injection site	75 (2)	1 (<1)	76 (1)
	Generalised muscle pain	302 (7)	41 (8)	343 (7)
	Generalised joint pain	163 (4)	26 (5)	189 (4)
	Headache	293 (6)	36 (7)	329 (6)
	Fever	331 (7)	44 (9)	375 (7)
	Chills	130 (3)	16 (3)	146 (3)
	Fatigue	531 (12)	65 (13)	596 (12)
	Vomiting	58 (1)	4 (1)	62 (1)
	Diarrhoea	38 (1)	4 (1)	42 (1)
	Unknown	5 (<1)		5 (<1)
AEs of special interest	Cardiovascular – arterial hypertension	4 (<1)	2 (<1)	6 (<1)
	Cardiovascular – arrhythmia	3 (<1)		3 (<1)
	Cardiovascular – coronary artery disease	2 (<1)		2 (<1)
	Cardiovascular – myocarditis and pericarditis	1 (<1)		1 (<1)
	Dermatologic – eczema, nodes and plaques	4 (<1)		4 (<1)
	Dermatologic – pruritus, injection site reaction, redness and burning	3 (<1)	2 (<1)	5 (<1)
	Gastrointestinal – liver injury	3(<1)	3 (1)	6 (<1)
	General conditions – hot flush, anxiety, lowered body temperature, loss and lack of appetite and night sweats	8 (<1)	1 (<1)	9 (<1)
	Haematological – peripheral deep vein thrombosis	2 (<1)		2 (<1)
	Haematological – haemorrhagic disease	1 (<1)		1 (<1)
	Haematological – thrombocytopenia	1 (<1)		1 (<1)
	Haematological – stroke	1 (<1)		1 (<1)
	Immunological – anaphylaxis	3 (<1)		3 (<1)
	Immunological – arthritis	4 (<1)	5 (1)	9 (<1)
	Immunological – skin and mucosal	8 (<1)	1 (<1)	9 (<1)
	Immunological – vasculitides	1 (<1)	2 (<1)	3 (<1)
	Lymphadenopathy	4 (<1)	2 (<1)	5 (<1)
	Malaise, fatigue and insomnia	5 (<1)	1 (<1)	6 (<1)
	Neurological – anosmia and ageusia	1 (<1)		1 (<1)
	Neurological – drowsiness, vertigo, dizziness, nausea, tinnitus, migraine and hallucination	21 (<1)	7 (1)	21 (<1)
	Other possible cardiac symptoms – ankle oedema, dyspnoea and dry cough	7 (<1)	2 (<1)	9 (<1)
	Pain/pain syndromes	2 (<1)	2 (<1)	4 (<1)
	Tendons and joints – tendinopathy, frozen shoulder and carpal tunnel syndrome	4 (<1)	2 (<1)	6 (<1)
	Viral infection – herpes, herpes zoster and shingles	9 (<1)	1 (<1)	10 (<1)
	Viral infection – influenza, flu-like episodes, rhinitis, cough and cold	7 (<1)	1 (<1)	8 (<1)
	Other	3 (<1)	3 (1)	6 (<1)
	Total of adverse events of special interest	112 (2)	37 (7)	149 (3)
AE seriousness	Non-serious	90 (2)	25 (5)	115 (2)
	Serious – important medical event	8 (<1)	8 (2)	16 (<1)
	Serious - hospitalisation (or prolongation of existing hospitalisation)	6 (<1)	2 (<1)	8 (<1)
	Serious – life threatening	3 (<1)		3 (<1)
	Unknown/missing	4 (<1)		4 (<1)
AE outcome	Ongoing/continuing	21 (<1)	6 (1)	27 (1)
	Recovered/resolved without sequelae	75 (1)	25 (5)	100 (2)
	Recovered/resolved with sequelae	6 (<1)	2 (<1)	8 (<1)
	Unknown/missing	9 (<1)	1 (<1)	10 (<1)

All values are n (%) unless stated otherwise.
AEs, adverse events; RMDs, rheumatic and musculoskeletal diseases.

Table 4 Flares and AEs stratified by inflammatory RMD disease group

		Inflammatory joint diseases (n=2977)	Connective tissue diseases (n=928)	Vasculitis (n=593)
Flare following vaccination	Yes	151 (5)	29 (3)	19 (3)
	No	2260 (76)	784 (85)	561 (95)
	Unknown/missing	566 (19)	115 (12)	13 (2)
Severity of flare	Mild/minor	51 (2)	13 (1)	5 (1)
	Moderate	77 (3)	12 (1)	8 (1)
	Severe/major without hospitalisation	15 (1)	1 (<1)	3 (1)
	Severe/major with hospitalisation	1 (<1)	2 (<1)	3 (1)
	Unknown/missing	7 (<1)	1 (<1)	
	New medication or dosage increase due to flare	44 (1)	10 (1)	11 (2)
Vaccine-related AEs	Yes	1092 (37)	382 (41)	175 (30)
	No	1885 (63)	546 (59)	418 (70)
AE severity (only collected for AEs of special interest)	Non-serious	55 (2)	21 (2)	13 (2)
	Severe – important medical event	4 (1)	4 (1)	
	Severe – hospitalisation (or prolongation of existing hospitalisation)	4 (1)		2 (<1)
	Severe – life threatening	2 (<1)	1 (<1)	
	Unknown/missing	2 (<1)		

All values are n (%) unless stated otherwise.
AEs, adverse events; RMD, rheumatic and musculoskeletal disease.

(12%) and Moderna (11%) cases had fever following vaccination (in comparison with 6% with other vaccines).

Forty-one per cent of connective tissue disease cases reported AEs, compared with 37% of inflammatory joint disease and 30% of vasculitis cases (table 4). When I-RMD cases were stratified by medication group (table 5), all groups reported similar AE percentages, expect for patients on other csDMARDs (42% vs 33%–35%).

In the NI-RMD group (online supplemental table 2), the prevalence of AEs was more variable across vaccine types, with

Table 5 Flares and adverse events in patients with inflammatory RMDs, stratified by medication

		MTX mono/ combi (no biologicals or tsDMARDs) (n=895)	Other csDMARD mono/ combi (no biologicals or tsDMARDs) (n=657)	TNF mono/ combi (n=1173)	RTX mono/ combi (n=260)	Other biologics mono/ combi (n=511)	tsDMARD mono/ combi (n=175)	Immunosuppressants mono/combi (no biologics or tsDMARDs) (n=995)
Flare following vaccination	Yes	32 (4)	31 (5)	65 (6)	7 (3)	27 (5)	8 (5)	27 (3)
	No	765 (85)	520 (79)	799 (68)	204 (78)	415 (81)	150 (86)	870 (87)
	Unknown/missing	98 (11)	106 (16)	309 (26)	49 (19)	69 (14)	17 (10)	98 (10)
Severity of flare	Mild/minor	13 (1)	14 (2)	19 (2)	3 (1)	6 (1)	4 (2)	11 (1)
	Moderate	14 (2)	11 (2)	39 (3)	1 (<1)	17 (3)	3 (2)	8 (1)
	Severe/major without hospitalisation	1 (<1)	3 (<1)	4 (<1)	1 (<1)	1 (<1)		2 (<1)
	Severe/major with hospitalisation	2 (<1)	2 (<1)		1 (<1)			6 (<1)
	Unknown/missing	2 (<1)	1 (<1)	3 (<1)	1 (<1)	3 (1)	1 (1)	
	New medication or dosage increase due to flare	12 (1)	11 (2)	16 (1)	1 (<1)	8 (2)		15 (2)
Vaccine-related AEs	Yes	314 (35)	276 (42)	412 (35)	87 (33)	172 (34)	61 (35)	352 (35)
	No	581 (65)	381 (58)	761 (65)	173 (67)	339 (66)	114 (65)	643 (65)
AE severity (only collected for AEs of special interest)	Non-serious	12 (1)	16 (2)	13 (1)	2 (1)	17 (3)	3 (2)	19 (2)
	Severe – Important medical event	1 (<1)	3 (<1)	1 (<1)		1 (<1)	3 (2)	4 (<1)
	Severe - Hospitalisation (or prolongation of existing hospitalisation)	1 (<1)	1 (<1)			1 (<1)	1 (1)	2 (<1)
	Severe - Life-threatening		1 (<1)		1 (<1)	1 (<1)		1 (<1)
	Missing	1 (<1)		2 (<1)				

All values are N (%) unless stated otherwise.

AEs, adverse events; combi, combination therapy; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; mono, monotherapy; MTX, methotrexate; RMD, rheumatic and musculoskeletal disease; RTX, rituximab; TNF, tumour necrosis factor; tsDMARD, targeted synthetic disease-modifying antirheumatic drug.

the most salient difference between vaccines being the lower percentages of Pfizer/BioNTech (13%) and AstraZeneca/Oxford (10%) cases that experienced pain at injection site compared with 50% of Moderna vaccinated cases.

There were 149 AEs of special interest (2.9% of all patients), 112 (2.4%) in the I-RMD group and 37 (7.2%) in the NI-RMD group, and most of the AEs resolved/recovered without sequelae (100 cases, 2.0% of all patients; n=75, 1.6% in the I-RMD group; n=25, 4.8% in the NI-RMD group). Both in the I-RMD and NI-RMD group, a larger diversity of AEs of special interest were seen following vaccination with Pfizer/BioNTech, reflecting the higher number of cases receiving this vaccine. However, there were no salient differences between vaccines or between patients with I-RMD and NI-RMD (table 6). Mean time between the most recent vaccine dose (prior to AE of special interest) and the AE of special interest was 7 days (SD 17), 7 days (SD 15) in the I-RMD group and 8 days (SD 21) in the NI-RMD group.

SAEs were rare (n=27, 0.5% of all patients) and more prevalent in the NI-RMD group (n=10, 1.9%) than in the I-RMD group (n=17, 0.4%). Among these 27 SAEs, three were life threatening, all occurring in Pfizer/BioNTech vaccine recipients in the I-RMD group. These were two cases of 'cardiac – coronary artery disease' events and one 'gastrointestinal – liver injury' event; all three events recovered/resolved, though one cardiac event and the 'gastrointestinal – liver injury' event recovered/resolved with sequelae (table 6).

There were six instances of SAEs resulting in hospitalisation in the I-RMD group, all in Pfizer/BioNTech vaccine recipients. One of these was a 'haematologic – peripheral deep vein thrombosis' event, one was a 'haematologic – stroke' event, one was an 'immunological - skin or mucosal' event (erythema nodosum), two were 'viral infection – herpes zoster/shingles' events and finally one 'other - neck swelling event' (table 6).

Eight SAEs classified as serious important medical events were seen in the I-RMD group, occurring in Pfizer/BioNTech (n=7) and AstraZeneca/Oxford (n=1) vaccine recipients. There were three 'immunological - skin or mucosal' events (gingivitis, pharyngitis and bullous leg rash), one 'cardiac - arterial hypertension', one 'malaise', one 'neurological – hemiparesis', one 'other – possible cardiac' event (dyspnoea) and one 'viral infection – herpes zoster/shingles' event (table 7).

There were two SAEs resulting in hospitalisation in the NI-RMD group: one an 'immunological – vasculitides' event (giant cell arteritis), in a Moderna vaccine recipient, and one other possible cardiac event (dyspnoea), in a Pfizer/BioNTech vaccine recipient. Eight events were classified as important medical events: one 'arterial hypertension' event, two 'immunological – arthritis' events, a 'gastrointestinal – liver injury' event, one 'immunological – vasculitides' event (polymyalgia rheumatica-like syndrome), one 'neurological – syncope', one 'neurological – vertigo' and one 'tendons and joints' event (frozen shoulder) (table 7).

SAEs resulting in death, persistent or significant disability/incapacity or congenital anomaly/birth defect were neither reported in the I-RMD group nor in the NI-RMD group.

Of note, we are not aware of any cases of vaccine-induced immune thrombotic thrombocytopenia in this cohort, an exceedingly rare complication described in the general population with the AstraZeneca and Janssen vaccines. One case of isolated thrombocytopenia after the first dose of the AstraZeneca vaccine was reported in a young (<30 years old) female patient with mixed connective tissue disease; however, this was a transient laboratory change without clinical repercussion. Regarding myocarditis and pericarditis, a rare complication associated with mRNA vaccines, this was reported after the second dose of the Pfizer vaccine in a young (<30 years old) female patient with systemic lupus erythematosus, and she recovered without sequelae from this event.

DISCUSSION

We created the largest international case series of people with I-RMDs vaccinated against SARS-CoV-2 and report that the safety profile of vaccines against SARS-CoV-2 in this population was reassuring. The overwhelming majority of patients tolerated their vaccination well with rare reports of I-RMD flare (4.4%, 0.6% severe) and very rare reports of SAEs (0.4%). Changes in medication due to flare were also rare (1.5% of I-RMD patients). Most AEs were the same and in similar proportion as observed in patients with NI-RMDs (and the general population); they were non-serious and involved transient local and systemic symptoms.

Regarding flares, the data suggest that the risk of I-RMD flare following vaccination is low and not more strongly associated with any particular type of vaccine, with observed percentages being compatible with the natural history of the disease rather than necessarily caused by vaccines against SARS-CoV-2.[17]

Regarding early AEs (reactogenicity), both the profile and frequency of AEs were similar between I-RMD and NI-RMD cases. The frequency and type of early AEs was also similar between vaccines, both for the I-RMD and NI-RMD groups, with the possible exception of a slightly higher proportion of pain at the injection site with the Moderna vaccine (both in the I-RMD and NI-RMD group). Both the flare and AE data are in line with previous smaller studies in patients with I-RMDs (13 to 2860 patients, with the largest cohort being patient reported rather than physician reported).[18–40]

Regarding AEs of special interest, they were infrequent and their proportion tended to be smaller in the I-RMD group compared with the NI-RMD group and in line with rates reported in trials in the general population. There was significant diversity in terms of AEs of special interest observed both in I-RMD and NI-RMD cases, particularly in I-RMD cases, reflecting the higher number of cases in this subgroup of patients; however, no salient differences between the I-RMD and NI-RMD groups were found, and no clustering of AEs of special interest was observed.

While the primary aim of our study was to collect safety data among I-RMD patients receiving vaccines against SARS-CoV-2, we also collected data regarding breakthrough infections and found that these occurred very infrequently, particularly in fully vaccinated patients (0.7% and 1.1% of cases in the I-RMD and NI-RMD group, respectively). A more detailed report describing cases of breakthrough infections in patients with I-RMDs from the EULAR COVAX and COVID-19 registries, including details about vaccines administered, exposure to anti-rheumatic medications and outcome of breakthrough infections, has previously been published.[40]

We found that temporary discontinuation of antirheumatic medications was infrequent. This attitude towards antirheumatic medications might reflect the fact that this is largely a European registry. Contrary to the American College of Rheumatology, who recommended holding methotrexate, JAK inhibitors, abatacept, mycophenolate mofetil and rituximab in certain patients with controlled disease,[12] EULAR did not advise temporarily stopping or adjusting the timing of any of these medications (with the exception of rituximab) relative to when the vaccine against SARS-CoV-2 is administered.[11] Future studies are needed to determine if changes in certain antirheumatic medication

Table 6 Adverse events of special interest possibly/probably related to COVID-19 vaccination among patient with inflammatory RMDs

AE type	Seriousness of AE	Outcome of AE	COVID-19 vaccine	RMD	RMD medication*	Medication held or reduced
Cardiovascular – arterial hypertension	Non-serious	Recovered/resolved without sequelae	Moderna	axSpA	TNFi	No
	Non-serious	Recovered/resolved without sequelae	AZ	RA	HCQ+GC	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	RA	MTX+GC	No
	Serious (important medical event)	Recovered/resolved without sequelae	Pfizer	pSpA	GC	No
Cardiac – arrhythmia	Non-serious	Recovered/resolved without sequelae	Pfizer	SLE	HCQ+AZA+GC	No
	Non-serious	UNK	Pfizer	RA	GC	Yes
	Non-serious	Ongoing/continuing	Pfizer	EDS	None	NA
Cardiac – coronary artery disease	Serious (life threatening)	Recovered/resolved without sequelae	Pfizer	RA	ABA+MTX+GC	No
	Serious (life threatening)	Recovered/resolved with sequelae	Pfizer	RA	RTX+LEF+GC	No
Cardiac – myocarditis and pericarditis	Non-serious	Recovered/resolved without sequelae	Pfizer	SLE	None	NA
Dermatological – eczema, nodes and plaques	Non-serious	Recovered/resolved without sequelae	Pfizer	RA	ABA	No
	Non-serious	Recovered/resolved without sequelae	Moderna	SjS	HCQ+GC	Yes
	Non-serious	Ongoing/continuing	AZ	PsA	Apremilast	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	pSpA	TNFi	No
Dermatological – pruritus, injection site reaction, redness and burning	Non-serious	Recovered/resolved without sequelae	Pfizer	pSpA	LEF+GC	No
	Non-serious	Ongoing/continuing	Pfizer	RA	MTX+GC	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	PMR	HCQ+GC	No
Gastrointestinal – liver injury	Non-serious	Recovered/resolved without sequelae	Pfizer	GCA	MTX	Yes
	Non-serious	Ongoing/continuing	Pfizer	RP	AZA+GC	No
	Serious (life threatening)	Recovered/resolved with sequelae	Pfizer	SLE	HCQ+GC	No
General conditions – hot flush, anxiety, lowered body temperature, loss and lack of appetite and night sweats	Non-serious	Recovered/resolved without sequelae	Pfizer	SSc	MMF+GC	No
	Non-serious	Recovered/resolved without sequelae	AZ	axSpA	TNFi	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	RA	IL-6	UNK
	Non-serious	Recovered/resolved without sequelae	Pfizer	PsA	IL-17	Yes
	Non-serious	Recovered/resolved without sequelae	Pfizer	GCA	None	NA
	Non-serious	Recovered/resolved without sequelae	AZ	RA	ABA+MTX	No
	Non-serious	Recovered/resolved without sequelae	Moderna	RA	None	NA
	Non-serious	Ongoing/continuing	AZ	axSpA	TNFi	No
Haematological – peripheral deep vein thrombosis	Non-serious	Ongoing/continuing	Pfizer	axSpA	IL-17	No
	Serious (hospitalisation)	Ongoing/continuing	Pfizer	AAV	AZA+GC	No
Haematological – haemorrhagic disease	Non-serious	Recovered/resolved without sequelae	AZ	RA	HCQ+GC	No
Haematological – stroke	Serious (Hospitalisation)	Recovered/resolved with sequelae	Pfizer	PMR	IL-6+GC	No
Haematological – thrombocytopenia	Non-serious	Recovered/resolved without sequelae	AZ	mCTD	None	NA
Immunological – anaphylaxis	Non-serious	Recovered/resolved without sequelae	Pfizer	RA	MTX	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	SSc	Sildenafil	No
	Non-serious	Recovered/resolved without sequelae	AZ	Other vasculitis	GC	Yes
Immunological – arthritis	Non-serious	Recovered/resolved without sequelae	AZ	SjS	HCQ	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	PMR	HCQ+GC	No
	Non-serious	UNK	AZ	GCA	IL-6+GC	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	SLE	HCQ+Belimumab	No
Immunological – skin or mucosal	Non-serious	Recovered/resolved without sequelae	Pfizer	SLE	AZA+GC	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	axSpA	SSZ+GC+NSAIDs	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	Myositis	HCQ+MTX+GC	No
	Non-serious	Ongoing/continuing	Moderna	SjS	None	NA
	Serious (hospitalisation)	Recovered/resolved without sequelae	Pfizer	PsA	None	NA
	Serious (important medical event)	Recovered/resolved without sequelae	Pfizer	RA	ABA+HCQ	No
	Serious (important medical event)	Recovered/resolved without sequelae	AZ	u-CTD	HCQ	No
	Serious (important medical event)	Ongoing/continuing	Pfizer	PsA	TNFi	No
Immunological – vasculitides	Non-serious	Recovered/resolved without sequelae	Pfizer	PsA	IL-17	Yes

Continued

Table 6 Continued

AE type	Seriousness of AE	Outcome of AE	COVID-19 vaccine	RMD	RMD medication*	Medication held or reduced
Lymphadenopathy	Non-serious	Recovered/resolved without sequelae	Pfizer	PsA	IL-17+MTX	No
	Non-serious	Recovered/resolved without sequelae	Moderna	mCTD	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	Other vasculitis	None	NA
	Non-serious	Ongoing/continuing	Pfizer	RA	MTX	No
Malaise, fatigue and insomnia	Non-serious	Recovered/resolved without sequelae	AZ	PMR	MTX+GC	No
	Non-serious	Recovered/resolved with sequelae	AZ	Monogenic AIS	Colchicine	No
	Serious (important medical event)	Ongoing/continuing	Pfizer	SSc	MMF	Yes
	UNK	UNK	Pfizer	PsA	None	NA
	UNK	UNK	Pfizer	PsA	TNFi+NSAIDs	No
Neurological – anosmia and ageusia	Non-serious	Recovered/resolved without sequelae	Pfizer	SjS	MTX	No
Neurological – drowsiness, vertigo, dizziness, nausea, tinnitus, migraine, hallucination and hemiparesis	Non-serious	Ongoing/continuing	Pfizer	Sarcoidosis	HCQ	No
	Non-serious	Recovered/resolved without sequelae	Unknown	Other IA	HCQ+GC	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	pSpA	Apremilast	No
	Non-serious	Recovered/resolved without sequelae	AZ	pSpA	IL-17	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	PsA	IL-17	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	axSpA	IL-17	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	SjS	MTX	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	PsA	MTX	No
	Non-serious	Recovered/resolved without sequelae	Moderna	RA	SSZ	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	axSpA	TNFi	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	Non-systemic JIA	TNFi	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	axSpA	TNFi	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	PsA	TNFi	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	axSpA	TNFi	Yes
	Non-serious	Recovered/resolved without sequelae	Moderna	pSpA	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	PsA	IL-12/23	No
	Non-serious	Ongoing/continuing	AZ	PsA	None	NA
	Non-serious	Recovered/resolved without sequelae	AZ	RA	SSZ	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	RA	MTX	Yes
	Non-serious	Recovered/resolved without sequelae	Pfizer	SSc	Unknown	NA
	Serious (important medical event)	Ongoing/continuing	Pfizer	SSc	HCQ+MMF	No (HCQ), UNK (MMF)
Other possible cardiac symptoms – ankle oedema, dyspnoea and dry cough	Non-serious	Recovered/resolved without sequelae	Pfizer	PsA	IL-17	No
	Non-serious	Recovered/resolved without sequelae	Moderna	RA	IL-6	No
	Non-serious	Recovered/resolved with sequelae	Pfizer	AAV	Benralizumab	No
	Non-serious	Ongoing/continuing	Moderna	RA	RTX+GC	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	RA	JAKi +GC	Yes (GC), No (JAKi)
	Serious (important medical event)	Recovered/resolved without sequelae	Pfizer	SjS	HCQ	No
	UNK	UNK	Pfizer	RA	TNFi	No
Pain/pain syndromes	Non-serious	UNK	Pfizer	SjS	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	PMR	GC	UNK
Tendons and joints – tendinopathy, frozen shoulder and carpal tunnel syndrome	Non-serious	UNK	Pfizer	RA	ABA+MTX	No
	Non-serious	Ongoing/continuing	Moderna	uCTD	None	NA
	Non-serious	Ongoing/continuing	Pfizer	PMR	GC	No
	UNK	UNK	AZ	RA	MTX	No

Continued

Table 6 Continued

AE type	Seriousness of AE	Outcome of AE	COVID-19 vaccine	RMD	RMD medication*	Medication held or reduced
Viral infections – herpes, herpes zoster and shingles	Non-serious	Ongoing/continuing	Pfizer	SjS	HCQ	No
	Non-serious	Ongoing/continuing	Moderna	RA	JAKi	No
	Non-serious	Ongoing/continuing	Pfizer	RA	JAKi	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	axSpA	TNFi	No
	Non-serious	Ongoing/continuing	Pfizer	PMR	GC	No
	Non-serious	Recovered/resolved with sequelae	AZ	RA	TNFi	No
	Serious (hospitalisation)	Recovered/resolved without sequelae	Pfizer	RA	JAKi	Yes
	Serious (hospitalisation)	UNK	Pfizer	RA	MTX+GC	Yes (MTX), No (GC)
	Serious (important medical event)	Recovered/resolved without sequelae	Pfizer	RA	MTX+GC	No
Viral infections – influenza, flu-like episodes, rhinitis, cough and cold	Non-serious	Recovered/resolved without sequelae	Pfizer+AZ	GCA	IL-6	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	Myositis	MTX	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	RA	MTX	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	RA	RTX+MTX	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	axSpA	TNFi	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	CRMO	TNFi+MTX	No
	Non-serious	Recovered/resolved without sequelae	Pfizer	SjS	None	NA
Other – GORD	Non-serious	Recovered/resolved without sequelae	Pfizer	RA	LEF	No
Other – neck swelling	Serious (hospitalisation)	Recovered/resolved without sequelae	Pfizer	RA	LEF	No
Other (UNK)	Non-serious	UNK	Pfizer	RA	JAKi+LEF+GC	No

*Immunosuppressive or immunomodulatory medication.

AAV, ANCA-associated vasculitis; ABA, abatacept; AE, adverse event; AIS, autoinflammatory syndrome; axSpA, axial spondyloarthritis; AZ, Oxford/AstraZeneca; AZA, azathioprine; CRMO, chronic recurrent multifocal osteomyelitis; EDS, Ehlers-Danlos syndrome; GC, glucocorticoids; GCA, giant cell arteritis; GORD, gastro-oesophageal reflux disease; HCQ, hydroxychloroquine; IA, inflammatory arthritis; IL-6, interleukin-6; IL-17, interleukin-17; IL-12/23, interleukin-12/23; JAKi, Janus kinase inhibitors; JIA, juvenile idiopathic arthritis; LEF, leflunomide; mCTD, mixed connective tissue disease; MMF, mycophenolate mofetil/mycophenolic acid; MTX, methotrexate; NSAIDs, nonsteroidal anti-inflammatory drugs; PMR, polymyalgia rheumatica; PsA, psoriatic arthritis; pSpA, peripheral spondyloarthritis; RA, rheumatoid arthritis; RMD, rheumatic and musculoskeletal disease; RP, relapsing polychondritis; RTX, rituximab; SjS, Sjogren's syndrome; SLE, systemic lupus erythematosus; SpA, spondyloarthritis; SSc, systemic sclerosis; SSZ, sulfasalazine; TNFi, tumour necrosis factor; uCTD, undifferentiated connective tissue disease; UNK, unknown/missing.

regimens might increase the effectiveness of vaccines against SARS-CoV-2 while balancing the risk of disease flare (and the need for additional treatment of the flare, such as GCs).

Strengths of this study include the rapid dissemination via European networks (EULAR, ERN ReCONNET and ERN RITA) that resulted in a large number of cases reported by rheumatologists, internists or associated healthcare professionals over a short period of time. However, our study has important limitations. The COVAX registry relies on voluntary case submission, leading to possible selection bias in the data, and concerns regarding the generalisability of the results. However, this could in principle have led to over-reporting of flares and AEs; therefore the low rate of flares/AEs consistent with other publications is reassuring. Moreover, the underlying risk of flare also differs among RMDs, which may influence the overall flare rate and differences between conditions. Furthermore, dissemination was more effectively achieved in certain European countries (eg, France, Italy and Portugal), and reporting was also influenced by differences in vaccine availability and access across European countries, which has resulted in a significantly higher proportion of cases vaccinated with the Pfizer vaccine, limiting comparisons between vaccines. Time between vaccination and case reporting is also variable and sometimes relatively short, limiting data interpretation and not allowing us to draw any conclusions regarding the long-term safety profile of vaccines against SARS-CoV-2. Moreover, a control group of patients with I-RMDs is not available, and the sample size of patients with NI-RMDs is substantially smaller. For some signs/symptoms, it can be difficult to determine if the event should be considered an I-RMD flare or simply a transient side effect of the vaccine (eg, polyarthralgia); in our study, this decision was left to the reporting physician, which can be considered a study limitation. Similarly, systemic flares were also based on the report of the physician without collection of more detailed evidence of the flare (eg, results of investigations). Finally, the information regarding SARS-CoV-2 infection after vaccination is based on the report of physicians/healthcare providers, and no information is provided concerning the presence or the titre of postvaccine antibodies. Importantly, no causal conclusions regarding vaccination and the development of flares/AEs can firmly be drawn from this dataset.

In conclusion, our findings should provide reassurance to rheumatologists, other health professionals and vaccine recipients and and promote confidence in SARS-CoV-2 vaccine safety in people with I-RMDs. The rate of severe flares was very low (0.6%). Likewise, the rate of SAEs in I-RMDs was 0.4%, comparable and even lower than in patients with NI-RMDs (1.1%), suggesting that the tolerance to the vaccine was not different between the groups. Interestingly, in clinical trials of mRNA, inactivated and non-replicating vector vaccines against SARS-CoV-2 in the general population, the pooled rates of SAEs were very similar to our study, ranging from 0.4% to 0.6% in the vaccine group, and from 0.5% to 0.6% in the control group,[41] suggesting that these SAEs are not necessarily causally related to the vaccine and might be coincidental observations. However, although the mean time between first vaccine dose and case reporting of 66 days in our report is not very different from the follow-up period in some of the vaccination trials, this is an indirect comparison that should be interpreted with caution, because

Table 7 Adverse events of special interest possibly/probably related to COVID-19 vaccination among patient with non-inflammatory RMDs

AE type	Severity of AE	Outcome of AE	COVID-19 vaccine	RMD	RMD medication	Medication held or reduced
Cardiovascular –arterial hypertension	Serious (important medical event)	Recovered/resolved with sequelae	Pfizer	OA	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	Other mechanical RMD	None	NA
Dermatological – pruritus, injection site reaction, redness and burning	Non-serious	Recovered/resolved without sequelae	Moderna	OA	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	OA	None	NA
Gastrointestinal – liver injury	Non-serious	Ongoing/continuing	Pfizer	OA	None	NA
	Non-serious	UNK	Moderna	Osteoporosis	None	No
	Serious (important medical event)	Ongoing/continuing	AZ	Fibromyalgia	None	
General conditions – hot flush, anxiety, lowered body temperature, loss and lack of appetite and night sweats	Non-serious	Recovered/resolved without sequelae	AZ	OA	None	NA
Immunological – arthritis	Non-serious	Recovered/resolved without sequelae	Pfizer	Other mechanical RMD	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	Osteoporosis	None	NA
	Serious (important medical event)	Ongoing/continuing	AZ	OA	None	NA
	Serious (important medical event)	Recovered/resolved with sequelae	Pfizer	OA	None	NA
	Non-serious	Ongoing/continuing	Moderna	Other mechanical RMD	None	NA
Immunological – skin and mucosal	Non-serious	Recovered/resolved without sequelae	AZ	OA	None	NA
Immunological – vasculitides	Serious (hospitalisation)	Recovered/resolved without sequelae	Moderna	OA	None	NA
	Serious (important medical event)	Ongoing/continuing	Pfizer	OA	None	NA
Malaise, fatigue and insomnia	Non-serious	Recovered/resolved without sequelae	Pfizer	OA	Colchicine	No
Lymphadenopathy	Non-serious	Recovered/resolved without sequelae	Pfizer	OA	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	Osteoporosis	None	NA
Neurological – drowsiness, vertigo, dizziness, nausea, tinnitus, migraine, hallucination and hemiparesis	Non-serious	Recovered/resolved without sequelae	Moderna	Osteoporosis	None	NA
	Non-serious	Recovered/resolved without sequelae	Moderna	OA	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	Other mechanical RMD	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	Gout	None	NA
	Non-serious	Recovered/resolved without sequelae	AZ	Other mechanical RMD	None	NA
	Serious (important medical event)	Recovered/resolved without sequelae	Pfizer	Fibromyalgia	None	NA
	Serious (important medical event)	Recovered/resolved without sequelae	Pfizer	Fibromyalgia	None	NA
Other possible cardiac symptoms – ankle oedema, dyspnoea and dry cough	Non-serious	Recovered/resolved without sequelae	Pfizer	OA	HCQ	No
	Serious (hospitalisation)	Recovered/resolved without sequelae	Pfizer	Osteoporosis	None	NA
Pain/pain syndromes	Non-serious	Recovered/resolved without sequelae	AZ	OA	None	NA
	Non-serious	Recovered/resolved without sequelae	Pfizer	OA	None	NA
Tendons and joints – tendinopathy, frozen shoulder and carpal tunnel syndrome	Non-serious	Recovered/resolved without sequelae	AZ	Osteoporosis	None	NA
	Serious (Important medical event)	Ongoing/continuing	AZ	Chronic mechanical back pain	None	NA
Viral infections – herpes, herpes zoster and shingles	Non-serious	Recovered/resolved without sequelae	Pfizer	OA	None	NA
Viral infections – influenza, flu-like episodes, rhinitis, cough and cold	Non-serious	Recovered/resolved without sequelae	AZ	Osteoporosis	None	NA
Other – epistaxis	Non-serious	Recovered/resolved without sequelae	AZ	OA	None	NA
Other (UNK)	Non-serious	Recovered/resolved without sequelae	Moderna	Fibromyalgia	None	NA
Other (UNK)	Non-serious	Recovered/resolved without sequelae	Pfizer	Fibromyalgia	None	NA

*Immunosuppressive or immunomodulatory medication.

AZ, Oxford/AstraZeneca; HCQ, hydroxychloroquine; OA, osteoarthritis; RMD, rheumatic and musculoskeletal disease; UNK, unknown/missing.

the follow-up period in our study was allowed to vary, and there are also important differences between follow-up periods among vaccination trials (that typically do not go beyond 6 months). Future studies should address the effectiveness and safety of vaccines against SARS-CoV-2 in patients with I-RMDs and/or patients taking immunosuppressive/immunomodulatory drugs, both in controlled and general surveillance settings.

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Acknowledgements We wish to thank all healthcare providers who entered data into the registry.

Collaborators In addition to the authors listed above, the following colleagues also contributed to the EULAR COVAX Registry by submitting at least 10 cases each: Viviane Queyrel, Julien Henry, Raphaele Seror, Eric Toussirot, Emoke Stenova, Azeddine Dellal, Vanda Mlynarikova, Romain Forestier, François Lamer, Hélène Maillard, Amélie Leurs, Thierry Zenone, Daniel Wendling, Amélie Florent, Theodoros Dimitroulas, Simona Rednic, Bernard Combe, Yves Piette, Jozef Odnoga, Giovanna Cuomo, Ioannis Raftakis, Jean-Camille Meric, Sylvain Lanot, Marion Mirabel, Mikhail Protopopov, Katalin Törõcsik, John Brockbank, Marion Jacob, Pascal Coquerelle, Christophe Richez, Elisabeth Gervais, Séverine Verlinden, Antoine Froissart, Fabienne Roux, Marion Couderc, Renaud Desbarbieux, Alojzija Hocevar, Pierre-Yves Jeandel, Sophie Rivière, Luciana Popa, Fabienne Coury, Inita Bulina, Jean-Jacques Dubost, Lionel Spielmann, Marie-Hélène Guyot, Nicolas Deseyne, Isabelle Amigues, Dagmar Mičeková, Loraine Gauzere, Gaëlle Viadere, Natalia de la Torre-Rubio, Victor Strotz.

Contributors PMM is responsible for the overall content as the guarantor, and accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish. PMM and SL-T had access to the study data, developed the figures and tables, wrote the first draft of the manuscript and vouch for the data and analyses. PMM, KLH, LG, AR, BR, CD, EH, EV, ES, GRB, GKY, JAG-P, JZ, LF, LK-F, MCU, MM, MCo, MS, NR, OB, PD, RC, TG and XM contributed to data collection and interpretation of the data. PMM, SL-T, AS, EFM, KLH, LG, LC, EH, MM, GRB, JWJB, IBM and XM contributed to study design and questionnaire development. PMM directed the work and had final responsibility for the decision to submit for publication. All authors contributed intellectual content during the drafting and revision of the work and approved the final version to be published.

Competing interests PMM has received consulting/speaker's fees from Abbvie, BMS, Celgene, Eli Lilly, Galapagos, Janssen, MSD, Novartis, Orphazyme, Pfizer, Roche and UCB, all unrelated to this manuscript, and is supported by the National Institute for Health Research (NIHR), University College London Hospitals (UCLH), Biomedical Research Centre. SL-T does not report conflicts of interest. AS has received personal fees from lectures for AbbVie, MSD, Lilly, Roche, BMS and Pfizer. EFM has received personal consultant fees from Boehringer Ingelheim Portugal, Lda; LPCDR received support for specific activities: grants from Abbvie, Novartis, Lilly Portugal, Amgen Biofarmacêutica, Grünenthal S.A., MSD, Medac and from A. Menarini Portugal - Farmacêutica, S.A.; grants and non-financial support from Pfizer, and non-financial support from Grünenthal GmbH, outside the submitted work. KLH has received non-personal speaker's fees from Abbvie and grant income from BMS, UCB and Pfizer, all unrelated to this manuscript, and is supported by the NIHR Manchester Biomedical Research Centre. LG has received personal consultant fees from AbbVie, Amgen, BMS, Galapagos, Gilead, Janssen, Lilly, Novartis, Pfizer, Samsung Bioepis, Sanofi-Aventis and UCB, and grants from Amgen, Galapagos, Lilly, Pfizer, Sandoz and Sanofi, all unrelated to this manuscript. LC has not received any fees or personal grants from any laboratory, but her institute works by contract for laboratories among other institutions, such as Abbvie Spain, Eisai, Gebro Pharma, Merck Sharp & Dohme España, S.A., Novartis Farmaceutica, Pfizer, Roche Farma, Sanofi Aventis, Astellas Pharma, Actelion Pharmaceuticals España, Grünenthal GmbH and UCB Pharma. AR has received research grants and consultant fees from Amgen and Pfizer, all unrelated to this manuscript. BR does not report conflicts of interest. CD does not report conflicts of interest. EH does not report conflicts of interest. EV reports personal consultant fees from Theramex, unrelated to this manuscript. ES does not report conflicts of interest. G-RRB reports personal consultant fees from AbbVie, Amgen, BMS, Galapagos, Gilead, Janssen, Lilly, Novartis, Pfizer, Sanofi-Aventis, UCB, all unrelated to this manuscript. GKY does not report conflicts of interest. JAG-P reports speaker fees from Abbvie, Astra-Zeneca, BMS, Galapagos, GSK, Janssen, Lilly, Novartis, Pfizer, Sanofi-Aventis and Roche, all unrelated to this manuscript. JZ reports speaker fees from Abbvie, Novartis, Janssen/Johnson & Johnson, all unrelated to this manuscript. LK-F does not report conflicts of interest. LT does not report conflicts of interest. MCu does not report conflicts of interest. MM does not report conflicts of interest. MCo does not report conflicts of interest. MS does not report conflicts of interest. NR does not report conflicts of interest. OB does not report conflicts of interest. PD does not report conflicts of interest. RC reports speaker's fees from Janssen, Roche, Sanofi, Abbvie, all unrelated to this work. TG does not report conflicts of interest. JWJB does not report conflicts of interest. IM does not report conflicts of interest. XM reports personal consultant fees from BMS, Galapagos, Gilead, Janssen, Novartis, Pfizer, Sanofi-Aventis, UCB and grant from Ose, all unrelated to this manuscript.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants but given that the Registry collects anonymous non-interventional data, the UK Health Research Authority (HRA) does not class the Registry as a research study (in line with the HRA decision tool) and patient consent is not required.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request. Applications to access the data should be made to the European Alliance of Associations for Rheumatology (EULAR).

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CLINICAL SCIENCE

Distinct impact of DMARD combination and monotherapy in immunogenicity of an inactivated SARS-CoV-2 vaccine in rheumatoid arthritis

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221735).

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Received 23 October 2021
Accepted 20 January 2022
Published Online First 8 February 2022

To cite: Medeiros-Ribeiro AC, Bonfiglioli KR, Domiciano DS, *et al*. *Ann Rheum Dis* 2022;**81**:710–719.

ABSTRACT

Objectives To evaluate the distinct impact of disease modifying antirheumatic drugs (DMARD) combination and monotherapy in immune response to an inactivated SARS-CoV-2 vaccine in patients with rheumatoid arthritis (RA).

Methods This phase 4 prospective study analysed seroconversion (SC) of anti-SARS-CoV-2 immunoglobulin G (IgG) and neutralising antibodies (NAb) induced by the inactivated vaccine (CoronaVac) in patients with RA in comparison to controls (CG). Disease activity and treatment were also assessed. Only participants with baseline negative IgG/NAb were included.

Results Patients with RA (N=260) and CG (N=104) had comparable median ages (59 years (50–65 years) vs 58 years (49.8–64 years), p=0.483). Patients with RA had moderate but lower SC (61.8% vs 94.2%, p<0.001) and NAb positivity (45% vs 78.6%, p<0.001) in comparison to CG after full vaccination. Baseline disease activity did not influence immunogenicity (p>0.05). After multivariate analyses, factors independently related to reduced SC were: older age (OR=0.79 (0.70–0.89) for each 5-year interval, p<0.001), methotrexate (OR=0.54 (0.29–0.98), p=0.044), abatacept (OR=0.37 (0.19–0.73), p=0.004) and number of DMARD (OR=0.55 (0.33–0.90), p=0.018). Regarding NAb, age (OR=0.87 (0.78–0.96) for each 5-year interval, p=0.007) and prednisone >7.5 mg/day (OR=0.38 (0.19–0.74), p=0.004) were negatively related to the presence of NAb. Further comparison of SC/NAb positivity among RA treatment subgroups and CG revealed that methotrexate/tofacitinib/abatacept/tocilizumab use, in monotherapy or in combination, resulted in lower responses (p<0.05), while tumour necrosis factor inhibitor and other conventional synthetic DMARD interfered solely when combined with other therapies.

Conclusions Patients with RA under DMARD have a moderate immunogenicity to CoronaVac. We identified that nearly all DMARD combinations have a deleterious effect in immunogenicity, whereas a more restricted number of drugs (methotrexate/tofacitinib/abatacept/tocilizumab) also hampered this response as monotherapy. These findings reinforce the need of a broader approach, not limited to specific drugs, to improve vaccine response for this population.

Trial registration details NCT04754698.

Key messages

What is already known about this subject?

- There is increasing evidence of the effect of rituximab, methotrexate, abatacept and corticosteroids on COVID-19 vaccine immunogenicity in overall autoimmune rheumatic diseases cohorts.

What does this study add?

- This is the first study to focus, exclusively in rheumatoid arthritis population, the impact of the different therapies and its combinations on the immunogenicity induced by the inactivated Sinovac-CoronaVac vaccine.
- We provided novel evidence of an overall reduced anti-SARS-CoV2 S1/S2 immunoglobulin G and neutralising antibodies responses for nearly all drugs used in different combinations and, for methotrexate, tofacitinib, abatacept and tocilizumab also in monotherapy.
- A cut-off point of 7.5 mg/day of prednisone was identified as deleterious for immunogenicity.
- The humoral response was not influenced by disease activity status at the time of vaccination.

How might this impact on clinical practice or future developments?

- Our findings show an overall deleterious effect of nearly all DMARD, either in monotherapy or in combination, reinforcing the need of a broader strategy, not limited to individual drugs, to improve vaccine response for this population which might include drug temporary discontinuation or booster doses.

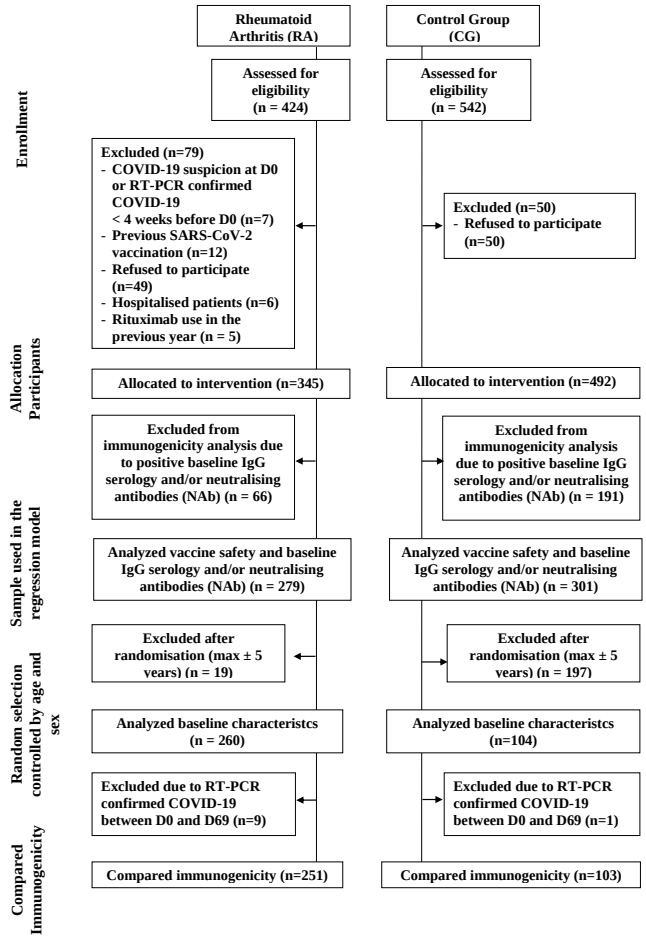

Figure 1 Modified CONSORT flow diagram. CONSORT, Consolidated Standards of Reporting Trials; IgG, immunoglobulin G; RT-PCR, reverse transcriptase PCR.

INTRODUCTION

The COVID-19 has proven to be a major threat to individuals worldwide.[1] Patients with rheumatoid arthritis (RA), one of the most common autoimmune rheumatic diseases (ARD),[2] are at a high risk of severe COVID-19 outcomes,[3–5] especially those with active disease, under immunosuppressive therapies, or with comorbidities.[5 6] In this context, mass vaccination is the main measure to control the pandemic.

The Sinovac-CoronaVac inactivated vaccine[7 8] has been one of the most widely used in the world, with an efficacy of 83.5% in reducing infections, according to the phase 3 trial.[9] Its effectiveness was demonstrated in Chile (10.2 million people studied), with a reduction of 87.5% in hospitalisation, 90.3% in intensive care unit admission and 86.3% for death.[10] Recently, an appropriate, but reduced, immune response with Sinovac-CoronaVac vaccine was described in a large population of overall patients with ARD.[11] Similarly, previous reports assessed safety and immunogenicity of other anti-SARS-CoV-2 vaccines, such as messenger RNA (mRNA) and viral vector vaccines, and demonstrated safety and lower, but adequate, immunogenicity, also in overall ARD populations.[12–22] Regardless of vaccine type, glucocorticoids,[11 14 21 22] rituximab,[11 13–16 20 21] methotrexate (MTX),[11 14 17 18 22] mycophenolate mofetil[11 13–15 21] and abatacept[11 14 15 21] have deleterious effects in immunogenicity.

None of these previous reports, however, focused specifically on patients with RA and the several combinations of distinct disease modifying antirheumatic drugs (DMARD) that they use. The only study to focus on such population was underpowered to show differences among treatments.[23] The small sample size of some drugs subgroups in monotherapy or combination in these studies and the scarce data on conventional synthetic DMARD (csDMARD) other than MTX precluded a definitive conclusion regarding the effect of these drugs on COVID-19 vaccine immunogenicity in the RA population.[11–23] The cut-off dose in which prednisone hampers vaccine-induced antibody response in patients with RA also needs to be established.

Therefore, the aim of the present study was to assess the impact of distinct DMARD, used in combination or in monotherapy, in the immunogenicity to Sinovac-CoronaVac vaccine in patients with RA, compared with age-balanced and sex-balanced controls. We also evaluated the safety and the influence of disease activity on immune response.

METHODS

Study design and participants

This was a subanalysis of a prospective, single centre, controlled phase 4 study (CoronavRheum, clinicaltrials.gov) that evaluated the immunogenicity/safety of Sinovac-CoronaVac vaccine in patients with ARD,[11] regularly followed at the Outpatient Clinics of Rheumatology Division (Hospital das Clinicas HCFMUSP, Faculdade de Medicina, Universidade de Sao Paulo, Brazil). For the original study,[11] adult (age: ≥18 years old) patients with ARD were invited to participate after their electronic chart review of the last 3 months (recruitment up to 3 weeks before enrolment). Subsequently, a control group (CG) was invited, including subjects without ARD among the maintenance/administrative hospital workers and relatives, with comparable sex and age to the overall ARD sample. For the current analysis, all patients with RA,[24] were included and, subsequently, a CG was randomly selected using an Excel programme (5 patients with RA to 2 controls), with comparable sex frequencies and ages (≤5-year difference).

Exclusion criteria were acute febrile illness/symptoms of COVID-19 at vaccination, history of anaphylaxis to vaccine components, demyelinating disease, decompensated heart failure (class III/IV), blood transfusion ≤6 months, inactivated virus vaccine ≤14 days, live virus vaccine ≤4 weeks, denial to participate, hospitalisation, previous vaccination with SARS-CoV-2 vaccine, rituximab therapy ≤12 months and prevaccination positive COVID-19 serology (anti-S1/S2 immunoglobulin G (IgG) and/or neutralising antibody (NAb)).

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

Vaccination protocol

The vaccination protocol included two doses of ready-to-use syringes with Sinovac-CoronaVac vaccine (Sinovac Life Sciences, Beijing, China, batch # 20200412),[7 8] containing 3 µg (0.5 mL) of β-propiolactone inactivated SARS-CoV-2 with aluminium hydroxide adjuvant, administered in the deltoid muscle. First dose was on 9–10 February 2021 (D0) and second dose on 9–10 March 2021 (D28), for both patients with RA and CG. Participants with suspicious COVID-19 were instructed to undergo a reverse transcriptase PCR (RT-PCR) test for SARS-CoV-2 in naso/oropharyngeal swabs, available at the hospital. Those with RT-PCR confirmed COVID-19 between doses were excluded from the immunogenicity analyses and received the second dose 4 weeks after the first symptoms.

Table 1 Baseline characteristics of patients with RA and CG, all with negative anti-S1/S2 IgG serology and NAb at baseline

	Patients with RA	CG	
	(n=260)	(n=104)	P value
Demographics			
Current age, years	59 (50–65)	58 (49.8–64)	0.483
Female sex	235 (90.4)	94 (90.4)	>0.999
Caucasian race	136 (52.3)	46 (44.2)	0.164
Comorbidities			
Arterial hypertension	136 (52.3)	39 (37.5)	0.011
Diabetes mellitus	46 (17.7)	21 (20.2)	0.578
Dyslipidaemia	93 (35.8)	10 (9.6)	<0.001
Obesity (BMI: ≥30.0 kg/m^2)	91 (35.1)	32 (31.1)	0.441
Chronic cardiomyopathy	12 (4.6)	2 (1.9)	0.366
Chronic renal disease	6 (2.3)	0 (0)	0.189
Current smoking	30 (11.5)	9 (8.7)	0.422
COPD	10 (3.8)	0 (0)	0.068
Asthma	12 (4.6)	5 (4.8)	0.937
Interstitial lung disease	22 (8.5)	0 (0)	0.001
Pulmonary hypertension	2 (0.8)	0 (0)	>0.999
Haemoglobinopathy	1 (0.4)	0 (0)	>0.999
Chronic hepatic disease	13 (5)	0 (0)	0.024
Current cancer	3 (1.2)	0 (0)	0.561
Previous stroke	7 (2.7)	0 (0)	0.199
Current tuberculosis	1 (0.4)	0 (0)	>0.999
HIV	0 (0)	0 (0)	>0.999
Disease parameters			
Duration of disease, years	19 (11–26)	–	–
RF positivity (n=257)	200 (77.8)	–	–
Anti-CCP positivity (n=152)	106 (69.7)	–	–
CDAI (n=256)	8.5 (4–16)	–	–
SDAI (n=246)	9.5 (5.2–17.6)	–	–
DAS28-CRP (n=207)	2.9 (2.2–4)	–	–
Current therapy			
Prednisone	157 (60.4)	–	–
Prednisone dose, mg/day	5 (5–10)	–	–
Number of DMARD	2 (1–2)	–	–
No current DMARD	7 (2.7)	–	–
DMARD monotherapy	66 (25.4)	–	–
Combination of DMARD	187 (71.9)	–	–
Conventional Synthetic DMARD			
MTX	117 (45.0)	–	–
MTX dose, mg/week	20 (15.6–25)	–	–
Leflunomide	91 (35)	–	–
Hydroxychloroquine	35 (13.5)	–	–
Sulfasalazine	30 (11.5)	–	–
Tofacitinib	19 (7.3)	–	–
Biologic DMARD			
TNFi	58 (22.3)	–	–
Abatacept	54 (20.8)	–	–
Tocilizumab	47 (18.1)	–	–

Results are expressed in median (IQRs) and n (%). Continuous data were compared using the Mann-Whitney U test, and categorical variables with the χ^2 or Fisher's exact tests, as appropriate, as two-sided analyses.
anti-CCP, anti-cyclic citrullinated peptides; BMI, body mass index; CDAI, Clinical Disease Activity Index; CG, control group; COPD, chronic obstructive pulmonary disease; DAS28-CRP, Disease Activity Score with 28 joints and C reactive protein; DMARD, disease modifying antirheumatic drugs; IgG, immunoglobulin G; MTX, methotrexate; NAb, neutralising antibodies; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; TNFi, tumour necrosis factor inhibitor.

Immunogenicity outcomes

Blood samples (20 mL) were collected immediately before each vaccine dose and 6 weeks after the last dose (D69) on 19 April 2021. Sera were stored at −70°C. The two co-primary outcomes were IgG seroconversion (SC) to anti-SARS-CoV-2 S1/S2 proteins and the presence of NAb at D69. Secondary outcomes were SC and the presence of NAb at D28, geometric mean titres (GMT) of anti-S1/S2 IgG and their factor increase in GMT (FI-GMT), and neutralising activity of NAb, also at D28 and D69.

IgG antibodies against the SARS-CoV-2 S1/S2 proteins were checked by a chemiluminescent immunoassay (Indirect ELISA, LIAISON, DiaSorin, Italy). SC was defined as positive serology (≥15.0 UA/mL).[25 26] GMT (95% CIs) were calculated, attributing the value of 1.9 UA/mL to undetectable levels (<3.8 UA/mL). FI-GMT is the ratio of the GMT after vaccination to the GMT before vaccination.

Circulating NAb were detected by the SARS-CoV-2 sVNT Kit (GenScript, Piscataway, New Jersey, USA). Positivity was defined as ≥30% inhibition of the linkage between the receptor-binding domain of the viral spike glycoprotein with the ACE2 cell surface receptor.[27] Medians (IQR) of the percentage of neutralising activity were only calculated for positive samples.

Vaccine adverse events

After each vaccine dose, participants (RA and CG) received a standardised diary to record prospectively local and systemic manifestations (online supplemental figure 1). These diaries were checked at the next evaluation. Additionally, participants were instructed to inform any moderate/severe adverse events (AE) after each vaccine dose (by telephone, smartphone instant messaging or email). AE severity was classified according to WHO definition (WHO 2021),.[28]

Medication and disease activity

Data regarding demography, disease characteristics/activity, medications and comorbidities of patients with RA were assessed by electronic chart review. Patients were not instructed to hold medications before or after vaccination, since ACR guidelines first version was uploaded on 8 February 2021, 1 day before the first vaccine dose (D0), with no time to submit changes in vaccination protocol to the ethics committee.[29] Patients were asked about their subjective perception of disease activity worsening after each vaccine dose.

Statistical analysis

The sample size calculation was based on the 24% reduction of SC after vaccination with the 2009 non-adjuvanted influenza A/H1N1 vaccine in patients with RA.[30] Expecting SC rates of 53% in patients and 77% in CG, with a 5% α error and 80% power (5:2 ratio), the minimum sample would be 110 patients with RA and 44 healthy subjects.

Categorical variables were presented as number (percentage) and compared using χ^2 or Fisher's exact tests, as appropriated. Continuous general data were presented as medians (IQRs) and compared using Mann-Whitney U test (two groups) or Kruskal-Wallis one-way analysis of variance on ranks (more than two groups). Only for patients with RA, multivariate logistic regression analyses were performed with SC or the presence of NAb at D69 as dependent variables, and variables with $p<0.2$ in each univariate analysis as independent variables. Subgroup analyses, including only patients with no DMARD or in DMARD monotherapy, were also performed with the same parameters. Comparisons of IgG titres were assessed as Napierian logarithm (ln) transformed data, using generalised estimating equations (GEE) with normal marginal distribution and gamma distribution, respectively, and identity binding function, assuming first-order autoregressive correlation matrix between moments, followed by Bonferroni's multiple comparisons to identify differences between groups (overall patients with RA and CG) and time points (D0, D28 and D69). Statistical significance was

Table 2 Data regarding anti-S1/S2 IgG (SC rates, anti-SARS-CoV-2 S1/S2 IgG titres and FI in titres, and frequency of NAb) and median percentage of neutralising activity in patients with RA and CG after the first (D28) and second (D69) doses of CoronaVac vaccine

	Before first dose			After first dose			After two doses		
	RA (n=251)	CG (n=103)	P value	RA (n=251)	CG (n=103)	P value	RA (n=251)	CG (n=103)	P value
Anti-S1/S2 IgG									
SC, n (%)	–	–	–	27 (10.8)	31 (30.1)	<0.001	155 (61.8)	97 (94.2)	<0.001
GMT	2.2 (2.1–2.3)	2.2 (2.0–2.4)	>0.999	3.7 (3.2–4.1)	9.0 (7.2–11.2)	<0.001	20.0 (16.8–23.7)	59.3 (51.0–69.0)	<0.001
FI-GMT	–	–	–	1.7 (1.5–1.9)	4.1 (3.4–5.1)	<0.001	9.2 (7.7–11.0)	27.5 (23.3–32.4)	<0.001
NAb									
Positivity, n (%)	–	–	–	35 (13.9)	33 (32.0)	<0.001	113 (45.0)	81 (78.6)	<0.001
Neutralising activity	–	–	–	40.7 (33.2–55.8)	46.8 (34.8–68.4)	0.200	55.1 (38.4–70.2)	62.5 (46.2–78.4)	0.033

SC is defined as post-vaccination titre ≥15 AU/mL by indirect ELISA, LIAISON SARS-CoV-2 S1/S2 IgG. Frequencies of SC are presented as number (%) and were compared using a two-sided χ^2 test between RA and CG at prespecified time points (D28 and D69). IgG antibody titres and FI-GMT are expressed as GMT with 95% CI.
Data regarding IgG titres were analysed in ln-transformed data using GEE with normal marginal distribution and gamma distribution, respectively, and identity binding function assuming first-order autoregressive correlation matrix between moments (D0, D28 and D69) in the comparison of the 2 groups (RA vs CG), followed by Bonferroni's multiple comparisons.
The behaviour of IgG titres was different for RA and CG groups between D28 and D69: mean titres increased at each time point for RA and CG (p< 0.001). FI-GMT values were compared using the Mann-Whitney U test for intergroup comparisons in ln-transformed data at prespecified time points (D28 and D69). All analyses were two-sided. Frequencies of subjects with positive NAb are expressed as number (%). Positivity for NAb was defined as neutralising activity ≥30% (cPass sVNT Kit). Data were compared using a two-sided χ^2 test between patients with RA and CG at prespecified time points (D28 and D69). Percentages of neutralising activity among subjects with positive NAb are expressed as median (IQR). Data were compared using a two-sided Mann-Whitney U test for comparison between patients with RA and CG, at prespecified time points (D28 and D69).
CG, control group; FI, factor increase; GEE, generalised estimating equations; GMT, geometric mean titre; IgG, immunoglobulin G; ln, logarithm; NAb, neutralising antibodies; RA, rheumatoid arthritis; SC, seroconversion.

defined as p<0.05. All statistical analyses were performed using SPSS, V.20.0 (IBM-SPSS for Windows V.20.0).

RESULTS

A total of 424 patients with RA and 542 controls were invited. After exclusion criteria, 279 seronegative patients with RA and 301 controls composed the final samples (figure 1). They were age balanced and sex balanced in a 5:2 ratio, and, finally, 260 patients with RA and 104 controls comprised the final comparison groups (figure 1, table 1). Most participants (n=339, 93.1%) were vaccinated on 9–10 February 2021, without differences between groups (91.5% vs 97.1%, p=0.07). Participants who could not attend on such days had up to 15 days for enrolment and vaccination.

Immunogenicity outcomes in patients with RA compared with CG

From the matched sample, 9 (3.5%) patients with RA and 1 (1%) control (p=0.293) were additionally excluded due to RT-PCR-confirmed COVID-19 during follow-up. Compared with controls, patients with RA had lower frequencies of SC and the presence of NAb, and lower GMT and neutralising activity at D69 (table 2, figure 2).

Assessment of factors associated with immunogenicity in patients with RA

Among the original 279 patients with RA, we analysed 266 (4 patients did not collect the last sample and 9 had COVID-19 during follow-up). Univariate and multivariate analyses pointed the following factors as negatively associated to SC: older age, number of DMARD, MTX and abatacept. Sulfasalazine was positively associated with SC only in univariate analyses (table 3). Regarding NAb, age and prednisone dose ≥7.5 mg/day

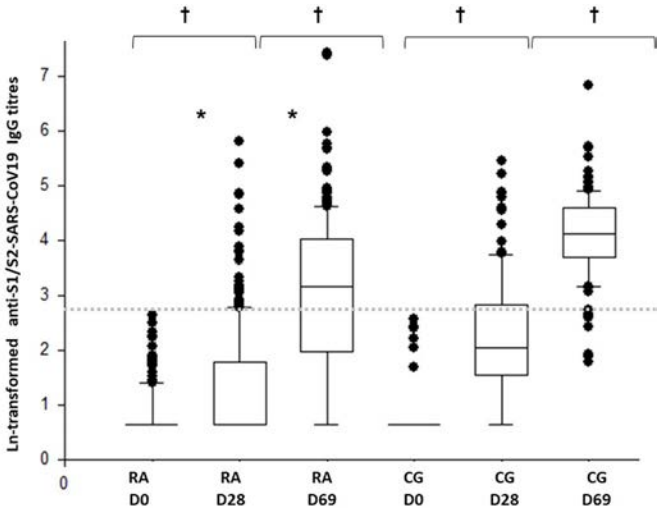

Figure 2 Box plots of ln-transformed IgG titres over time in patients with RA (n=251) and controls (CG, n=103). Data were analysed in ln-transformed data using GEE with normal marginal distribution and gamma distribution, respectively, and identity binding function assuming first-order autoregressive correlation matrix between moments (D0, D28 and D69) in the comparison of the 2 groups (RA vs CG), followed by Bonferroni's multiple comparisons. Tests were two-sided. RA and CG were comparable only at D0 (P > 0.999). The mean behaviour of the ln-transformed IgG titres was different in RA and CG groups at D28 (*p<0.001) and D69 (*p<0.001). Mean titres increased at each time point for RA and CG (†p<0.001). Dotted line denotes the cut-off level for positivity (ln 15 AU/mL=2.71 by indirect ELISA, LIAISON SARS-CoV-2 S1/S2 IgG). CG, control group; GEE, generalised estimating equations; IgG, immunoglobulin G; ln, logarithm; RA, rheumatoid arthritis

Table 3 Unadjusted and adjusted logistic regression models examining the factors associated with positive anti-S1/S2 IgG antibodies and NAb after two doses of CoronaVac (at D69) in 266 patients with RA

	Positive anti-S1/S2 IgG				Positive NAb			
	Unadjusted		Adjusted		Unadjusted		Adjusted	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Demographics								
Age (5-year period)	0.81 (0.72 to 0.91)	<0.001	0.79 (0.70 to 0.89)	<0.001	0.88 (0.80 to 0.97)	0.012	0.87 (0.78 to 0.96)	0.007
Age ≥60 years	0.42 (0.25 to 0.70)	<0.001	–	–	0.60 (0.37 to 0.97)	0.039	–	–
Female sex	0.68 (0.30 to 1.56)	0.364	–	–	1.33 (0.61 to 2.89)	0.468	–	–
Caucasian race	0.64 (0.38 to 1.05)	0.077	0.64 (0.37 to 1.13)	0.124	0.65 (0.40 to 1.05)	0.077	0.58 (0.35 to 1.05)	0.056
Disease characteristics and activity								
RF positivity	0.99 (0.53 to 1.84)	0.969	–	–	1.10 (0.61 to 2.01)	0.748	–	–
Anti-CCP positivity	1.22 (0.60 to 2.52)	0.583	–	–	1.29 (0.63 to 2.63)	0.485	–	–
CRP	1.02 (1.00 to 1.05)	0.084	1.03 (1.00 to 1.06)	0.103	1.00 (0.99 to 1.02)	0.784	–	–
CDAI	1.00 (0.98 to 1.02)	0.973	–	–	0.99 (0.97 to 1.02)	0.560	–	–
SDAI	1.00 (0.98 to 1.03)	0.815	–	–	0.99 (0.97 to 1.02)	0.572	–	–
DAS28	0.97 (0.79 to 1.19)	0.761	–	–	0.96 (0.78 to 1.17)	0.659	–	–
Current therapy								
Prednisone	0.67 (0.40 to 1.13)	0.131	0.66 (0.37 to 1.19)	0.167	0.60 (0.37 to 0.99)	0.046	–	–
Prednisone ≥7.5 mg/day	0.76 (0.42 to 1.38)	0.361	–	–	0.39 (0.20 to 0.73)	0.003	0.38 (0.19 to 0.73)	0.004
≥2 DMARD	0.51 (0.29 to 0.90)	0.021	–	–	0.72 (0.43 to 1.22)	0.225	–	–
Number of DMARD	0.52 (0.34 to 0.80)	0.003	0.55 (0.33 to 0.90)	0.018	0.77 (0.52 to 1.13)	0.173	0.75 (0.43 to 1.30)	0.298
MTX	0.47 (0.28 to 0.77)	0.003	0.54 (0.29 to 0.98)	0.044	0.74 (0.46 to 1.21)	0.230	–	–
Leflunomide	1.23 (0.72 2.09)	0.449	–	–	1.34 (0.81 to 2.24)	0.255	0.38 (0.19 to 0.73)	0.004
Hydroxychloroquine	0.72 (0.35 to 1.46)	0.363	–	–	1.19 (0.59 to 2.40)	0.627	–	–
Sulfasalazine	2.65 (1.04 to 6.72)	0.041	2.86 (0.97 to 8.41)	0.056	0.88 (0.41 to 1.88)	0.735	0.75 (0.43 to 1.30)	0.298
Tofacitinib	3.21 (0.91 to 11.39)	0.071	2.31 (0.61–8.76)	0.219	0.93 (0.35 to 2.42)	0.874	–	–
TNFi	0.80 (0.45 to 1.44)	0.460	–	–	1.12 (0.63 to 1.98)	0.712	–	–
Abatacept	0.33 (0.18 to 0.60)	<0.001	0.37 (0.19 to 0.73)	0.004	0.52 (0.28 to 0.97)	0.039	0.68 (0.35 to 1.29)	0.237
Tocilizumab	1.36 (0.68 to 2.70)	0.388	–	–	0.86 (0.45 to 1.66)	0.656	–	–

Results are expressed in OR (95% CI) regarding the positivity for anti-S1/S2 IgG and for NAb.
Adjusted analyses included the factors with p>0.20 at unadjusted analyses.
anti-CCP, anti-cyclic citrullinated peptides; CDAI, Clinical Disease Activity Index; CRP, C reactive protein; DAS28-CRP, Disease Activity Score with 28 joints and C reactive protein; DMARD, disease modifying antirheumatic drugs; IgG, immunoglobulin G; NAb, neutralising antibodies; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; TNFi, tumour necrosis factor inhibitors.

were negatively related to the presence of NAb in univariate and multivariate analyses (table 3).

Assessment of factors associated with immunogenicity in patients with RA without combination therapy

A subgroup analysis, including only patients with no DMARD or in DMARD monotherapy, was performed and pointed older age and abatacept use as negatively associated to SC (table 4), while prednisone use in a dose ≥7.5 mg/day was related to the absence of NAb (table 4).

Direct comparisons of patients under distinct DMARD combinations with the original CG

Direct assessments of subgroups of patients under distinct treatments with the original CG were performed at D69 (online supplemental tables 1 and 2; figures 3 and 4). For each subgroup

Table 4 Unadjusted and adjusted logistic regression models examining the factors associated with positive anti-S1/S2 IgG antibodies and NAb after 2 doses of CoronaVac (at D69) in 71 patients with RA under any DMARD monotherapy (n=64) or without DMARD (n=7)

	Positive anti-S1/S2 IgG				Positive NAb			
	Unadjusted		Adjusted		Unadjusted		Adjusted	
	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value	OR (95% CI)	P value
Demographics								
Age (5-year period)	0.69 (0.53 to 0.91)	0.009	0.66 (0.49 to 0.88)		0.95 (0.81 to 1.12)		–	–
Age ≥60 years	0.10 (0.02 to 0.45)	0.003	–	–	0.75 (0.31 to 1.83)	0.522	–	–
Female sex	0.35 (0.07 to 1.72)	0.197	0.29 (0.05 to 1.81)	0.185	0.93 (0.30 to 2.86)	0.899	–	–
Caucasian race	0.45 (0.16 to 1.27)	0.131	0.24 (0.07 to 1.13)	0.054	1.04 (0.43 to 2.49)	0.934	–	–
Disease characteristics and activity								
RF positivity	0.44 (0.11 to 1.69)	0.230	–	–	0.96 (0.34 to 2.70)	0.942	–	–
Anti-CCP positivity	1.25 (0.26 to 6.00)	0.780	–	–	1.67 (0.38 to 7.29)	0.497	–	–
CRP	1.01 (0.97 to 1.06)	0.553	–	–	0.97 (0.92 to 1.02)	0.217	–	–
CDAI	1.03 (0.96 to 1.11)	0.400	–	–	0.97 (0.92 to 1.03)	0.369	–	–
SDAI	1.03 (0.97 to 1.10)	0.350	–	–	0.97 (0.92 to 1.02)	0.250	–	–
DAS28	1.26 (0.76 to 2.10)	0.371	–	–	0.75 (0.49 to 1.17)	0.208	–	–
Current therapy								
Prednisone	1.19 (0.45 to 3.16)	0.734	–	–	0.94 (0.39 to 2.26)	0.895	–	–
Prednisone ≥7.5 mg/day	0.65 (0.12 to 3.36)	0.605	–	–	0.08 (0.01 to 0.69)	0.022	0.06 (0.01 to 0.75)	0.028
MTX	1.02 (0.36 to 2.91)	0.974	–	–	0.90 (0.35 to 2.28)	0.819	–	–
Leflunomide	2.83 (0.33 to 24.41)	0.345	–	–	7.60 (0.89 to 64.92)	0.064	10.69 (0.78 to 146)	0.076
Sulfasalazine	0.36 (0.33 to 24.41)	0.509	–	–	1.90 (0.17 to 21.8)	0.606	–	–
Tofacitinib	1.50 (0.15 to 15.2)	0.732	–	–	0.21 (0.02 to 2.00)	0.176	0.65 (0.05 to 8.54)	0.741
TNFi	0.93 (0.17 to 5.16)	0.930	–	–	0.67 (0.14 to 3.22)	0.620	–	–
Abatacept	0.19 (0.05 to 0.77)	0.020	0.16 (0.03 to 0.84)	0.030	0.58 (0.15 to 2.23)	0.427	–	–
Tocilizumab	0.71 (0.19 to 2.63)	0.604	–	–	0.92 (0.27 to 3.12)	0.889	–	–

Results are expressed in OR (95% CI) regarding the positivity for anti-S1/S2 IgG and for NAb.
Adjusted analyses included the factors with p>0.20 at unadjusted analyses.
anti-CCP, anti-cyclic citrullinated peptides; CDAI, Clinical Disease Activity Index; CRP, C-reactive protein; DAS28-CRP, Disease Activity Score with 28 joints and C reactive protein; DMARD, disease modifying antirheumatic drugs; MTX, methotrexate; NAb, neutralising antibodies; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; TNFi, tumour necrosis factor inhibitors.

assessed, age and sex distribution persisted comparable to CG (p>0.05), except for those under 'other csDMARD' (sulfasalazine) in monotherapy (online supplemental table 1). Disease activity was similar among major groups (p>0.05) (online supplemental table 3). Lower SC and the presence of NAb rates were observed among patients under MTX, abatacept and tocilizumab, both in monotherapy and in combination with other drugs. Patients under non-MTX-csDMARD and tumour necrosis factor inhibitor (TNFi) had decreased rates only in combination with other therapies. Tofacitinib use, alone or in combination, impacted on NAb presence (online supplemental table 1; figure 3). Most patients with RA under any DMARD (alone or in combination) had lower GMT than CG (figure 4; online supplemental table 4).

Vaccine tolerance and safety

No moderate or severe AE was reported. After the first dose, patients had more overall and systemic reactions, headache and arthralgia, than CG (online supplemental table 4). After the second dose, no difference was observed. After first vaccine dose, 11 patients (4.2%) reported worsening of disease activity perception, while 14 (5.6%) reported after the second shot.

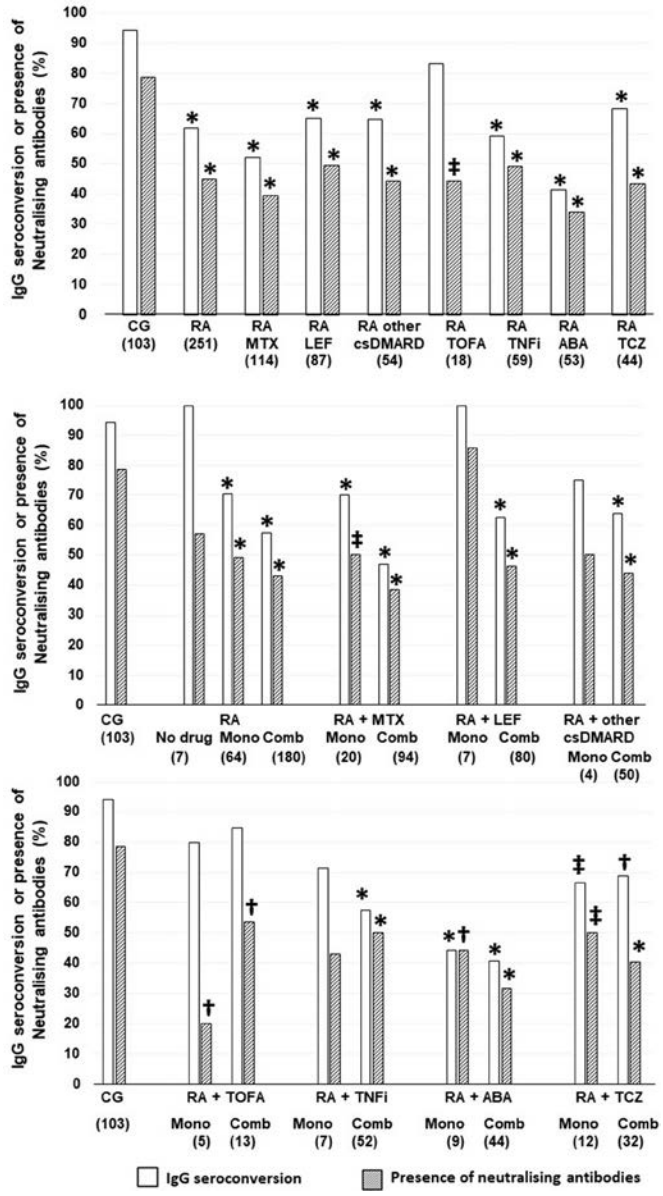

Figure 3 Anti-SARS-CoV-2 S1/S2 IgG SC and the presence of NAb at D69, according to RA treatments in comparison to CG, using a two-sided χ^2 or Fisher's exact tests, as appropriate. All the analyses are two-sided. Data are shown as percentages. The number of patients in groups is depicted under their designations. ABA, abatacept; CG, control group; Comb, combination therapy; csDMARD, conventional synthetic disease modifying antirheumatic drugs; DMARD, disease modifying antirheumatic drugs; IgG, immunoglobulin G; LEF, leflunomide; Mono, monotherapy; MTX, methotrexate; NAb, neutralising antibodies; RA, rheumatoid arthritis; TCZ, tocilizumab; TNFi, tumour necrosis factor inhibitor; TOFA, tofacitinib. Other csDMARD: sulfasalazine (n=29) or hydroxychloroquine (n=25). *p<0.001 vs CG; †p<0.05 vs CG; ‡p<0.01 vs CG.

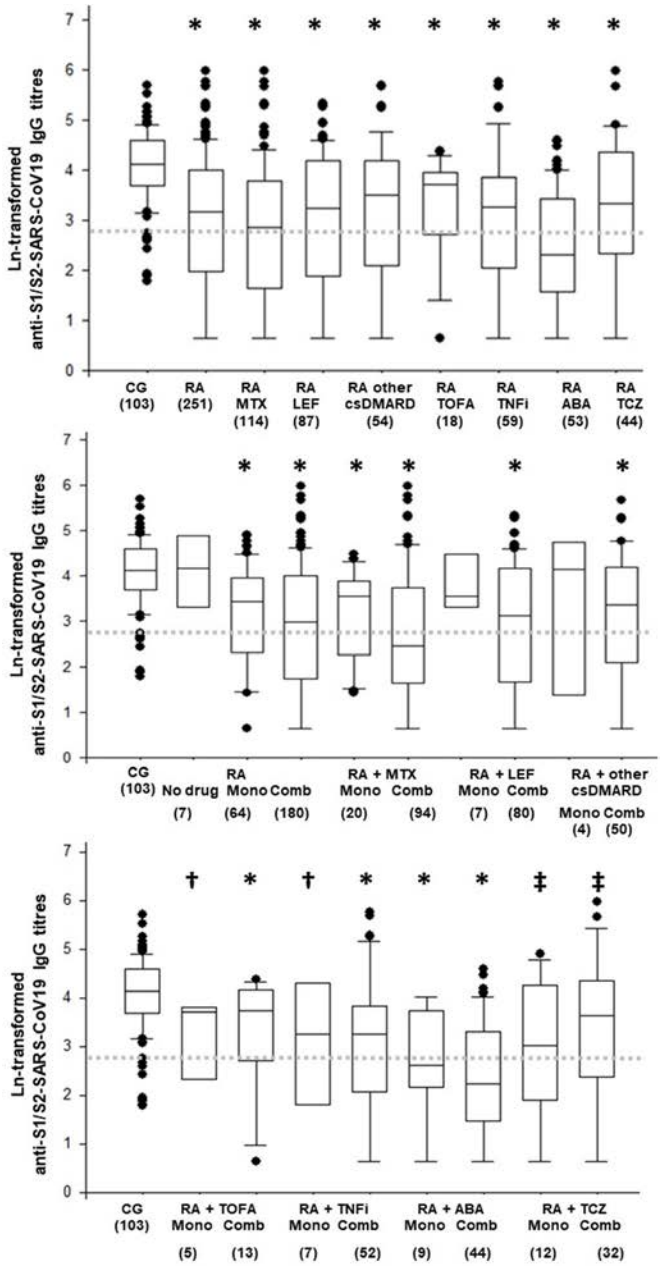

Figure 4 Box plots show Napierian ln-transformed anti-SARS-CoV-2 S1/S2 IgG titres at D69, according to RA treatments in comparison to CG, using the Mann-Whitney U test. Analyses were two-sided. Dotted line denotes the cut-off level for positivity (ln 15 AU/mL=2.71 by Indirect ELISA, LIAISON SARS-CoV-2 S1/S2 IgG). The number of patients in groups is depicted under their designations. ABA, abatacept; CG, control group; Comb, combination therapy; csDMARD, conventional synthetic disease modifying antirheumatic drugs; DMARD, disease modifying antirheumatic drugs; LEF, leflunomide; ln, logarithm; Mono, monotherapy; MTX, methotrexate; RA, rheumatoid arthritis; TCZ, tocilizumab; TNFi, tumour necrosis factor inhibitor; TOFA, tofacitinib. Other csDMARD: sulfasalazine (n=29) or hydroxicloroquine (n=25). *p<0.001 vs CG; †p<0.05 vs CG; ‡p<0.01 vs CG.

DISCUSSION

To the best of our knowledge, this is the first study to focus on the impact of the different therapies and its combinations, exclusively in patients with RA, on the immunogenicity induced by inactivated Sinovac-CoronaVac vaccine. This detailed analysis provided novel evidence of an overall reduced immune response in many different combinations, and, in monotherapy, for abatacept, MTX, tocilizumab and tofacitinib.

The main strength of this study is the inclusion of a robust RA population under representative distribution of different drug categories, allowing a precise analysis of the influence of specific mechanisms on humoral response, even in monotherapy. Inclusion of a paired CG was also essential to avoid the well-known effects of age and sex on vaccine immunogenicity.[31 32] Moreover,

disease activity was evaluated by validated scores. The isolated analysis of patients with RA prevented interference from drugs not commonly used in RA in the regression models. We also deliberately avoided patients under rituximab because of its well-known influence on humoral immunogenicity and heterogeneous phases of the cycles at the vaccination period.[11 13–15 17 20 21 30] Furthermore, immunogenicity was assessed by two validated methods.[25–27] In this context, anti-SARS-CoV-2 S1/S2 IgG was used in other large trials of COVID-19 vaccination in patients with ARD.[14 22] Although higher titres of NAb were associated to increased COVID-19 protection,[33 34] further prospective studies with continuous disease surveillance are necessary to better evaluate possible cut-off levels and persistence of protection.

Among csDMARD, virtually any combination therapy was associated with decreased responses for both IgG and NAb. Although 62.5% of these combinations included MTX, other associated csDMARD also reduced immunogenicity when in combination with biologic DMARD (bDMARD) or other sDMARD. In fact, the number of DMARD was independently related to reduced SC in a similar magnitude to MTX (45% decrease). These novel findings of reduced immunogenicity with non-MTX-csDMARD associations were not appropriately evaluated in other studies due to the small sample size of subgroups of patients using such combinations.[12–23]

Of note, in monotherapy, MTX was the only csDMARD associated with reduced response both to IgG and NAb in comparison to controls. Although Braun-Moscovici *et al*[15] suggested that impairment of the humoral response might be attributed to the concomitant treatment, our findings point that this also occurs with MTX monotherapy at a lesser degree. This result reinforces those findings with other COVID-19 vaccines.[14 17–19 22] Interestingly, tofacitinib, alone or in combination, had a negative impact mainly on NAb, but also in IgG GMT, which is in line with previous findings about the BNT162b2 mRNA[14] and anti-pneumococcal vaccines.[35] We reinforce that non-MTX csDMARD in monotherapy (leflunomide and sulfasalazine) had no negative impact on immunogenicity, and this is probably a subgroup of patients who do not need drug discontinuation, as observed in studies with other COVID-19[20 22] and influenza[30 36] vaccines. However, the small representation in monotherapy of these drugs precludes a definitive conclusion.

Regarding the role of biological therapies, any combination with bDMARD was deleterious. This finding extended previous results on relevance of combination therapy as the main cause of poor vaccine response.[14 15] Regarding abatacept, it was the drug with the greatest impact on immunogenicity herein: IgG SC rate and NAb positivity were, respectively, limited to 25% and 20% of patients under abatacept in combination with MTX. This fact is in accordance with previous findings of up to 90% reduction of anti-SARS-CoV-2 IgG response to the BNT162b2 mRNA vaccine, especially in combination with MTX.[14 15 22] We extended these observations, demonstrating a harmful effect of abatacept in combination with other csDMARD and in monotherapy. Specifically, the exclusive evaluation of patients without combination therapy highlighted abatacept as related to vaccine non-response, even in monotherapy, in comparison to other DMARD. The impact of abatacept on vaccination confirms previous evidence from influenza A/H1N1,[36–38] and pneumococcal conjugate vaccines,[39] probably due to the attenuation of co-stimulating signal of naïve T cells, inhibition of T cell proliferation, and inadequate stimulation of B cells.[40 41]

Other bDMARD, TNFi and tocilizumab, also decreased SC, GMT and NAb responses, in combination not only with MTX but also with other csDMARD, adding information to previous data with other COVID-19 vaccines, solely for combination with MTX.[14 17] However, in monotherapy, TNFi did not have deleterious effects on vaccination response, in accordance with previous findings.[13 15 17 21 22] In contrast, the negative impact of tocilizumab, both in combination and in monotherapy, was not previously reported. This finding may be related to the weaker immunogenicity of the inactivated vaccine in comparison with mRNA or virus vector vaccines.[33] Alternatively, patients evaluated herein were all seronegative at baseline, while other studies did not exclude pre-exposed patients,[14–16] who are known to have a greater immune response magnitude.[17 42]

Of note, more than half of our RA population was using prednisone. In this regard, some trials[11 14 21 22] evidenced a negative effect of steroids on immunogenicity of different SARS-CoV-2 vaccines but without a clear minimum daily dose threshold. Recently, doses greater than 10 mg/day were pointed as a possible cut-off in patients with lupus.[43] Herein, we found that lower doses of 7.5 mg/day of prednisone are a major factor associated with impairment of NAb response.

Safety was demonstrated, with only mild AE reported. However, higher frequencies of AE occurred in patients with RA, as previously described for other COVID-19,[44] influenza[30 45–47] and yellow fever[48] vaccines. This is possibly due to a greater awareness of symptoms among patients, and probably not related to recall or reporting bias, since diaries were given after each vaccine dose and collected in the next visit, and not at the end of the study.

Disease activity status did not interfere with the immune response as observed by different composite activity indexes, in accordance with previous findings on influenza A/H1N1 vaccine.[30] However, data were obtained from up to 3 months before vaccination, precluding a definitive conclusion. Of note, most patients were on low activity status at the first vaccine dose. Therefore, the results may not be generalised for those with high disease activity.

The present study has some limitations. Disease Activity Scores with 28 joints (DAS28) were not systematically assessed after immunisation, although less than 6% of patients reported the perception of disease worsening. Similarly, no flare-up assessed by DAS28 was observed in two prior cohorts of patients with ARD who received mRNA vaccines.[12 15] In contrast, Furer *et al* demonstrated worsening in Simplified Disease Activity Index score in 20% of patients with RA after complete vaccination with the BNT162b2 mRNA in a short period of follow-up,[14] while 20% got better and 60% remained unchanged. It is not clear if the effect was due to vaccination itself, and further studies are necessary to clarify this point. Another limitation was the absence of T cell response evaluation. In addition, the comparison of small sample size subgroups with controls may be underpowered to draw definite conclusions.

In summary, we provided novel evidence that the RA moderate response to Sinovac-CoronaVac vaccine is associated with a distinct impact of drugs, with nearly all DMARD combinations presenting a deleterious effect in immunogenicity. A more restricted number of drugs (abatacept, MTX, tocilizumab and tofacitinib) also hampered this response even as monotherapy, while TNFi and non-MTX csDMARD did not. In addition, we identified that prednisone at a dosage of ≥7.5 mg/day decreased NAb response to vaccine. Altogether, these findings reinforce the need of a broader approach, not limited to a specific drug temporary suspension, to improve vaccine response for this population.

Acknowledgements We thank the contribution of the Central Laboratory Division, Registry Division, Security Division, IT Division, Superintendence, Pharmacy Division

and Vaccination Centre for their technical support. We also thank the volunteers for participating in the three in-person visits of the protocol and for handling the biological material, and those responsible for the follow-up of all participants.

Contributors ACM-R, CGSS, EFNY, SGP, EGK, NEA and EB conceived and designed the study. EB is responsible for the overall content as the guarantor. ACM-R, CGSS, EFNY, SGP, CAS, TP, LdVKK, NEA and EB participated in data collection and analysis and supervised clinical data management, writing of the manuscript and revision of the manuscript. LdVKK, TP and EB organised and supervised blood collection and vaccination protocol. Pasoto supervised serum processing, SARS-CoV-2-specific antibody ELISA/neutralisation assays and SARS-CoV-2 RT-PCR. ACM-R, KRB, DSD, AYS, HCdS, CGSS, EFNY, SGP, CAS, TP, LdVKK, CSRA, MSRS, TLN, GGMB, EGK, NEA and EB collected epidemiological and clinical data and assisted with the identification of SARS-CoV-2 infection and follow-up of patients. All authors helped to edit the manuscript.

Funding This study was sponsored by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (number: 2015/03756–4 to NEA, SGP, CAS and EB; number: 2017/14352–7 to TP; number: 2019/17272–0 to LdVKK; and number: 2021/06613–0 to TLN); Conselho Nacional de Desenvolvimento Científico e Tecnológico (number: 305242/2019–9 to EB and number: 304984/2020–5 to CAS) and B3, Bolsa de Valores do Brasil. Instituto Butantan supplied the study product and had no other role in the trial.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants. The protocol was approved by the National (Comissão Nacional de Ética em Pesquisa) and Institutional Ethical Committee (Comissão de Ética para Análise de Projetos de Pesquisa) of Hospital das Clinicas HCFMUSP, Faculdade de Medicina, Universidade de Sao Paulo, Brazil (ID CAAE: 42566621.0.0000.0068). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

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

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CLINICAL SCIENCE

Immunogenicity of BNT162b2 vaccine against the Alpha and Delta variants in immunocompromised patients with systemic inflammatory diseases

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221508).

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Received 17 September 2021
Accepted 20 December 2021
Published Online First 12 January 2022

To cite: Hadjadj J, Planas D, Ouedrani A, *et al*. *Ann Rheum Dis* 2022;**81**:720–728.

ABSTRACT

Objectives The emergence of strains of SARS-CoV-2 exhibiting increase viral fitness and immune escape potential, such as the Delta variant (B.1.617.2), raises concerns in immunocompromised patients. We aimed to evaluate seroconversion, cross-neutralisation and T-cell responses induced by BNT162b2 in immunocompromised patients with systemic inflammatory diseases.

Methods Prospective monocentric study including patients with systemic inflammatory diseases and healthcare immunocompetent workers as controls. Primary endpoints were anti-spike antibodies levels and cross-neutralisation of Alpha and Delta variants after BNT162b2 vaccine. Secondary endpoints were T-cell responses, breakthrough infections and safety.

Results Sixty-four cases and 21 controls not previously infected with SARS-CoV-2 were analysed. Kinetics of anti-spike IgG after BNT162b2 vaccine showed lower and delayed induction in cases, more pronounced with rituximab. Administration of two doses of BNT162b2 generated a neutralising response against Alpha and Delta in 100% of controls, while sera from only one of rituximab-treated patients neutralised Alpha (5%) and none Delta. Other therapeutic regimens induced a partial neutralising activity against Alpha, even lower against Delta. All controls and cases except those treated with methotrexate mounted a SARS-CoV-2 specific T-cell response. Methotrexate abrogated T-cell responses after one dose and dramatically impaired T-cell responses after two doses of BNT162b2. Third dose of vaccine improved immunogenicity in patients with low responses.

Conclusion Rituximab and methotrexate differentially impact the immunogenicity of BNT162b2, by impairing B-cell and T-cell responses, respectively. Delta fully escapes the humoral response of individuals treated with rituximab. These findings support efforts to improve BNT162b2 immunogenicity in immunocompromised individuals (ClinicalTrials.gov number, NCT04870411).

Key messages

What is already known about this subject?
- The course of COVID-19 is less favourable in patients with systemic inflammatory diseases.
- Rituximab and methotrexate decrease seroprotection rate following vaccination against influenza, pneumococcus, and ancestral and Alpha variants of SARS-CoV-2.
- Sensitivity of Delta variant to antibody neutralisation is reduced in vitro.

What does this study add?
- This study describes that 95% of sera from patients treated with rituximab did not neutralise Alpha and Delta variants after two doses of BNT162b2.
- In contrast, these patients have similar SARS-CoV-2 specific T-cell response that controls.
- Methotrexate completely abrogated T-cell responses after one dose and dramatically impaired T-cell responses after two doses of BNT162b2.
- Third dose improved immunogenicity in patients with low responses after two doses but had no effect in those with no responses.

How might this impact on clinical practice or future developments?
- This differential impairment of immunogenicity after BNT162b2 vaccine according to the treatments received is critical to identify patients in which optimisation of vaccine strategies should be evaluated.
- The administration of a third dose of mRNA-based vaccine should be proposed in patients with low responses after two doses.
- Other strategies should be considered in patients with no response after two doses.

INTRODUCTION

The course of COVID-19 is less favourable in patients with systemic inflammatory diseases. Older age, male gender, cardiovascular disease and obesity are risk factors of severe forms and COVID-19-related death in this immunocompromised population,[1–4] as it is in the general population.[5 6] Disease-specific factors including disease activity and treatments, especially glucocorticoids,

mycophenolate mofetil and rituximab, are additional risk factors.[1–3]

BNT162b2 and mRNA-1273 COVID-19 vaccines have been developed using a novel liposomal mRNA-based delivery platform. These vaccines have a good safety profile, induce strong and persistent B-cell and T-cell responses,[7 8] and are highly effective to prevent SARS-CoV-2 infection, hospitalisation and death with the ancestral strain and the Alpha (B.1.1.7) variant.[9]

The efficacy of vaccine has been recently questioned by variants of SARS-CoV-2 exhibiting increase viral fitness and immune escape potential. Among them, the Delta variant (B.1.617.2) was first identified in India in October 2020 and rapidly became the predominant strain across the globe.[10] While in vitro data indicate reduced sensitivity of Delta variant to antibody neutralisation,[11] only modest differences in vaccine effectiveness are noted with Delta as compared with Alpha.[12] In patients with systemic inflammatory diseases, the use of rituximab and methotrexate, commonly used to induce and maintain remission, decreases seroprotection rate after vaccination against influenza, pneumococcus, and ancestral and Alpha variants of SARS-CoV-2.[13–16] Yet, how the different immunosuppressive or immunomodulatory drugs tune humoral and cellular responses, and how the Delta variant impacts vaccine effectiveness in this population remains unclear.

In this study, we measured seroconversion, cross-neutralisation of Alpha and Delta variants and T-cell responses induced by BNT162b2 in immunocompromised patients with systemic inflammatory diseases according to the treatments received.

METHODS

Study design

The prospective COVADIS study (NCT04870411) included patients with systemic inflammatory diseases managed in Cochin Hospital, University of Paris (Paris, France). Healthcare immunocompetent workers from the same hospital were included as controls. Patients with a positive COVID-19 serology at baseline were excluded from the main analysis. Cases and controls received two doses of BNT162b2 vaccine 28 days apart. Four groups of patients receiving different immunosuppressive or immunomodulatory drugs were defined: patients receiving rituximab ('rituximab' treatment group), methotrexate ('methotrexate' group), immunosuppressive drugs such as mycophenolate mofetil or azathioprine ('immunosuppressive drugs' group), and those receiving other strategies described to have limited impact on vaccine immunogenicity ('other' treatment group).

Clinical and laboratory data

Clinical data were collected at baseline and during follow-up until month 6. To evaluate vaccine immunogenicity, blood samples were collected before the first dose of vaccine (M0), 1 month later just before the second dose (M1), at 3 months (M3) and 6 months (M6).

Outcomes

Primary endpoints were BNT162b2 immunogenicity and cross-neutralisation of Alpha and Delta variants at 3 months, that is, after two vaccine doses, defined by neutralisation titre (median of the half maximal effective dilution, ED50) for both virus with ED50 above 30. Secondary endpoints were the proportion of patients with positive anti-SARS-CoV-2 antibodies (define as an antibody binding unit (BU) above 1.1 for IgG and 0.2 for IgA) at M1, M3 and M6, cross-neutralisation of Alpha and Delta variants at 6 months, T-cell response defined by the number of circulating SARS-CoV-2-spike-specific interferon-γ (IFNγ)-producing T cells at M1, M3 and M6, breakthrough infections and safety.

T and B cell immunophenotyping

Extended B cell and T cell immunoprofiling were performed on whole blood as described in the online supplemental appendix 1 and online supplemental figures 1 and 2.

S-Flow assay

The S-Flow assay was used to detect antibodies bound to 293T cells stably expressing the spike protein (S) at their surface using *flow* cytometry. This assay is highly sensitive and allows quantification of antibodies through a standardised mean fluorescence intensity (MFI, referred to as binding unit, BU), which is calculated using an anti-spike monoclonal antibody as reference. The cut-off value of 1.1 BU was established using pre-pandemic sera. The method is described in the online supplemental appendix 1 and online supplemental figure 3.

Virus strains

The Alpha (B.1.1.7) variant originated from an individual returning from the UK. The Delta (B.1.617.2) variant originated from a hospitalised patient returning from India. The variant strains were isolated from nasal swabs using Vero E6 cells and amplified by two passages. Additional information is described in the online supplemental appendix 1.

S-Fuse neutralisation assay

The S-Fuse neutralisation assay was used to assess the neutralising activity of sera against emerging variants. The method is described in the online supplemental appendix 1.

T-cell response using enzyme-linked immunospot (ELISpot)

SARS-CoV-2-specific IFNγ-producing T cells were identified by using commercially available pools derived from a peptide scan through SARS-CoV-2 N-terminal (pool S1) and C-terminal (pool S2) fragments of spike glycoprotein (JPT Peptide Technologies GmbH, BioNTech AG, Berlin, Germany). Results are expressed as spot forming unit (SFU)/10^6 CD3+ T cells after subtracting background values from wells with non-stimulated cells. The method is described in the online supplemental appendix 1.

Statistical analysis

No statistical methods were used to predetermine sample size. The experiments were performed in blind regarding to the allocation groups. Flow cytometry data were analysed with FlowJo V.10 software (TriStar). Calculations were performed using Excel V.365 (Microsoft). Figures were drawn using GraphPad Prism V.9. Statistical analyses were conducted using GraphPad Prism V.9. Statistical significance between different groups was calculated using the tests indicated in each figure legend. Detailed statistical analysis is described in the online supplemental appendix 1.

RESULTS

Patients characteristics

Between January and April 2021, 77 cases and 28 controls were included in the study. Twenty participants (13 cases and 7 controls) with positive SARS-CoV-2 serological tests at baseline were excluded from the main analysis (figure 1). Finally, 64 cases and 21 controls were analysed. One patient and two controls were not sampled before the second dose of BNT162b2 vaccine. Baseline characteristics of patients are shown in table 1. Median age in controls and cases was 56 (39.5–59.5) and 52 (37.8–66.3) years, respectively. In

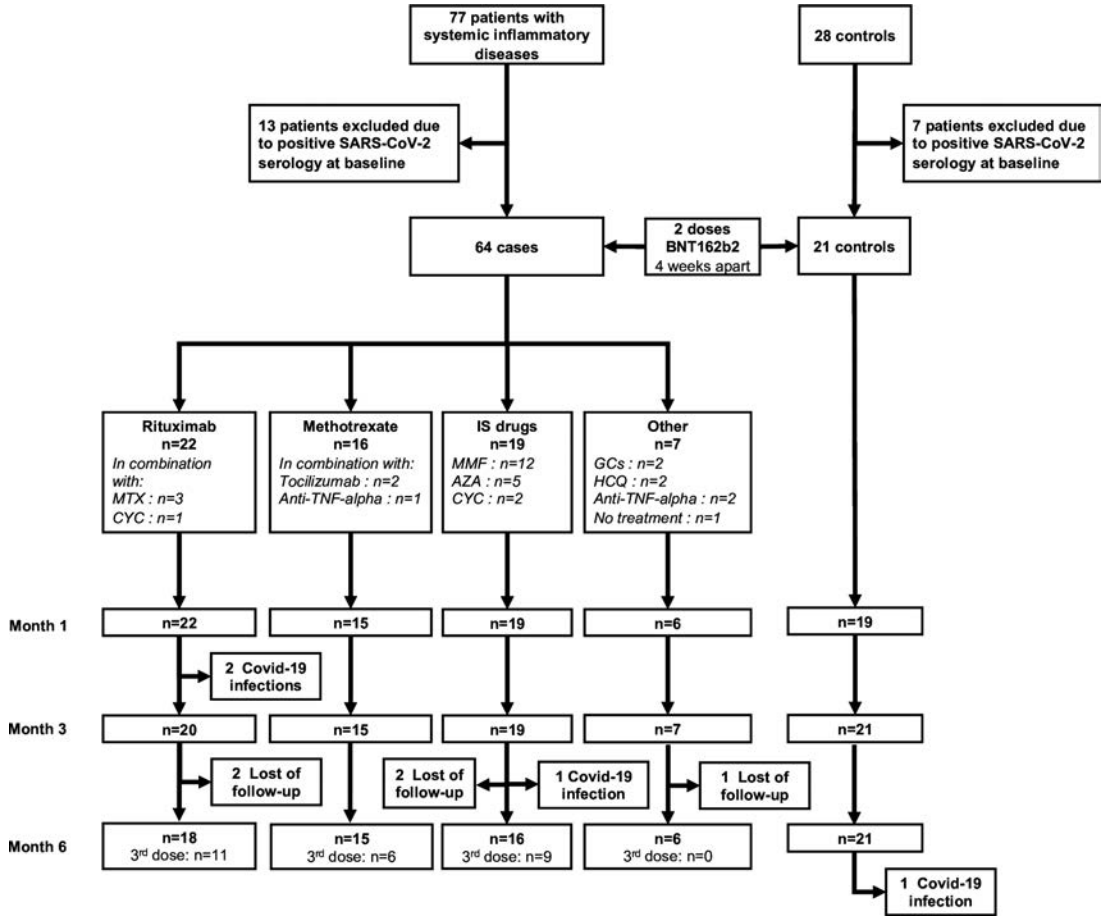

Figure 1 Flowchart of the study. AZA, azathioprine; CYC, cyclophosphamide; GCs, glucocorticoids; HCQ, hydroxychloroquine; IS, immunosuppressive; MMF, mycophenolate mofetil; MTX, methotrexate.

patients in the 'rituximab' group, median time since the last infusion was 13.5 (0–117.5) days. The immunological characteristics are shown in the online supplemental table 1 and online supplemental figures 4 and 5. Compared with controls, cases showed lower total lymphocytes count. As expected, in the 'rituximab' treatment group, circulating B cells were not detected (except in one patient) and levels of IgG, IgA and IgM were significantly lower.

Induction of anti-spike antibodies after BNT162b2 vaccine

First, we analysed the kinetics of induction of anti-spike IgG in patients' sera after the first and second dose of BNT162b2 vaccine. We observed a delayed response in cases compared with controls. Anti-spike IgG inductions were detectable mainly after the second dose in cases, whereas it was noted from the first dose in controls (online supplemental figure 6). On samples collected after the two doses, at 3 months, all treatment groups except the 'other' group showed significantly lower anti-spike IgG levels than controls (figure 2A). The 'rituximab' group showed the lowest response. Then, we categorised individuals who seroconvert in IgG at M3 as 'responders'. All controls and cases from the 'other' treatment group seroconverted in IgG (figure 2B). 'Rituximab' showed again the lowest response, with only 50% of individuals who seroconverted at M3 (figure 2B). 'Methotrexate' and 'immunosuppressive drugs' treatment groups showed intermediate levels of anti-spike'IgG levels at 3 months, with 93% and 68% of individuals who seroconverted, respectively (figure 2A,B). A large interindividual variability was observed in these two groups. The use of azathioprine or mycophenolate mofetil did not discriminate between responders and non-responders in the immunosuppressive drugs group. Analysis of the circulating follicular helper CD4+ T cells after the first and second dose showed a delayed increase in cases compared with controls, occurring mainly after the second dose in patients treated by methotrexate and immunosuppressive drugs and detected after the first dose in controls. No difference was observed in the proportion of plasmablast and memory B cells (online supplemental figure 7).

Neutralisation of Alpha and Delta variants by sera after BNT162b2 vaccine

We next examined whether BNT162b2 vaccine-elicited antibodies at month 3 neutralised the Alpha and Delta variants in cases and controls (figure 2C,D). Median ED50 for Alpha in controls and in cases from the 'rituximab', 'methotrexate', 'immunosuppressive drugs' and 'other' treatment groups were 1942,<7.5, 199, 65 and 2173, respectively; and 539, <7.5, 31, <7.5 and 270 for Delta (figure 2C). Delta was fourfold less sensitive to neutralisation than Alpha in the controls, confirming previous observation.[11] Among cases, titres were reduced by sixfold between Delta and Alpha in the 'methotrexate' group, ninefold in the 'immunosuppressive drugs' group, eightfold in the 'other' group. The lack of neutralisation in the 'rituximab' group impaired the calculation of a fold decrease.

Then, we arbitrarily classified individuals as neutralisers according to the detection of neutralising antibodies at a serum dilution of 1:30 and non-neutralisers. Administration of two doses of BNT162b2 generated a neutralising response against the Alpha and Delta variants in 100% of controls. Only one individual in the 'rituximab' group neutralised Alpha (5%) and

Table 1 Patients' characteristics at vaccination

	All n=64	Rituximab n=22	Methotrexate n=16	Immunosuppressive drugs n=19	Others n=7
Age, years					
Median (IQR)	52 (37.8–66.3)	58.5 (48.3–67.8)	50 (38.5–72.3)	34 (30–53.5)	51 (44–58.5)
>50 year, n (%)	35 (54.7)	16 (72.7)	8 (50)	7 (36.8)	4 (57)
Female, n (%)	48 (75)	15 (68.2)	11 (68.8)	15 (79)	7 (100)
Diagnosis					
Vasculitis					
ANCA-associated vasculitis	18 (28.1)	18 (81.8)	0	0	0
Behçet's	2 (1.6)	0	0	2 (10.5)	0
Cryoglobulinemia vasculitis	2 (1.6)	2 (9.1)	0	0	0
Large vessel vasculitis	4 (6.3)	0	4 (25)	0	0
Connective tissue disease					
Systemic lupus erythematosus	15 (23.4)	0	4 (25)	9 (47.4)	2 (28.6)
Systemic sclerosis	7 (10.9)	1 (4.5)	0	4 (21.1)	2 (28.6)
Sjogren syndrome	2 (1.6)	1 (4.5)	1 (6.3)	0	0
Myositis	5 (7.8)	0	3 (18.8)	2 (10.5)	0
Inflammatory rheumatic diseases*	3 (4.7)	0	2 (12.5)	0	1 (14.3)
Sarcoidosis	3 (4.7)	0	1 (6.3)	1 (5.3)	1 (14.3)
Others	3 (4.7)	0	1 (6.3)	1 (5.3)	1 (14.3)
Disease duration (years), mean (SD)	9.5 (9)	9.2 (9.1)	10.1 (8)	8.4 (8.9)	12 (11.9)
Disease activity status					
Active disease, n (%)	17 (26.5)	4 (18.2)	6 (37.5)	7 (36.8)	0 (0)
Renal involvement, n (%)	19 (29.7)	9 (41)	3 (18.8)	6 (31.6)	1 (14.3)
Ongoing treatments, n (%)					
Prednisone	45 (70.3)	13 (59.1)	12 (75)	17 (89.5)	3 (42.9)
Median, mg/day (IQR)	7.5 (5–15)	5 (5–13.8)	7.5 (5–13.8)	10 (5–25)	5 (5–12.5)
cDMARDs					
Methotrexate	19 (29.7)	3 (13.6)	16 (100)	0	0
Azathioprine	5 (7.8)	0	0	5 (26.3)	0
Mycophenolate mofetil	12 (18.8)	0	0	12 (63.2)	0
Cyclophosphamide	3 (4.7)	1 (4.5)	0	2 (10.5)	0
Biological therapies					
Anti-TNF-α	6 (9.4)	0	1 (6.3)	3 (15.8)	2 (28.6)
Rituximab	22 (34.4)	22 (100)	0	0	0
Tocilizumab	3 (4.7)	0	3 (18.8)	0	0
Belimumab	1 (1.6)	0	1 (6.3)	0	0
Hydroxychloroquine	15 (23.4)	2 (9.1)	4 (25)	7 (36.8)	2 (28.6)
No DMARDs, biologics or prednisone	1 (1.6)	–	–	–	1 (14.3)
Number of lines of previous treatments, n, median (IQR)	2 (1–3.8)	2 (1–4.3)	2 (1–4)	2 (1–3)	2 (1–2)

*Inflammatory rheumatic diseases: rheumatoid arthritis (n=2), spondyloarthritis (n=1).
cDMARDs, conventional disease-modifying antirheumatic drugs; TNF-α, tumour necrosis factor.

none neutralised Delta (figure 2C,D). Of note, despite a seroconversion in 50% of vaccinated individuals, IgG levels were particularly low and probably insufficient to display any detectable neutralising activity. Sera of 87% of patients in the 'methotrexate' group neutralised Alpha, dropping to 57% against Delta (figure 2D). Sera from patients in the 'immunosuppressive drugs' group neutralised Alpha and Delta in 53% and 42%, respectively. Nine (14%) cases neutralised Alpha but not Delta, including five patients treated with methotrexate, two with immunosuppressive drugs, one with rituximab and one with anti-TNF-α therapy. Correlation between Alpha and Delta neutralisation titres, and between IgG production and ED50 of Alpha variant was strong in all participants except for those receiving rituximab and immunosuppressive drugs (online supplemental figure 8).

The lack of neutralisation of Delta was associated with active disease (p<0.001), the use of rituximab (p<0.001), glucocorticoids (p=0.007) and low IgM (p=0.047) and IgG2 (p=0.05) levels (online supplemental table 3). In multivariate analysis, ED50 of Delta remained negatively associated with rituximab (p<0.001), methotrexate (p<0.001) and immunosuppressive drugs (p<0.001) (table 2).

Overall, B-cell response to BNT162b2 vaccine was impaired in immunocompromised patients at different levels depending on the treatments received. The effect was further amplified when evaluating the efficacy of sera to neutralise the Delta variant.

Seroconversion and neutralisation of Alpha and Delta variants in convalescent vaccinated individuals

We then quantified anti-spike IgG and neutralisation activity 3 months after vaccination in the 7 controls and 13 cases

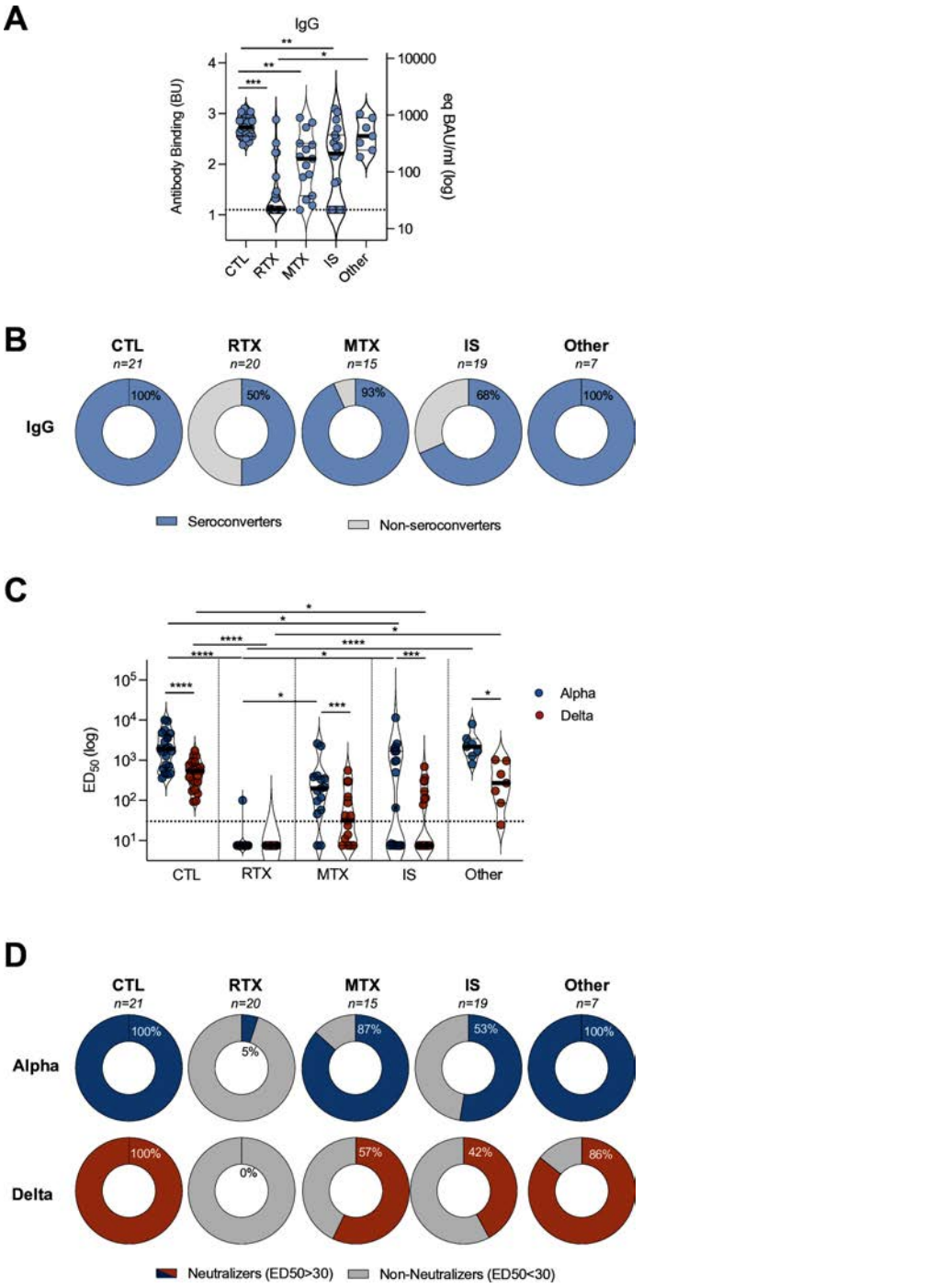

Figure 2 Humoral immune response to SARS-CoV-2 3 months after BNT162b2 vaccine. (A) Levels of anti-S IgG antibodies in the indicated groups after full vaccination at 3 months (M3) as determined by the S-Flow assay. The binding unit (BU), in a log scale, is calculated using a serially diluted anti-S monoclonal antibody as standard. Dotted lines indicate threshold of positivity (BU=1.1). Two-sided Kruskal-Wallis test with Dunn's test for multiple comparisons were performed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (B) In each group, individuals were defined as a 'seroconverter' (blue) if antibodies were detected above the threshold or 'non-responders' (grey) otherwise. Numbers of individuals in each group and percentages of responders are indicated. (C) Neutralising titres of sera against Alpha and Delta variants are expressed as ED50 values, in a log scale. Dotted line indicates the limit of detection (ED50=30). Data are mean of two independent experiments. In each group, Wilcoxon paired t-test was performed to compared ED50 of Alpha vs Delta variants. Two-sided Kruskal-Wallis test with Dunn's test for multiple comparisons between group of treatment was performed. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (D) In each group, individuals were defined as a 'neutralisers' (blue for Alpha; orange for Delta) if neutralisation was detected at the dilution 1:30 or 'non-neutralisers' (grey) otherwise. Numbers of individuals in each group and percentages of neutralisers are indicated. CTL, controls; IS, immunosuppressive; MTX, methotrexate; RTX, rituximab.

who had been previously infected with SARS-CoV-2 and excluded from the main analysis (online supplemental figure 9). In convalescent controls, vaccination boosted levels of anti-spike IgG as well as neutralising antibody titres against both variants, as compared with the uninfected vaccinated control group. In previously infected cases under immunosuppressive or immunomodulatory drugs, a low response remained after vaccination.

Table 2 Multivariate linear regression models assessing the association between patient's characteristics and quantitative humoral and cellular response

	ED50 alpha		ED50 delta		SARS-CoV-2-specific IFNγ-producing T cells	
	β coefficient(95% CI)	P value	β coefficient(95% CI)	P value	β coefficient(95% CI)	P value
Age, years	7.49 (–25.17 to 40.14)	0.649	2.16 (–1.91 to 6.22)	0.294	–1.61 (–4.41 to 1.18)	0.253
Treatment group controls	Ref		Ref		Ref	Ref
Immunosupressants	–1809.56 (–3590.36 to to 28.77)	0.047	–434.85 (–669.67 to 200.03)	<0.001	26.40 (–126.56 to 179.35)	0.731
Methotrexate	–2729.50 (–4485.78 to 973.23)	0.003	–462.83 (–701.35 to 224.31)	<0.001	–70.95 (–227.87 to 85.97)	0.370
Rituximab	–3153.98 (–4823.90 to 1484.06)	<0.001	–583.41 (–803.88 to 362.95)	<0.001	77.73 (–62.81 218.26)	0.273
Other	–398.73 (–2351.02 1553.55)	0.685	–190.32 (–440.08 59.43)	0.133	–35.00 (–198.04 to 128.04)	0.669
Glucocorticoids (%)	–50.01 (–1332.51, 1232.50)	0.938	–48.87 (–207.06 to 109.31)	0.540	–44.34 (–153.91 to 65.23)	0.4222
IgA, g/L	31.61 (–279.05 to 342.27)	0.840	0.10 (–41.14 to 41.34)	0.996	45.96 (14.67 to 77.25)	0.005
IgG2, g/L	189.43 (–183.08 to 561.95)	0.314	246.59 (–29.18 to 522.36)	0.079	–11.57 (–38.53 to 15.38)	0.394

T-cell response to BNT162b2 vaccine

We next investigated whether controls and cases mounted a SARS-CoV-2-specific T-cell response following the first and second doses of BNT162b2 vaccine (figure 3 and online supplemental figure 10). All controls (except one) and cases except those from the 'methotrexate' treatment group had similar levels of specific T-cells in response to S1 pool (figure 3A). Methotrexate completely abrogated T-cell responses after one dose and dramatically impaired T-cell responses after two doses of BNT162b2 compared with controls and cases from other treatment groups (figure 3A,B). Similar results, but less pronounced, were observed for S2 peptide pool (figure 3A and online supplemental figure 10). Importantly, despite the absence of neutralising activity in response to BNT162b2, patients receiving rituximab showed increased levels of specific T-cell responses that reached after a delay the same levels as controls (figure 3A,B). No correlation between B-cell and T-cell responses within the rituximab and the methotrexate groups was observed. The relationship between humoral and cellular immune responses against SARS-CoV-2 is shown in online supplemental figure 11, highlighting the impact of the different treatment groups on both humoral and cellular responses. The lack of T-cell response was associated with the use of methotrexate (p=0.045) and glucocorticoids (p=0.012) (online supplemental table 4). In multivariate analysis, no variable correlated with SARS-CoV-2-specific IFNγ-producing T cells (table 2). Also, no significant differences in the proportion of circulating CD4+ memory T cells and Th1 T cells after the first and second dose of BNT162b2 were found in all groups (online supplemental figure 7).

Overall, T-cell responses to S1 and S2 peptide pools were similar in cases compared with the controls except methotrexate treated patients showing significantly decreased T-cell responses.

Impact of booster vaccination at 6 months

Lastly, we evaluated in controls and cases how B-cell and T-cell responses persisted at 6 months after the two first doses of vaccine and the impact of a third booster vaccination in some of the patients (figures 1 and 4). In controls who did not receive a third dose, anti-spike IgG levels were stable at 6 months, and neutralisation titres against Alpha and Delta waned by 3.5-fold and 5-fold, respectively (figure 4A,B). A similar dynamic of anti-S antibodies and neutralisation was observed in patients from the 'other group' who were not eligible for a booster dose in France (figure 4A,B). A third dose was administered in 26 cases (all from RTX, MTX and immunosuppressive drugs groups) after a median time since the first dose of 102 (88–127) days. This third injection had no effect on humoral response in patients treated with rituximab but significantly increased anti-spike IgG levels and neutralisation against both variants in patients with methotrexate and immunosuppressive drugs compared with those that received only two doses of vaccine (figure 4A,B). Number of circulating B cells in the 'rituximab group' at the time of the third dose was not available. Conversely, booster vaccination increased levels of specific T-cells in the 'rituximab group', whereas methotrexate still dramatically impaired T-cell responses after three doses (figure 4C).

At 6 months of follow-up, one control and three patients from the cohort developed symptomatic COVID-19. Two individuals belonged to the 'rituximab' and one to 'immunosuppressive drugs' treatment groups. Four patients (6.3%) experienced a disease flare within the 3 months after the first dose of vaccine, two patients with systemic lupus erythematosus and two with systemic vasculitis, leading to modification of immunosuppressive regimen.

DISCUSSION

As the Delta variant spreads across the globe, aggregating data on the effectiveness of COVID-19 vaccines in specific immunocompromised populations is a critical issue. Data from solid organ transplant recipients, patients with malignant hemopathy or with chronic inflammatory arthritis suggested that risk factors for reduced SARS-CoV-2 vaccine immunogenicity included older age and treatments with glucocorticoids, rituximab, mycophenolate mofetil and abatacept.[15 17–19] However, levels of anti-spike antibodies were mainly measured and few studies used neutralisation assay or assessed T-cell response.

Additional studies specifically reported that B cell depletion by rituximab blocked humoral but not T cell response to vaccination, using anti-RBD IgG measurement and IFNγ ELISpot T-cell response. The time since the last infusion of rituximab and the number of circulating B cells are major predictive factors of humoral response.[20] SARS-CoV-2 antibody response was reported in 0% –39% of the vaccinated B-cell-depleted patients, whereas T cell responses were noted in 58%–100%.[20 21] This early assessment showed that humoral immunity to one or two doses of BNT162b2 was also impaired by methotrexate treatment.[22–24] However, conflicting results were found for cellular responses showing either preserved[22] or impaired T-cell activation.[24] Most of these studies assessed very early timepoints that may not allow an appropriate assessment of immune response after complete vaccination.

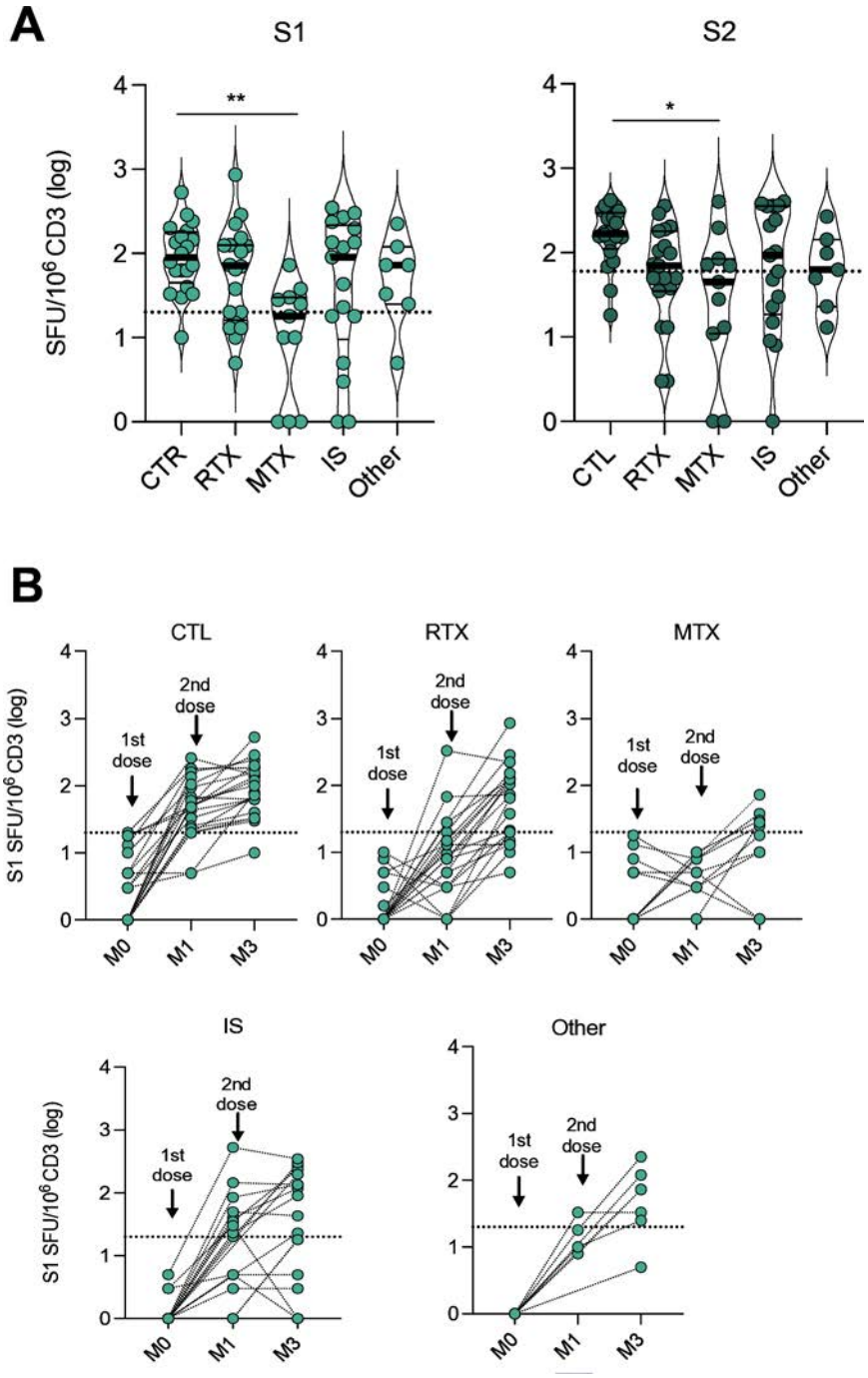

Figure 3 Cellular immune response to SARS-CoV-2 after BNT162b2 vaccine. (A) Quantification of SARS-CoV-2-specific T-cell responses using ELISpot at M3 in the indicated groups. Results were expressed as spot forming unit (SFU)/10^6 CD3+ T cells after subtraction of background values from wells with non-stimulated cells, in a log scale. Negative controls were PBMC in the culture medium. Positive controls were PHA-P and CEFX Ultra SuperStim Pool. SARS-Cov-2 peptide pools tested were derived from a peptide scan through SARS-CoV-2 Spike glycoprotein (left S1, N-terminal fragment, right: S2, C- terminal fragment). P values were determined with two-sided Kruskal-Wallis test with Dunn's test for multiple comparisons were performed. *p<0.05; **p<0.01; ***p<0.001. (B) Kinetic of specific T-cell responses against the SARS-CoV-2 S1 peptide before the first dose (M0), before the second dose (M1) and after full vaccination at 3 months (M3) according to the treatments received. Data indicate median. Each dot represents a single patient. CTL, controls; MTX, methotrexate; RTX, rituximab; IS, immunosuppressive.

Sera from convalescent and vaccinated individuals neutralise less efficiently the Delta variant than the Alpha.[11] However, this was studied in the general population and assessing the sensitivity of the Delta variant to antibody neutralisation in immunocomprised populations is thus necessary.

In this study, we focused on patients with systemic inflammatory diseases that were receiving rituximab, methotrexate and/or other immunosuppressive drugs, and provided important data regarding sensitivity to Delta variant according to the treatments used. We analysed patients after the first and the second doses of the BNT162b2 vaccine. We report a delayed and lower induction of anti-spike IgG compared with controls, much more pronounced with rituximab. While two doses of BNT162b2 generated a neutralising response against Alpha and Delta

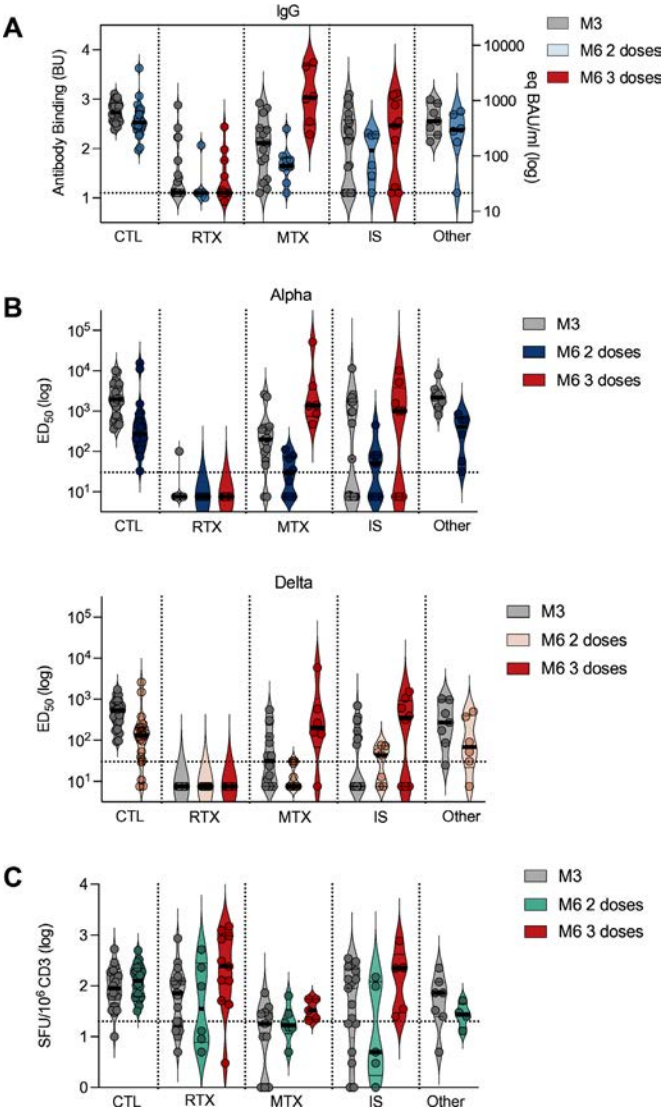

Figure 4 Impact of booster vaccination on immune response at 6 Months. (A) Levels of anti-S IgG antibodies in the indicated groups at 3 and 6 months (M3/M6) as determined by the S-Flow assay. (B) Neutralising titres of sera against Alpha and Delta variants at M3 and M6 are expressed as ED50 values, in a log scale. Dotted line indicates the limit of detection (ED50=30). Data are mean of two independent experiments. (C) Quantification of SARS-CoV-2-specific T-cells responses using ELISpot at M3 and M6 in the indicated groups. Results were expressed as spot forming unit (SFU)/10^6 CD3+ T cells. CTL, controls; MTX, methotrexate; RTX, rituximab; IS, immunosuppressive.

variants in 100% of controls, 95% of sera from patients treated with rituximab did not neutralise these two variants. Of note, we observed that 50% of RTX-treated individuals have seroconverted despite an almost complete lack of neutralisation in this group. It is likely explained by our serological assay, which measures total anti-S antibodies (ie, targeting RBD and non-RBD epitopes). The hypothesis that RTX-treated seroconverters have an antibody response biased towards non-neutralising epitopes deserves further investigation. In contrast, SARS-CoV-2-specific T-cell response was similarly measured in controls and cases with the exception of methotrexate-treated patients. This differential impairment of immunogenicity after BNT162b2 vaccine according to the treatments received, mainly for rituximab and methotrexate, is critical to identify patients in which optimisation of vaccine strategies should be evaluated.

To counteract this impaired immunogenicity, the administration of a third dose of mRNA-based vaccine has been proposed. Recent data in solid-organ transplant recipients showed that a third dose of BNT162b2 vaccine increased the prevalence of seroconversion and antibody titres, without serious adverse events.[25–27] A third dose also increased specific cellular response even in patients who remained seronegative, but the impact of this cellular response remains to be determined.[27] We analysed B-cell and T-cell responses at 6 months in 40% of our immunocompromised patients having received a third dose of vaccine. A third dose of vaccine had no effect on B-cell response in patients treated with rituximab but it significantly increased anti-spike IgG levels and neutralisation activity against both variants in patients with methotrexate and cDMARDs compared with those receiving only two doses. In a cohort of 33 patients treated with rituximab who did not respond to two injection, only 21% harbour neutralising antibodies after a booster vaccination.[28] The discrepancy in response is most likely due to variation in the extent of B-cell depletion as suggested by other studies.[20 29 30] Our results are in line with these observations, and suggest that a third dose is needed, mainly in patients with low responses after two doses, but not sufficient, in most RTX-treated individuals. Finally, a third dose increased levels of specific T-cells in the 'rituximab group', whereas methotrexate still dramatically impaired T-cell responses after three doses.

Our study has several limitations. The findings are observational and based on small numbers and should be interpreted with caution. Differences in treatment groups were highly associated with the type of underlying inflammatory disease, and there may be differences among the populations. Especially, 82% of patients on rituximab were patients with antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis, limiting the generalisation of the findings to patients with rheumatoid arthritis. However, except for more frequent renal involvement at diagnosis in the 'rituximab' group and younger age in the 'immunosuppressive drugs' group, patients' characteristics were comparable between treatment groups. Lastly, ELISpot is a less sensitive assay than intracellular staining and could have played a role if in the detection of T-cell response.

Overall, we found that rituximab and methotrexate differentially impact the immunogenicity of BNT162b2 vaccine, by impairing B-cell and T-cell responses, respectively. The Delta variant fully escapes the suboptimal humoral response of individuals treated with rituximab. Our findings support efforts to improve effectiveness of mRNA vaccines in this immunocompromised population.

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Acknowledgements We thank all the patients who participated and all the nurses who performed the samples. We thank all the clinicians of the department who included patients in the study.

Collaborators Philippe Blanche, Benjamin Chaigne, Pascal Cohen, Nathalie Costedoat-Chalumeau, Bertrand Dunogué, Laure Frumholtz, Gaelle Guettrot-Imbert, Véronique Le Guern, Loic Guillevin, Claire Le Jeunne, Xavier Puechal, Tali-Anne Szwebel

Contributors All authors contributed to manuscript preparation. BT is responsible for the overall content as the guarantor. JH and BT contributed to the study design. DP, IS and TB performed antibody measurement, neutralisation assay and data analysis. AO and LC contributed to cellular assays, data analysis and manuscript preparation. JH, BT, DP, TB, LD, YN, SB, OS and LC performed data analysis. LD and MCS performed lymphocytes phenotyping.

Funding This study was supported by the Fonds IMMUNOV, for Innovation in Immunopathology. Work in OS lab is funded by Institut Pasteur, Urgence COVID-19 Fundraising Campaign of Institut Pasteur, Fondation pour la Recherche Médicale, ANRS, the Vaccine Research Institute (ANR-10-LABX-77), Labex IBEID (ANR-10-LABX-62-IBEID), ANR/FRM Flash COVID-19 PROTEO-SARS-CoV-2 and IDISCOVR. DP is supported by the Vaccine Research Institute

Competing interests TB, IS and OS are coinventors on provisional patent no. US 63/020,063 entitled 'S-Flow: a FACS-based assay for serological analysis of SARS-CoV-2 infection' submitted by Institut Pasteur.

Patient consent for publication Not applicable.

Ethics approval Ethics approval was obtained by the local ethics committee Comité de Protection des Personnes Nord-Ouest II. The Study was registered under the clinical trial number NCT04870411.

Provenance and peer review Not commissioned; externally peer reviewed.

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

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EPIDEMIOLOGICAL SCIENCE

Accounting for missing data caused by drug cessation in observational comparative effectiveness research: a simulation study

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Handling editor Josef S Smolen

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221477).

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Received 10 September 2021
Accepted 20 December 2021
Published Online First 13 January 2022

To cite: Mongin D, Lauper K, Finckh A, *et al*. *Ann Rheum Dis* 2022;**81**:729–736.

ABSTRACT

Objectives To assess the performance of statistical methods used to compare the effectiveness between drugs in an observational setting in the presence of attrition.

Methods In this simulation study, we compared the estimations of low disease activity (LDA) at 1 year produced by complete case analysis (CC), last observation carried forward (LOCF), LUNDEX, non-responder imputation (NRI), inverse probability weighting (IPW) and multiple imputations of the outcome. All methods were adjusted for confounders. The reasons to stop the treatments were included in the multiple imputation method (confounder-adjusted response rate with attrition correction, CARRAC) and were either included (IPW2) or not (IPW1) in the IPW method. A realistic simulation data set was generated from a real-world data collection. The amount of missing data caused by attrition and its dependence on the 'true' value of the data missing were varied to assess the robustness of each method to these changes.

Results LUNDEX and NRI strongly underestimated the absolute LDA difference between two treatments, and their estimates were highly sensitive to the amount of attrition. IPW1 and CC overestimated the absolute LDA difference between the two treatments and the overestimation increased with increasing attrition or when missingness depended on disease activity at 1 year. IPW2 and CARRAC produced unbiased estimations, but IPW2 had a greater sensitivity to the missing pattern of data and the amount of attrition than CARRAC.

Conclusions Only multiple imputation and IPW2, which considered both confounding and treatment cessation reasons, produced accurate comparative effectiveness estimates.

Key messages

What is already known about this subject?

- Attrition, defined as an increased selection due to participants leaving the study, is a source of bias for comparative effectiveness research, but it is often not acknowledged.
- Several imputation methods exist to account for attrition in comparative effectiveness research with observational data, but comparison of these methods is lacking

What does this study add?

- This study provides an extensive comparison of the statistical methods used to compare treatment effectiveness in an observational setting in the presence of attrition.
- Omitting to consider confounding, treatment cessation or dropouts leads to biased estimation of effectiveness. The present article provides advices, methods and code to report effectiveness properly.

How might this impact on clinical practice or future developments?

- This work suggests that comparative effectiveness results in observational data may have large over or underestimation depending on the method used.

INTRODUCTION

In rheumatology, as in other specialties, randomised controlled trials are the gold standard when evaluating treatment efficacy. However, because of the highly selected populations, their conclusions are difficult to generalise to routine clinical practice. For this reason among others, evaluation of the effectiveness of treatments in the real-world patient population is needed.[1] Comparative effectiveness between treatments when using observational real-world data requires overcoming several difficulties.

In addition to confounding, a recurring difficulty is missing data.[2] The data used to define drug effectiveness can be missing at the follow-up time of interest, while patients are still on treatment, and the missing data may have to be imputed to avoid selection bias,[3] using proper imputation methods.[4 5] But the data of interest can also be missing because of attrition (an increasing selection due to participants leaving the study).[6–8] Considering effectiveness among those remaining on therapy after a certain set of time (complete case analysis; CC[9]) is known to be a source of bias,[3 10] because it excludes from the analysis patients who stopped the drug for an adverse event or lack of effect, thus resulting in a selection bias in favour of responders.[11] Although 'intention to treat' analysis intends to avoid this bias in controlled trials,[12] there is no consensus regarding how this should be handled in an observational study.[13] Several statistical methods exist allowing to account for both attrition and confounding, such as inverse probability weighting (IPW)[7 14] or multiple imputation (MI) of the outcome.[15 16] In rheumatic diseases cohort studies, a popular method to account

for potential attrition bias is the LUNDEX index.[17] Analogously to non-responder imputation (NRI),[18] it corrects for the attrition bias by assuming that all patients stopping their treatment are non-responders. It, therefore, multiplies the estimation of effectiveness by the estimate of each drug survival, which may result in underestimating true drug effectiveness.

Depending on how it is handled, attrition may lead to biased conclusions in comparative effectiveness research. Therefore, the characterisation and comparison of existing statistical methods are needed. The reasons to stop the treatment are often available in registers and, to our knowledge, are generally not employed in standard statistical analyses when accounting for attrition.[8]

The aim of this research is, thus, to perform an extensive simulation study to compare the ability of different statistical approaches to account for missing data caused by attrition, when studying comparative effectiveness using rheumatology observational data.

METHODS

Our study simulates a comparative effectiveness study of two treatments in patients with potentially different baseline characteristics and attrition rates. In this simulation, we compare the effectiveness of tumour necrosis factor inhibitors (TNFi) versus a biologic disease-modifying antirheumatic drugs (bDMARD) with another mode of action (OMA) in patients with rheumatoid arthritis (RA) using the clinical disease activity index (CDAI) definition of low disease activity (LDA) at 1-year follow-up as outcome. We adjust for the following characteristics at treatment start (hereafter referred to as baseline covariates): disease duration, concomitant treatment with conventional synthetic DMARD (csDMARD), the number of previous bDMARDs (prev_bDMARD), and CDAI ($CDAI_0$). A collection of registers is used to generate a single simulation dataset with all CDAI values at 1 year. The simulation study consists in generating, in four iterative steps, missing data of CDAI at 1 year caused by attrition to then compare how different statistical methods estimate effectiveness. The sensitivity of the results is studied by changing the amount and the pattern of the missing data.

Creation of the simulation dataset

An original data set composed of a collaboration of RA registers including TNFi and OMA, including >45 000 treatment courses[19] (see table 1) was used to construct a realistic open cohort simulation data set composed of 10 000 treatment courses. The variables of this simulation data set were treatment (TNFi or OMA), CDAI value at treatment start and at 12 months ($CDAI_0$, $CDAI_{12}$, respectively), all confounders cited above and the treatment status, which indicates the last available status about the patient' treatment. It is equal to 'ongoing' if the patient is still on treatment at the time of data extraction and to the reason for treatment cessation otherwise (see online supplemental material for more details).

The simulation data set was constructed to have the same proportion of treatments (OMA and TNFi) as the whole data collection. For each treatment group, $CDAI_0$, disease duration, prev_bDMARD, csDMARD and the treatment status were independently randomly drawn from the entire collection. The treatment duration values in the simulation data set were then randomly sampled while matching exactly the treatment, the treatment status, and prev_bDMARD. $CDAI_{12}$ was then randomly sampled from the entire data set while matching exactly the treatment, the treatment status, prev_bDMARD, and the categories of CDAI0 (see online supplemental figure 1 for a graphical representation of the data generation). By doing so, we recreated the association between $CDAI_{12}$ and the reason to discontinue using the reasons to discontinue that happened between 12 and 36 months in the original register collection. Our simulation, thus, imposed this same association between $CDAI_{12}$ and discontinuations before 12 months. In the resulting data set for the 12-month follow-up, more than 55% of the treatment status are 'ongoing' (table 2).

Table 1 Characteristics of the patients under treatment in the initial real-world register collection

	Other mode of actions	*TNF inhibitor*
N	6067	40 767
Disease duration (median (IQR) in year)	9.8 (4.6, 17.9)	7.4 (2.9, 14.6)
Treatment duration (median (IQR) in year)	1.2 (0.5, 2.7)	1.7 (0.6, 4.3)
Number of previous bDMARD (number (%))		
0	1451 (23.9)	23 016 (56.5)
1	2019 (33.3)	12 269 (30.1)
2	1383 (22.8)	3896 (9.6)
3+	1214 (20.0)	1586 (3.9)
Concomitant csDMARD (number (%))		
MTX	2982 (49.2)	20 062 (49.2)
MTX +other	68 (1.1)	1080 (2.6)
None	1907 (31.4)	15 047 (36.9)
Other	1110 (18.3)	4578 (11.2)
$CDAI_0$ (median (IQR))	23.4 (16.5, 32.0)	23.0 (13.9, 33.5)
$CDAI_{12}$ (median (IQR))	10.0 (5.0, 17.3)	7.0 (2.9, 14.0)
Treatment status (number (%))		
Ongoing	3336 (55.0)	24 968 (61.2)
Stopped for adverse event	459 (7.6)	1876 (4.6)
Stopped for ineffectiveness	748 (12.3)	3369 (8.3)
Stopped for pregnancy	5 (0.1)	53 (0.1)
Stopped for remission	62 (1.0)	189 (0.5)
Stopped for other	313 (5.2)	1089 (2.7)
Stopped for unspecified reason	1144 (18.9)	9223 (22.6)
Missing $CDAI_{12}$ (number (%))	1680 (27.7)	9415 (23.1)
Number (%) of patients stopping after 1 year (number (%))	1017 (16.8)	6081 (14.9)
LDA at 12 month (number (%))	1811 (51.4)	15 873 (63.7)

Number of observations (N), number and proportion of patients having 0, 1, 2, 3 and more (3+) previous biological DMARD, of patient having methotrexate alone (MTX), methotrexate with other csDMARD (MTX +other), at least an csDMARD other than MTX (other) or no concomitant synthetic DMARD treatment (none), median (IQR) value of baseline CDAI ($CDAI_0$) and CDAI at 12 months ($CDAI_{12}$). CDAI, Clinical Disease Activity Index; csDMARD, conventional synthetic disease-modifying antirheumatic drugs; TNF, tumour necrosis factor.

Generation of missing data

Data can be missing completely at random when the probability of missing is independent of observed and unobserved data, missing at random (MAR) when the probability of missing depends on observed data, and missing not at random (MNAR) when the probability of missing depends on unobserved data. The patients

Table 2 Characteristics of the patients in the simulated dataset

	Other mode of action	*TNF inhibitor*
N	1295	8705
Disease duration (median (IQR) in year)	9.7 (4.5, 17.8)	7.3 (2.9, 14.3)
Treatment duration (median (IQR) in year)	1.2 (0.5, 2.7)	1.6 (0.5, 4.2)
Number of previous bDMARD (number (%))		
0	326 (25.2)	4973 (57.1)
1	409 (31.6)	2618 (30.1)
2	294 (22.7)	775 (8.9)
3+	266 (20.5)	339 (3.9)
Concomitant csDMARD (number (%))		
MTX	611 (47.2)	4330 (49.7)
MTX +other	18 (1.4)	248 (2.8)
None	427 (33.0)	3143 (36.1)
Other	239 (18.5)	984 (11.3)
$CDAI_0$ (median (IQR))	23.2 (16.4, 32.4)	23.0 (14.0, 33.3)
$CDAI_{12}$ (median (IQR))	10.2 (5.3, 17.0)	8.0 (3.4, 15.1)
treatment status (number (%))		
Ongoing	740 (57.1)	5355 (61.5)
Stopped for adverse event	95 (7.3)	407 (4.7)
Stopped for ineffectiveness	159 (12.3)	755 (8.7)
Stopped for pregnancy	1 (0.1)	11 (0.1)
Stopped for remission	17 (1.3)	41 (0.5)
Stopped for other	57 (4.4)	215 (2.5)
Stopped for unspecified reason	226 (17.5)	1921 (22.1)
LDA	639 (49.3)	5210 (59.9)

Number of observations (N), number and proportion of patients having 0, 1, 2, 3 and more (3+) previous biological DMARD, of patient having methotrexate alone (MTX), methotrexate with other csDMARD (MTX +other), at least an csDMARD other than MTX (other) or no concomitant synthetic DMARD treatment (none), median (IQR) value of age, body mass index (BMI), baseline CDAI ($CDAI_0$) and CDAI at 12 months ($CDAI_{12}$).
CDAI, Clinical Disease Activity Index; csDMARD, conventional synthetic disease-modifying antirheumatic drugs; LDA, low disease activity; TNF, tumour necrosis factor.

whose CDAI values at 12 months were set to missing in the simulated data set were chosen by sampling from the patients having a treatment status different from 'ongoing' with the conditional probability of having a missing $CDAI_{12}$ value in the original real data. These probabilities were extracted from a generalised linear model applied on the initial 'real-world' register collection estimating the probability of having a missing outcome as a function of the treatment, prev_bDMARD, csDMARD, $CDAI_0$, $CDAI_{12}$ and the treatment status. Before applying this generalised linear model, all predictors were imputed using MI by chained equation (mice) with 40 iteration, 40 samples and predictive mean matching.

Different ways of deleting data were applied, leading to different types of missing patterns for the different estimation methods considered (see table 3):

- A reference scenario (missingness condition 1) with 30% treatment cessation in both treatments and no association between effectiveness at 1 year and treatment cessation.
- To test the dependence of the observed comparative effectiveness on the amount of attrition, the proportion of missing $CDAI_{12}$ was set to 10%, 15%, 20%, 25% and 30% in OMA only, and then in both treatments.
- To assess the sensitivity of the estimators to MNAR data, we changed the association between $CDAI_{12}$ values and missingness of $CDAI_{12}$ caused by attrition by modifying the OR for $CDAI_{12}$ yielded by the generalised linear model predicting the missing values of $CDAI_{12}$ (ie, the effect of $CDAI_{12}$ values on the odds of having a missing $CDAI_{12}$). It was set to 1 for the reference treatment, and to 1.07 or 1.14 (probability of having a $CDAI_{12}$ missing multiplied by 2 or 4 for an increase in 10 points of $CDAI_{12}$) for OMA only, and then in both treatments.

For treatment courses with $CDAI_{12}$ set to missing, the treatment durations were imputed with plausible values (treatment duration between 0 and 12 months) using MI with predictive mean matching, including $CDAI_0$, disease duration and treatment as covariates.

Simulation

For each condition, the simulation consisted in generating 1000 samples with missing data caused by treatment cessation and estimating the difference in LDA proportion between the two treatments with different statistical methods.

We report bias as the difference between the value estimated in the simulation sample and the true value. The true value is defined here as the LDA rate given by the treatment effect of a linear model predicting LDA on the complete simulation data set (ie, before inducing missing values) adjusting for baseline covariates. In addition, we estimate coverage as the percentage of CIs in the simulation samples which included the true value.

All code to generate simulation data, estimate measures of LDA, and generate the manuscript figures and tables is available in a repository.[20] All the simulation, statistical analysis and figures were made in R V.4.1.0,[21] using the library *ipw*[22] for IPW, *mice*[23] for MI with chained equation, *geepack*[24] for the generalised estimating equations.

Methods to estimate the LDA

The dataset analysed contained one line per patient's treatment course. All methods used a generalised linear model with Huber-White robust standard errors predicting a binary outcome as a function of the baseline covariates (referred hereafter as the adjusted model).[25–27] In line with Cheung and co-authors[26 27], we use a Gaussian identity link, and the coefficient for treatment provides the increase of LDA rate compared with the reference treatment. The following estimation methods were considered.

Complete case analysis

CC consists of restricting the analysis only to available data. We considered here the adjusted estimation of the LDA difference between treatments.

Last observation carried forward

All the missing $CDAI_{12}$ values were set to the last available value of CDAI, which could be the baseline value. The LDA rate is then estimated with the adjusted model.

LUNDEX

LDA rate ($P_{LDA\text{-}LUNDEX}$) is estimated by the proportion of patients reaching LDA (P_{LDA}) obtained by the adjusted model multiplied by the Kaplan Meyer estimates of the drug survival P_{LDA} at the time of outcome evaluation[17] :

Table 3 Missingness conditions for the simulation, 1 being the reference condition

Condition	Attrition for OMA (%)	Attrition for TNFi (%)	$CDAI_{12}$ OR for OMA	$CDAI_{12}$ OR for TNFi	Missingness for CC, LOCF, LUNDEX, NRI, IPW1	Missingness for IPW2 and CARRAC
1	20	20	1	1	MNAR	MAR
2	10	20	1	1	MNAR	MAR
3	15	20	1	1	MNAR	MAR
4	25	20	1	1	MNAR	MAR
5	30	20	1	1	MNAR	MAR
6	10	10	1	1	MNAR	MAR
7	15	15	1	1	MNAR	MAR
8	25	25	1	1	MNAR	MAR
9	30	25	1	1	MNAR	MAR
10	20	20	1.07	1	MNAR	MNAR for OMA, MAR for TNFi
11	20	20	1.14	1	MNAR	MNAR for OMA, MAR for TNFi
12	20	20	1.07	1.07	MNAR	MNAR
13	20	20	1.14	1.14	MNAR	MNAR

Attrition for OMA of TNFi indicates the percentage of missing CDAI at 12 months for the other mode of action treatment (OMA) of the reference treatment TNFi, $CDAI_{12}$ OR are the OR of the CDAI value at 12 months ($CDAI_{12}$) in the generalised linear model predicting missingness of $CDAI_{12}$ used to create missing values in the simulation. A CDAI12 OR of 1.07 or 1.14 implies that the odds of having a CDAI12 missing is multiplied by 2 or by 4 for an increase of 10 points of CDAI12. The column 'Missingness for CC, LOCF, LUNDEX, NRI, IPW1' indicates if the missing data are MNAR or MAR for the methods CC, LOCF, LUNDEX, NRI, IPW not accounting for the reasons for treatment cessation in the attrition weights (IPW1). The column 'Missingness for IPW2 and CARRAC' does the same for IPW accounting for the reasons for treatment cessation in the attrition weights (IPW2) and CARRAC.
CARRAC, confounder-adjusted response rate with attrition correction; CC, complete case analysis; CDAI, Clinical Disease Activity Index; IPW, inverse probability weighting; LDA, low disease activity; LOCF, last observation carried forward; MAR, missing at random ; MNAR, missing not at random ; NRI, non-responder imputation; OMA, another mode of action; TNF, tumor necrosis factor.

$$P_{\mathrm{LDA-LUNDEX}} = S_{\mathrm{KM}} \times P_{\mathrm{LDA}} \qquad \text{(equation 1)}$$

CIs were calculated by bootstrap using the quantiles of 1000 samples.

Non-responder imputation

All missing values caused by attrition were set to a non-responder value. The LDA proportion was then estimated using the adjusted model.

Inverse probability weighting 1 and 2

The inverse probability weights for treatment (ipwt) and for attrition (ipwc) were computed using a generalised linear model. Weights for treatment (ipwt) included the baseline covariates. Weights for censoring (ipwc) included the same baseline covariates in the first version of this method (IPW1), or additionally included the treatment status (IPW2). The LDA proportion was then computed using the adjusted model with weights equal to ipwt × ipwc.

Multiple imputation

Missing disease activity values at 12 months were imputed using MI using chained equations (mice) with the predictive mean matching algorithm, with baseline covariates and the treatment status.

For each of the imputed samples, the LDA difference between treatments was calculated with the adjusted model. The overall estimate and its SE are then pooled from the 10 mice samples using Rubin's rule.[28] The overall method is hereafter named confounder-adjusted response rate with attrition correction (CARRAC).

RESULTS

In the reference scenario, CARRAC and IPW2 provided almost unbiased LDA for each treatment (figure 1), thereby estimating almost unbiased LDA difference for these two methods. CC and IPW1 overestimated LDA in both treatments. As their overestimations were similar in OMA and in the reference treatment, the absolute LDA difference was almost unbiased. Last observation carried forward (LOCF), LUNDEX and NRI strongly underestimated LDA in both treatments. Because the underestimation was much lower in OMA, the absolute LDA difference was underestimated by these methods. The coverage was 95% or above in individual treatments only with CARRAC, but due to their smaller bias when considering the LDA difference, the two IPW methods and CC retained good coverage for the comparative effectiveness.

When increasing the association between missingness and the true value of effectiveness at 1 year for both treatments (figure 2A and online supplemental figure 2), LUNDEX, NRI, IPW1, LOCF and CC estimations were almost unaffected, while IPW2 and CARRAC started to overestimate the absolute LDA difference. When the association between missingness of $CDAI_{12}$ and $CDAI_{12}$ values existed only in one treatment (figure 2B and online supplemental figure 3), all methods overestimated the difference in LDA, leading to inadequate coverage.

When changing the amount of missingness in both treatments, CARRAC, CC and IPW1 provided unchanged LDA difference estimation when compared with the reference situation (figure 2C and online supplemental figure 4). IPW2 estimations remained approximately unbiased, but their dispersion increased significantly. The bias yielded by NRI and LUNDEX, and to a lesser extent by LOCF, was increased when the amount of missing data increased equally in both treatments.

Increasing the amount of missingness in OMA but not in TNFi decreased the absolute LDA difference between the treatments provided by CC and IPW1, while the LDA proportion difference yielded by LOCF, LUNDEX and NRI increased strongly (figure 2D and online supplemental figure 5). IPW2 stayed unbiased but started to produce more dispersed estimations when the percentage of missingness was 30%. CARRAC estimations

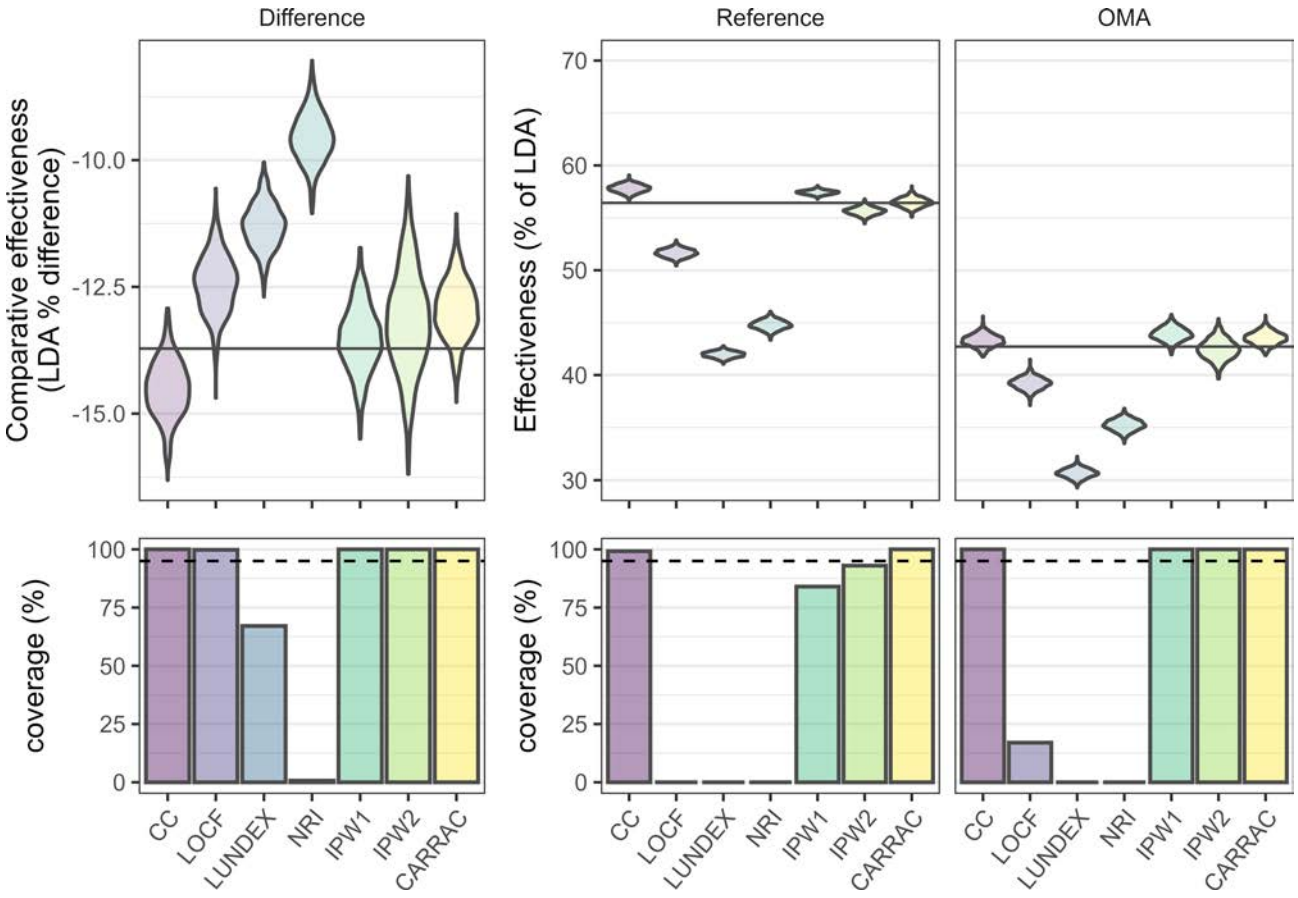

Figure 1 Distribution of the effectiveness measured by the low disease activity (LDA) proportion (upper panels) and the associated coverage (percentage of CIs in the simulation samples which include the true value, lower panels) for each treatment (reference and another mode of action (OMA)—other modes of action, middle and right panels) and for the difference between the treatments (difference, left panels). The methods analysed are complete case analysis (CC), last observation carried forward (LOCF), LUNDEX, non-responder imputation (NRI), inverse probability weighting accounting for the reasons for treatment cessation in the attrition weights (IPW2) or not (IPW1), and confounder-adjusted response rate with attrition correction by reason for drug cessation (CARRAC). The widths of the violins are fixed, so the area of the violin does not represent the number of counts. The true value is represented as a black horizontal line.

of LDA difference slightly underestimated the absolute true difference when increasing the amount of attrition but retained coverage for all missingness conditions.

DISCUSSION

Using a simulation data set generated from a real-world data collection, the present simulation study addresses the issue that some patients may stop the treatment not because it does not work but for some complex reasons including adverse events, remission, etc, which could be taken into account to accurately assess effectiveness. We, therefore, investigated several methods used to compare the response rates of two treatments in presence of confounding and attrition. We focused on attrition, by manipulating the missingness pattern to create greater treatment discontinuation in one treatment versus the other or by increasing the risk of treatment discontinuation. Because observational studies usually use adjusted models to estimate the causal effect of treatments, we used as the true estimate the difference in LDA proportion based on an adjusted model applied on the simulation data set without missing data.

We first observed that the bias in the difference of effectiveness was always lower than the strongest bias observed for the effectiveness estimated in individual treatments. This will always be the case if the estimation of effectiveness is biased in the same direction in both treatments. As expected, methods including a model for attrition and based on MIs and IPW performed well[29 30] as long as missingness was dependent only on known covariates (MAR data), but not on unmeasured information (MNAR data). Although an important part of the treatment cessation reasons was unknown, this information was still valuable when estimating effectiveness in the presence of attrition. Including the treatment status in the calculation of the censoring weights permitted IPW to estimate more precisely LDA for each treatment. On the other hand, it led to a higher sensibility to the amount of attrition and to the association between missingness and effectiveness value. This result highlights the importance of model specification for the missingness pattern and the difference between IPW and MI. For instance, the model used for missingness in IPW did not include an interaction term between treatment and variables predicting effectiveness at 1 year (such as baseline disease activity for instance), thus misspecifying the differential effect introduced in the data. MI using predictive mean matching, in the CARRAC method, is less sensitive to misspecification because the model defines the distribution of missing data, which has less variation between the treatments.

CC and IPW1 were biased in each individual treatment, indicating a persistent association between $CDAI_{12}$ values and their missingness. This association, reflecting the fact that patients remaining on treatment tend to have a better response than those stopping,[31] caused the LDA estimated by CC in each

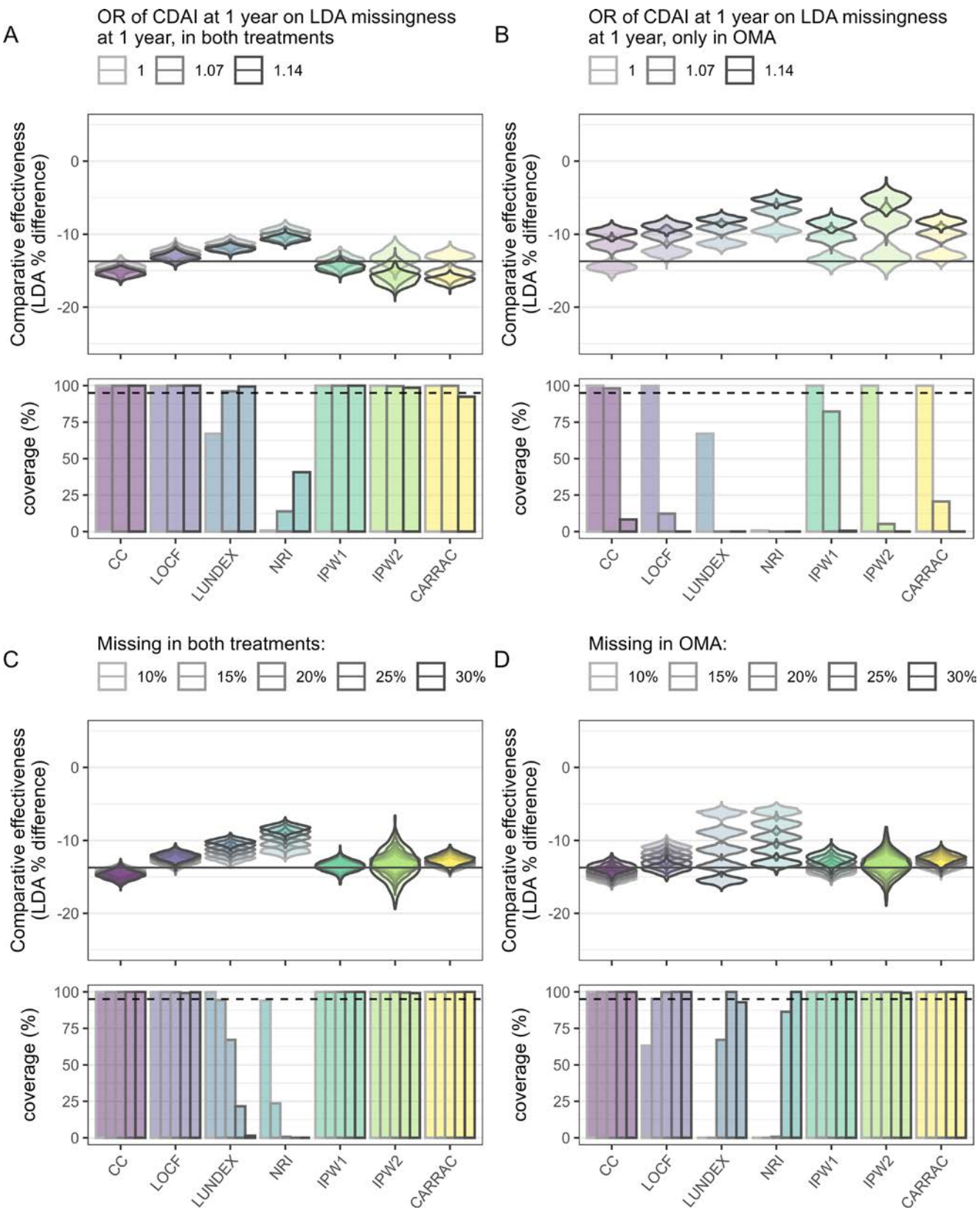

Figure 2 Low disease activity (LDA) difference between treatments and the associated coverage (percentage of CIs in the simulation samples that include the true value) of the reference situation (condition 1) and when having missing not at random in both treatments (condition 12, A), when having missing not at random in one treatment (conditions 10 and 11, B), when having a changing proportion of missing due to attrition in both treatments conditions (conditions 6–9, C) and when having a changing proportion of missing due to attrition in only one treatment (conditions 2–5, D). The methods analysed are complete case analysis (CC), last observation carried forward (LOCF), LUNDEX, non-responder imputation (NRI), inverse probability weighting accounting for the reasons for treatment cessation in the attrition weights (IPW2) or not (IPW1) and confounder-adjusted response rate with attrition correction by reason for drug cessation (CARRAC). The widths of the violins are fixed, so the area of the violin do not represent the number of counts. The true value is represented as a black horizontal line.

treatment to increase with the amount of missing data at 1 year or with the increase in association between CDA_{12} values and their missingness. IPW1 did so to a smaller extent, as its model partially accounts for the attrition. When the amount of attrition was similar in the two treatments, the biases were similar, and these two methods correctly estimated the difference in LDA. But when the missingness differed between the two treatments, these methods yielded strongly biased estimations of the differential effectiveness.

LOCF, LUNDEX, and NRI underestimated LDA in each treatment. LOCF assumes that the missing values of CDAI are identical to the last available values, although disease activity is known to globally decrease with time.[32 33] LUNDEX and NRI considered all patients without information at 1 year as non-responders, although studies show that treatment cessation is not only due to ineffectiveness[34] but also due to various other reasons, such as adverse events, pregnancy, or even remission. Therefore, when increasing the proportion of missing values, the LDA proportion yielded by NRI and LUNDEX converge to 0%, which is the LDA when all data at 1 year are missing, and the one provided by LOCF converge to an intermediate value comprised between the baseline proportion of LDA and the one at 1 year.

Three main groups of methods with different ways of handling missing data caused by attrition emerged from our discussion: those including a model for attrition (IPW2 and CARRAC), those considering patients who stopped treatment as non-responders (LUNDEX and NRI) or as keeping the same disease activity in time (LOCF) and those not adjusting for attrition (IPW1 and CC). Within each group, these methods did not handle confounding the same way either, thus causing a residual difference between them.

Strength and limitations

The use of data stemming from a real register to generate a simulation data set is a strength of this study, as it allowed to test the statistical methods on close to real-world data. The large variety of methods studied and the release of the code on an open-access repository are also assets to the present work, which guarantee the accessibility of the methods. These methods are also generalisable for any disease or treatments, where treatments may be stopped for different reasons. However, as in every clinical study, the quality of the imputation will depend on model specification, so careful thought should be given to the covariates included in the model for each particular case. Nevertheless, a limitation of any simulation study is that results depend on the model used to generate the data. In the case of this study, our simulation design favours both CARRAC and IPW2, as they are the only ones making use of the treatment status used to generate the data. Thus, we may underestimate the impact of model misspecification. Another limitation is the use of only baseline disease activity, baseline confounders, and reason for stopping to estimate response rate. Though this choice corresponds to the reality of registry data, which usually have few intermediate visits, further models including repeated measurements could be informative. Finally, we used the association between the disease activity at 1 year and the reasons to stop in the future to recreate in the simulation data set the link between the reasons to stop before 1 year and the value of CDAI a patient would have had at 1 year. This procedure may underestimate the real association between reasons for cessation and disease activity. Therefore, the difference between the statistical methods presented here may be more pronounced in real applied analysis.

CONCLUSION

Correct estimation of effectiveness requires considering confounding, treatment cessation and dropouts. While CARRAC and IPW can produce proper estimates, methods omitting one of those, such as LUNDEX, NRI or CC, yield biased estimation, depending on the amount of attrition in the treatments. While the choice of methods is important, and some methods make stronger assumptions than others, model specification remains crucial. Careful justification of the model used for both missingness and confounding is necessary to obtain trustworthy and accurate results.

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Acknowledgements The authors thank all the rheumatologists and patients who participated in the registries used to initially create the simulation dataset.

Contributors DM performed the simulation study, the data analysis, participated in the interpretation of the results, and designed and wrote the manuscript; KL participated in the study design and contributed to the interpretation of the results; AF contributed to the interpretation of the results; TF contributed to the study design and to the interpretation of the results; DSC designed the study, contributed to the implementation of the simulation study and the data analysis, participated in the interpretation of the results and is the garantor of this work. All authors revised critically the manuscript and approved its current version.

Funding This project was supported by an unrestricted grant from AbbVie.

Competing interests None declared.

Patient and public involvement statement Because the present work is a methodological simulation study, it was not appropriate to involve patients or the public in its design, conduct or reporting.

Patient consent for publication Not applicable.

Ethics approval Because the study is a retrospective study using completely deidentified data, it was exempted from ethical approval. Approval of each local ethical committee for the collection of clinical data in each register was obtained for the register collection used to create the simulation dataset.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. The simulated dataset and all the code used to generate the data, perform the simulation study and create the figures and tables have been made openly available in the following repository: https://gitlab.com/dmongin/comparative-effectiveness. Concerning the original register collection used to create the simulation dataset, all the data belong to the registries. Anonymised data can be shared as long as agreements are made with all participating registries.

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Attenuated response to fourth dose SARS-CoV-2 vaccination in patients with autoimmune disease: a case series

Severe, occasionally fatal breakthrough COVID-19 infections despite vaccination have been reported in patients with autoimmune disease,[1] bringing vaccine efficacy in this population into question. Recently, the Food and Drug Administration authorised a third vaccine dose in immunocompromised patients who previously received two mRNA vaccines. We previously reported augmented antibody titers in 89% of patients with autoimmune disease after third SARS-CoV-2 vaccination dose[2]; herein, we describe antibody response in patients who received two additional SARS-CoV-2 vaccine doses after completion of initial series.

Patients with autoimmune diseases were recruited for our observational study as previously reported.[3] We identified 18 patients ≥18 years of age who completed initial SARS-CoV-2 vaccine series (mRNA or adenovirus vector) and subsequently obtained two additional doses (AD) of SARS-CoV-2 vaccine between 30 April 2021 and 8 July 2021, six of whom were included in a previous report on response after three dose-vaccination.[2] Participants with prior COVID-19 infection were excluded. Serial semiquantitative SARS-CoV-2 antibody testing

Table 1 Vaccines administered, autoimmune diagnoses, immunosuppressive regimens and perivaccination management with serial antispike antibody responses

Age/sex	Diagnosis	Immunosuppression	Initial vaccine series	Meds held or modified preinitial Vaccine	Pre-AD1 antibody U/mL*	Additional vaccines	PostAD1 antibody U/mL*	PostAD2 antibody U/mL*	Therapy held periAD†
62F	Myositis	Mycophenolate‡ Prednisone	Pfizer	No	Negative§	AD1: Pfizer AD2: Pfizer	–	<0.4	No
56F	Mucous membrane pemphigoid	Mycophenolate‡	Pfizer	No	<0.4	AD1: J&J AD2: Moderna	<0.8	<0.4	No
67F	Systemic sclerosis	Mycophenolate‡	Pfizer	No	<0.4	AD1: Pfizer AD2: Pfizer	<0.4	2.1	Yes
73M	Myasthenia gravis	Mycophenolate‡ Prednisone	Moderna	No	<0.4	AD1: Pfizer AD2: Pfizer	–	21.8	–
44F	Inflammatory arthritis¶	Abatacept Hydroxychloroquine Methotrexate Prednisone	Pfizer	No	<0.4	AD1:Pfizer AD2: J&J	<0.4	27.1	Yes
55M	Inflammatory arthritis¶	Infliximab Mycophenolate‡	Pfizer	No	Negative§	AD1: Pfizer AD2: Pfizer	<0.8	46.5	Yes
64F	Myositis	Mycophenolate‡	Pfizer	No	Negative§	AD1: Pfizer AD2: Pfizer	38.1	120.9	Yes
53F	Inflammatory arthritis¶	Adalimumab Mycophenolate‡ Prednisone	Pfizer	No	<0.4	AD1: Moderna AD2: Moderna	229	134	No
55M	Sarcoidosis	Infliximab Mycophenolate‡ Prednisone	Pfizer	No	<0.4	AD1: Moderna AD2: Moderna	2.40	1276	Yes
40F	Inflammatory bowel disease	Adalimumab Hydroxychloroquine Methotrexate	Pfizer	No	178.4	AD1: Moderna AD2: Pfizer	601.2	1750	No
49F	Overlap CT disease**	Belimumab Methotrexate Prednisone	Pfizer	No	<0.4	AD1: Pfizer AD2: Pfizer	16.4	>2500	–
68F	Proliferative nephritis	Mycophenolate‡ Prednisone	Moderna	No	<0.4	AD1: J&J AD2: Moderna	714	>2500	–
53F	Sjögren's syndrome	Azathioprine	J&J	No	<0.4	AD1: Pfizer AD2: Pfizer	>250	>2500	Yes
55F	Minimal change disease	Mycophenolate‡	J&J	No	<0.4	AD1: Moderna AD2: Moderna	>2500	>2500	No
74M	Myositis	Mycophenolate‡	Pfizer	No	Negative§	AD1: Moderna AD2: Moderna	>2500	>2500	Yes
42F	Overlap CT disease**	Hydroxychloroquine Mycophenolate‡	Pfizer	No	<0.4	AD1: Pfizer AD2: Pfizer	–	>2500	Yes
65F	Inflammatory arthritis	Abatacept	J&J	No	Negative§	AD1: Pfizer AD2: Pfizer	–	>2500	Yes
52M	Overlap CT disease**	Hydroxychloroquine Mycophenolate‡	J&J	No	18.6	AD1: Pfizer AD2: Pfizer	>2500	>2500	Yes

- denotes missing data.
*Roche Elecsys anti-RBD pan-Ig≥0.8 units/mL is considered positive (upper ceiling expanded from>250 to >2500 U/mL per manufacturer).
†Pre-AD1 median number of doses held for mycophenolate 6, 23 doses of azathioprine held by one patient, and two abatacept infusion held by one patient. Pre-AD2 median number (IQR) of doses of mycophenolate 14 (10–14), 23 doses of azathioprine and 2 abatacept infusion held by one patient.
‡Mycophenolate includes mycophenolic acid and mycophenolate mofetil.
§Self-reported values.
¶Rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, reactive arthritis, or inflammatory bowel disease associated arthritis.
**Denotes a combination of two or more defined rheumatic diagnoses.
AD, additional dose; J&J, Johnson and Johnson; RBD, receptor binding domain.

was completed on the Roche Elecsys anti-SARS-CoV-2 S enzyme immunoassay, which measures total antibody to the SARS-CoV-2 S-receptor binding domain protein (positive ≥0.8 U/mL) and a consistent correlate of plasma neutralising capacity.[4] Participants provided informed consent electronically.

Thirteen participants were female, with a median (IQR) age of 56 (52–66) years (table 1). The most common autoimmune diagnoses included inflammatory arthritis (n=4), myositis (n=3) and overlap connective tissue disease (n=3). Participants completed initial vaccine series with two doses of Pfizer (n=11), Moderna (n=2) or single dose of Janssen/Johnson and Johnson (J&J) (n=5). Mycophenolate was the most common immunosuppressive therapy (13/18) with median (IQR) daily dose of 2500 mg (1125, 3000 mg). All participants reported continuation of immunosuppression without interruption or modification during the initial vaccine series.

There were 16/18 participants with negative anti-spike antibody response at a median of 84 (31–90) days after initial vaccine series. Participants reported the following additional vaccinations: AD 1 (AD1): Pfizer (n=11), Moderna (n=5), J&J (n=2), followed by AD 2 (AD2) of Pfizer (n=11) or Moderna (n=6) or J&J (n=1). Most participants (11/18) reported temporarily withholding of immunosuppressive therapy in the period surrounding the AD. Among those who completed antibody testing after AD1 (12/18), antispike antibodies increased above the threshold of positivity in eight participants and remained negative in two participants at a median (IQR) of 24 (14–31) days. Antibody testing was performed at a median (IQR) of 32 (28–34) days after AD2 in all participants, with median (IQR) antispike antibody titre of 1750 U/mL (26–2500). Both participants with persistently negative response reported use of mycophenolate and did not undergo perivaccination interruption of therapy.

This study has several limitations including small sample size, convenience sampling and lack of data on cellular response. Furthermore, most participants continued immunosuppressive therapy during initial vaccine series but modulated therapy around the time of AD which confounds results and limits interpretation of our findings; larger studies are required for systematic evaluation. We cannot exclude asymptomatic COVID-19 infection as we did not complete antinucleocapsid testing. Participants who initially received the J&J vaccine received a total of three doses while those who initially received mRNA vaccine received a total of four doses, which limits comparability. We did not routinely collect baseline disease activity or severity and the reason for participants receiving two AD, as opposed to a single AD, is unknown.

This is the first case series describing antibody responses to two AD of SARS-CoV-2 vaccines in patients with autoimmune disease on immunosuppression. While most patients demonstrated an augmented antibody response, our findings suggest that a subset of patients who do not withhold immunosuppression continue to have an impaired vaccine response despite four vaccine doses; this is similar to findings in other immunosuppressed populations.[5] Both non-responders reported use of mycophenolate and continued therapy during the peri-vaccination period, which is consistent with findings that temporary interruption in immunosuppression can augment the humoral response,[2 6] although, a recent case report demonstrated seroconversion following four vaccine doses without interruption of immunosuppression.[7] More studies are needed to identify patients who may benefit from antibody monitoring, refinement in vaccination schedule, adjustment of perivaccination immunosuppression, or other strategies such as prophylactic therapies to better protect this vulnerable population.

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Acknowledgements We would like to acknowledge the contributions of: Jake A. Ruddy BS, Allan B. Massie PhD, and Jacqueline M. Garonzik-Wang MD PhD.

Contributors Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work. MT, CMC, SF, BJB, JA, TP-YC, AAS, JA, LC-S, WAW, DLS and JJP. Drafting the work or revising it critically for important intellectual content. MT, CMC, SF, BJB, JA, TP-YC, AAS, JA, LC-S, WAW, DLS and JJP. Final approval of the version to be published MT, CMC, SF, BJB, JA, TP-YC, AAS, JA, LCS, WAW, DLS and JJP. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. CMC, MT, JA, DLS and JJP.

Funding This work was made possible by the generous support of the Ben Dov family. This work was supported by grant number F32DK124941 (Boyarsky), T32DK007713 (Alejo) from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), K24AI144954 (Segev), U01AI138897 and K23AI157893 (Werbel) from National Institute of Allergy and Infectious Diseases (NIAID), K23AR073927 (Paik) from National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS).

Disclaimer The analyses described here are the responsibility of the authors alone and do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organisations imply endorsement by the US Government.

Competing interests DLS has the following financial disclosures: consulting and speaking honoraria from Sanofi, Novartis, CSL Behring, Jazz Pharmaceuticals, Veloxis, Mallincrodt, Thermo Fisher Scientific. LC-S has the following financial disclosures: consultant fees from Janssen, Boehringer-Ingelheim, Mallinckrodt, EMD-Serono, Allogene and ArgenX. The other authors of this manuscript have no financial disclosures or completing interest to disclose as described by Annals of the Rheumatic Diseases.

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 applicable.

Ethics approval This study was approved by the Johns Hopkins Institutional Review Board (IRB00248540).

Provenance and peer review Not commissioned; externally peer reviewed.

MT and CMC contributed equally.

DLS and JJP are joint senior authors.

To cite Teles M, Connolly CM, Frey S, *et al*. *Ann Rheum Dis* 2022;**81**:738–740.

Received 8 October 2021
Accepted 6 January 2022
Published Online First 17 January 2022

Ann Rheum Dis 2022;**81**:738–740. doi:10.1136/annrheumdis-2021-221641

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Management of contemporary early undifferentiated arthritis: data on EULAR's recommendation on the risk of persistent disease

Early treatment initiation is crucial to improve long-term outcomes in rheumatoid arthritis (RA). This may also apply to undifferentiated arthritis (UA), patients at high risk of persistent arthritis/RA. Therefore, the EULAR recommendations for early arthritis recommend assessing the following risk factors for disease persistency in early UA: number of swollen joints, acute phase reactants (ie, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)), rheumatoid-factor (RF), anti-citrullinated protein antibodies (ACPA) and imaging findings/erosions.[1 2] This recommendation was based on markers identified as predictive in a systematic literature review.[2] Importantly however, prognostic research in UA is based on an outdated definition of UA: not meeting 1987-RA-classification criteria and having no alternative diagnosis ('conventional UA'). A proportion of these patients with conventional UA meet the 2010-RA-criteria and is currently considered to have RA.[3–5] Contemporary UA, in contrast, is defined as neither meeting the 1987-RA-criteria nor the 2010-RA-criteria and having no other clinical diagnosis. In addition, for some of the recommended risk factors data were lacking in the systematic literature review (polyarthritis) or it was concluded that adequately designed studies were lacking (CRP and ESR).[2] Since predictors may be disease stage or population dependent, and because predictors for persistent disease identified in conventional UA may not be applicable to contemporary UA, we conducted a large cohort study in contemporary UA to assess risk factors for persistent disease mentioned in the current EULAR recommendation. Conventional UA was studied for comparison.

In short, 710 patients with contemporary UA, not fulfilling 1987-RA-criteria or 2010-RA-criteria and having no alternative diagnosis, consecutively included in the Leiden Early Arthritis Clinic cohort between 2006–2019, when disease-modifying anti rheumatic drug (DMARD) start in UA was recommended, were studied (online supplemental figure 1). The cohort is described in detail elsewhere.[6] At inclusion swollen joint counts and laboratory procedures were performed, including: ACPA (EliA CPP (anti-CCP2),

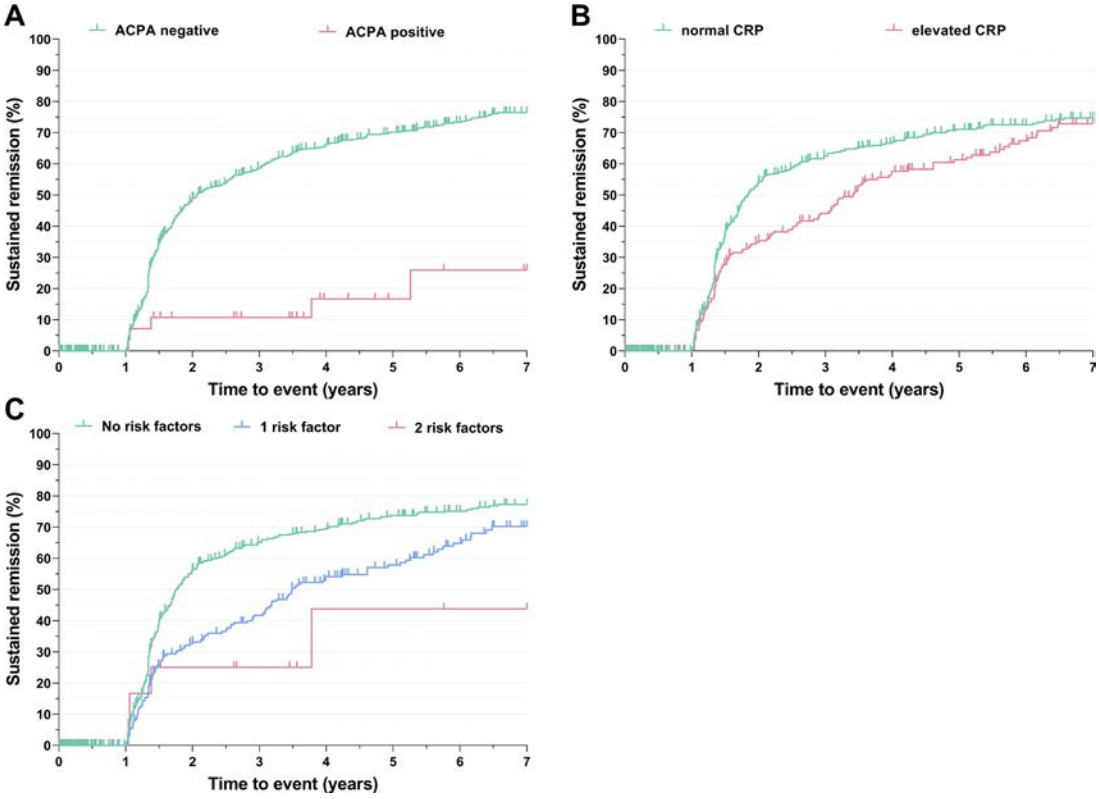

Figure 1 Cumulative incidence of sustained remission in contemporary undifferentiated arthritis for presence of ACPA positivity (A), elevated CRP (B), 0, 1 and 2 of these factors (C). (1A) Of all the patients with contemporary UA, 5% was APCA positive (≥10 U/mL); (1B) in total 31% of the patients with contemporary UA had an elevated CRP (≥10 mg/L); (1C) of all the patients with contemporary UA 67% had no risk factors, 31% had one risk factor and only 2% had two risk factors. ACPA, anti-citrullinated protein antibodies; CRP, C-reactive protein; UA, undifferentiated arthritis.

Phadia, the Netherlands, elevated ≥10 U/mL)), IgM-RF (in-house ELISA, elevated ≥5.0 IU/mL), CRP (elevated ≥10 mg/L) and ESR. Patients were assessed after 4 months, 12 months and yearly thereafter. The outcome was sustained remission, thus absence of disease persistence, defined as sustained absence of clinical synovitis without DMARDs (including corticosteroids) for at least 1 year and the entire follow-up. The cumulative incidence was visualised using Kaplan-Meier. Cox regression analysis was used to test risk factors. Radiographic erosions at baseline were rare (1.8%) and not included in analyses. Within the same inclusion period, 1004 patients with conventional UA were included (not fulfilling the 1987-RA-criteria and having no other diagnosis); analyses were also performed in this population.

Patients with contemporary UA presented with a median of two swollen joints and were mostly ACPA-negative (online supplemental table 1 for baseline characteristics). The median follow-up was 6 years (IQR 3–9 years). DMARDs were started by 48% of the patients. Sustained remission after a median of 1.5 years (IQR 1–3) was achieved by 60% of the patients with UA, after which they were followed for another 5.5 years (IQR 3–8) without recurrence of arthritis, demonstrating the sustained absence of disease. Univariable analyses showed that CRP, ESR, ACPA and RF were associated with time to sustained remission (HR 0.77 (95% CI: 0.62 to 0.95), HR 0.79 (95% CI: 0.64 to 0.97), HR 0.18 (95% CI: 0.08 to 0.44) and HR 0.49 (95% CI: 0.31 to 0.77), respectively), while polyarthritis (HR 0.76 (95% CI: 0.57 to 1.01)) was not statistically significant. In multivariable Cox regression ACPA (HR 0.095 (95% CI: 0.03 to 0.32); figure 1A) and CRP (HR 0.67 (95% CI: 0.50 to 0.91); figure 1B) remained significantly associated (online supplemental table 2). Assessing the number of these remaining two risk-factors (0, 1, 2 factors; figure 1C) showed that patients with UA without any of these two factors (67% of patients with UA) achieved sustained remission in 77%. Patients with two risk-factors in contrast, were rare (2%) and had persistent disease in 56%.

For comparison, patients with conventional UA were more often ACPA positive (online supplemental table 1 for baseline characteristics). Multivariable Cox regression analysis in these patients with UA revealed that ACPA, RF, CRP and polyarthritis were associated with sustained remission (online supplemental table 2).

Concluding, the population with contemporary UA is different from conventional UA and risk factors for disease persistence are partly dissimilar. ACPA and CRP remain to be predictive in contemporary UA. Other factors included in the current EULAR recommendation are uninformative (RF, ESR and polyarthritis) or rare (erosions). Further prognostic studies in contemporary UA are warranted, after which risk factors recommended in future EULAR recommendations may require revision.

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Handling editor Josef S Smolen

Contributors All authors contributed to the conception and study design. NKdH contributed to acquisition of the data and analysed the data. All authors contributed to interpretation of the data and the development of the manuscript. All authors approved the final version of the manuscript.

Funding The research leading to these results has received funding from the Dutch Arthritis Foundation and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (starting grant, agreement No 714312).

Disclaimer The funding source had no role in the design and conduct of the study; collection, management, analysis and interpretation of the data; preparation, review or approval of the manuscript; or decision to submit the manuscript for publication.

Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by 'Commisie Medische Ethiek' of the Leiden University Medical Centre (B19.008). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-221821).

To cite den Hollander NK, Verstappen M, Huizinga TWJ, *et al*. *Ann Rheum Dis* 2022;**81**:740–741.

Received 9 November 2021
Accepted 12 January 2022
Published Online First 27 January 2022

Ann Rheum Dis 2022;**81**:740–741. doi:10.1136/annrheumdis-2021-221821

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Managing the selection of placebo group switched to experimental treatment group in post-randomised controlled trial extension studies

Well-designed clinical trial extension studies (TES) can provide robust and meaningful information on the long-term safety, tolerability and efficacy of emergent drugs. A previous 'European Alliance of Associations for Rheumatology (EULAR) initiative established recommendations on the conduct, design and

analysis of TES to mitigate potential bias and distortion of long-term outcomes,[1] which subsequent post-randomised controlled trial (RCT) TES have applied. As prior participants to this initiative, we highlight an area that needs further consideration, namely the selection of the control group that takes place to switch to experimental therapy.

The objective of TES is to capture outcomes of patients exposed to the experimental drug. The recommendations emphasise the importance of intention-to-treat (ITT) analysis, the denominator being the original number entering the RCT. In the original trial, the purpose of ITT is to capture outcomes for the complete randomised population, regardless of exposure to the intervention, as we can only assume balance in prognostic factors immediately after randomisation. ITT is often 'modified' as in the case of a TES to include only patients that have taken at least one dose of study medication, although this can jeopardise ITT analysis if a substantial number of patients is excluded.

For the experimental group, the denominator should be the number of subjects from time of randomisation exposed to at least one dose of study medication. For placebo or active comparator, however, the appropriate denominator is the number of subjects switching to experimental treatment—rather than the number starting placebo in the original RCT. The recommendations stated (with 100% agreement) that the start of a TES should be at the 'point of exposure to the experimental drug of interest. For the experimental randomised arm, this will be the start of the original RCT, while for those randomised to placebo/active comparator arm, this point will be on switching to experimental treatment.' This raises clear challenges.

Selection occurs in the placebo group switching to experimental treatment group (underscoring why the number and proportion of placebo patients not starting experimental treatment should be noted). The original intention of the recommendation on what constitutes the start of a TES was to ensure inadvertent comparisons are not made to the original placebo group. Including only (all) the patients that enter the TES cohort means in essence, combining two (or more) selected patient groups: the original experimental treatment group minus the dropouts (from lack of efficacy, safety or other reasons), and a selection of original placebo group that satisfy criteria to switch to experimental treatment. The selected group would not necessarily have the same prognostic features as the patients in the original experimental group.

In addition, trial designs of RA, psoriatic arthritis and non-radiographic axial spondyloarthritis often offer the possibility of rescue, with switch from placebo to experimental treatment. However, this may be at multiple time points on application of (a combination of) criteria. For example, a primary outcome may be disease activity score-28 joint count (DAS28) <2.6 at 12 weeks with start of TES at 24 weeks; but the possibility to switch may be set as 'no improvement in swollen or tender joints' at 12 weeks, with switch also permitted if less than 20% improvement in swollen or tender joint is observed by week 16–20. In this scenario, selection of 'total non-responders' to placebo that stay in and switch at 12 weeks has occurred, with a further selection of 'partial responders' that stay in and switch between 16 and 20 weeks; with also all remaining patients that were randomised to placebo switching to active therapy at 24 weeks.

The optimal approach to resolve these issues is not clear. For now, we recommend that the original 'experimental' group (starting active treatment on randomisation) and the 'placebo-switch' group be handled separately, and only combined if no meaningful differences exist in prognostic factors and outcome.

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Handling editor Josef S Smolen

Contributors MHB drafted and WPM and MB revised the manuscript. All authors approved the final submitted manuscript.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Disclaimer MHB: The views expressed are those of the author(s) and not necessarily those of the NIHR or the Department of Health and Social Care.

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 applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

To cite Buch MH, Maksymowych WP, Boers M. *Ann Rheum Dis* 2022;**81**:741–742.

Received 1 November 2021
Accepted 30 December 2021
Published Online First 13 January 2022

Ann Rheum Dis 2022;**81**:741–742. doi:10.1136/annrheumdis-2021-221775

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Anti-RuvBL1/2 autoantibodies in patients with systemic sclerosis or idiopathic inflammatory myopathy and a nuclear speckled pattern

The International Consensus on ANA Patterns (ICAP) initiative recently described the clinical relevance and associated autoantibodies of HEp-2 indirect immunofluorescence (HEp-2 IIF) patterns.[1] We here contend that autoantibodies to RuvBL1/2 are associated with a nuclear speckled HEp-2 IIF pattern, potentially conjointly with a cytoplasmic pattern, an association not included in ICAP so far.

Anti-RuvBL1/2 autoantibodies have recently been described in patients with systemic sclerosis (SSc) and SSc-myositis overlap syndrome, primarily in patients without other known autoantibodies.[2–6] Thirteen of 15 anti-RuvBL1/2-positive patients (87%) with description of HEp-2 IIF results in literature had a nuclear speckled pattern. In this study, we evaluated the presence of

anti-RuvBL1/2 autoantibodies in patients with SSc or idiopathic inflammatory myopathy (IIM) with a nuclear speckled pattern on HEp-2 IIF without other known associated autoantibodies from two tertiary referral centres in Belgium, the University Hospitals Leuven and Ghent University Hospital.

We performed immunoprecipitation (IP) of HeLa nuclear extract with human sera, followed by western blotting with rabbit polyclonal anti-RuvBL1 and anti-RuvBL2 antibodies in 51 patients classifiable as SSc according to the 2013 European Alliance of Associations for Rheumatology/American College of Rheumatology (EULAR/ACR) classification criteria or early SSc according to the Leroy criteria (n=41), IIM according to the 2017 EULAR/ACR classification criteria (n=8), or both (n=2).[7–9] All patients had a nuclear fine or coarse speckled pattern (ICAP AC-4 or AC-5) with a fluorescence intensity corresponding to a titer ≥1/80 on HEp-2 IIF and were negative for known SSc-associated or IIM-associated autoantibodies.

We detected anti-RuvBL1/2 autoantibodies in 8 of 51 patients (16%, online supplemental figure 1 and online supplemental table 1). Five of those eight patients had an SSc-myositis overlap syndrome, with two additional patients presenting transient asymptomatic creatine kinase elevation during the course of their disease. Interstitial lung disease was a frequent manifestation (seven out of eight). Six out of eight patients had a combined nuclear and cytoplasmic speckled pattern on HEp-2 IIF. HEp-2 IIF patterns and IP-western blot results of two patients are shown in figure 1. In a control group of patients with autoantibody-positive SSc or IIM (n=30), systemic lupus erythematosus (n=10), primary Sjögren's syndrome (n=10) and blood donors (n=10) no anti-RuvBL1/2 autoantibodies were detected (online supplemental figure 2).

Hitherto, only 43 cases with anti-RuvBL1/2 autoantibodies have been described, of which 24 had SSc with myositis, 18 SSc and 1 SSc with Sjögren's syndrome (online supplemental table 2). Presentation with manifestations not specific for SSc is possible as three of our patients first presented with myositis or articular involvement. Out of 15 patients with known HEp-2 IIF status in literature, 13 had a nuclear speckled pattern, of which 4 also had an additional cytoplasmic granular pattern. Including our patients with anti-RuvBL1/2 autoantibodies, 10 in 21 patients with known HEp-2 IIF status heretofore described had a combined nuclear and cytoplasmic HEp-2 IIF pattern, with the caveat that our cohort was selected based on presence of a nuclear speckled pattern. This dual pattern is plausible as the RuvBL1/2 complex is present in both the nucleus and cytosol with specific functions ascribed to the complex in each subcellular compartment.[10]

Presence of a compatible HEp-2 IIF pattern may prompt the clinician to pursue testing for anti-RuvBL1/2 autoantibodies. However, anti-RuvBL1/2 autoantibody testing is limited as a high-throughput detection method is not yet available. With a recombinant protein-based ELISA for RuvBL1 and RuvBL2 separately Kaji *et al* could not find the association with an SSc or SSc-myositis phenotype as established by their immunoprecipitation-based detection method.[2] This discordance between detection methods could stem from the presumably conformational nature of the RuvBL1/2 autoantigen. Our IP-western blot approach offers an immunoprecipitation-based detection method without the need for radioactive-labelled cell extract.

To conclude, RuvBL1/2 merits consideration as a relevant autoantigen associated with the ICAP AC-4 pattern, potentially conjointly with a cytoplasmic speckled pattern (AC-19 or AC-20), in patients in the extended SSc-myositis spectrum.

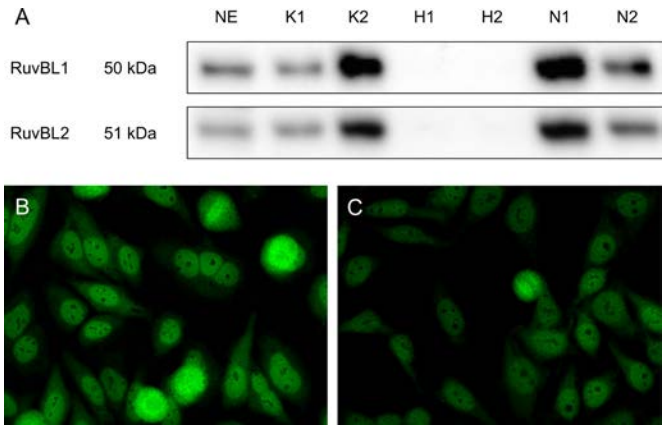

Figure 1 Immunoprecipitation-western blot and HEp-2 indirect immunofluorescence of two anti-RuvBL1/2 autoantibody-positive patients (A) Immunoprecipitation-western blot for RuvBL1 and RuvBL2, NE HeLa nuclear extract (25 µg), K1 and K2 are positive controls from Kanazawa University, H1 and H2 are healthy controls, N1 and N2 are anti-RuvBL1/2-positive patients from Leuven and Ghent. Immunoprecipitation was performed by incubating Pierce A/G magnetic beads (Thermo Fisher Scientific, USA) with human serum (1/20 Tris-buffered saline) for 1 hour at room temperature with subsequent cross-linking with 5 mM BS3 according to manufacturer's protocol (Thermo Fisher Scientific, USA) and overnight incubation at 4°C with a HeLa nuclear extract (100 µg, extraction with NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo Fisher Scientific, USA). Polyclonal rabbit anti-RuvBL1 antibodies (1/2000 in 5% non-fat milk/TBST; OriGene, USA) and anti-RuvBL2 antibodies (1/2000 in 5% non-fat milk/TBST; OriGene, USA) were used as primary antibody and Veriblot IgG (1/1000 in 5% non-fat milk/TBST; Abcam, UK) as secondary antibody. Membranes were developed with SuperSignal West Femto maximum sensitivity substrate (Thermo Fisher Scientific, USA), visualised with Chemidoc XRS+System (Bio-Rad, USA) and analysed with ImageLab software (Bio-Rad, USA). (**B, C**) HEp-2 indirect immunofluorescence analysis of two anti-RuvBL1/2 autoantibody-positive patients (N1 and N2 from panel a), 1/80 dilution at ×40 magnification with NOVA View (Inova, USA).

A high-throughput detection method is needed to make anti-RuvBL1/2 autoantibody testing more widely available.

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Funding JB Vulsteke holds a Research Foundation—Flanders (FWO) SB Fellowship (1S62419N).

Competing interests WAW has received research grants from Roche, Boehringer-Ingelheim and Galapagos. VS has received research grants and support from Boehringer-Ingelheim and Janssen-Cilag, and research support from Accord Healthcare, UCB, and Celgene. XB was part of a scientific advisory committee for Inova Diagnostics and Thermo Fisher (outside the scope from the submitted work).

Patient consent for publication Not applicable.

Ethics approval The study was approved by the local Ethical Committees: S60347/B32220071204 (University Hospitals Leuven); B67020084358 (Ghent University Hospital).

Provenance and peer review Not commissioned; externally peer reviewed.

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2021-220004).

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To cite Vulsteke J-B, Piette Y, Bonroy C, *et al*. *Ann Rheum Dis* 2022;**81**:742–744.

Received 26 January 2021
Accepted 5 January 2022
Published Online First 13 January 2022

Ann Rheum Dis 2022;**81**:742–744. doi:10.1136/annrheumdis-2021-220004

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Obinutuzumab in connective tissue diseases after former rituximab-non-response: a case series

In the following case series, we present four patients with different connective tissue diseases (CTD) showing a remarkably positive response on treatment with Obinutuzumab despite former rituximab-non-response in three cases. Demographic data including, age, gender, disease duration, type of involvement, previous as well as concomitant treatments are shown in table 1. Efficacy of treatment was assessed by clinical, laboratory and radiologic findings or global patient assessment for rheumatological symptoms, respectively. Clinical response was defined by an improvement of involved organ functions as well as a reduction of the severity of symptoms. Global tolerance was evaluated.

SLE

Two patients with SLE and active glomerulonephritis were treated with Obinutuzumab after rituximab failure. One patient each additionally suffered from antiphospholipid syndrome and neuropsychiatric lupus, respectively. After one cycle with obinutuzumab (1 g, day 0, 14), both patients came off dialysis and showed a stable kidney function over a time period of at least 6 months. One patient had cardiac involvement and highly elevated NT-pro-BNP which markedly decreased after treatment with obinutuzumab. Serological markers such as anti-ds-DNA antibodies and C3-complement consumption strongly improved after therapy.

ANTI-JO1 SYNDROME

We further included a patient with anti-Jo1-syndrome who did not respond to her previous treatments including Rituximab, IVIG, Cyclophosphamide and repeated prednisolone pulse therapies. Her disease was manifested by myositis (creatine-kinase (CK) max. 8946 U/L) and CT-confirmed interstitial lung disease with a decreased CO-diffusion capacity of 57.3% expected. After one cycle of obinutuzumab, muscle weakness improved and CK and lactate dehydrogenase levels markedly decreased.

CREST SYNDROME

In this patient, CREST syndrome was diagnosed with sclerodactyly, Raynaud's phenomenon, oesophageal hypomotility, teleangiectasia, calcinosis cutis and pulmonary arterial hypertension and an ANA-titre of 1:10 000 in 2006. In 2013, she

Table 1 Patient demographics and history of diseases and treatments

	SLE 1	SLE 2	Anti-Jo1 syndrome	CREST-syndrome/CLL
Age	33	52	46	80
Gender	F	F	F	F
Year of diagnosis	2019	2008	2019	2006/ 2013
Clinical manifestations	Nephritis, polyserositis, pancytopaenia, pancarditis	Nephritis, CNS-involvement, Libman-Sacks endocarditis, APS, ILD	Myositis, ILD	Sclerodactylia, teleangiectasia, Raynaud's, PAH, calcinosis cutis
Previous therapies and dosage of prednisolone before treatment with OBI	CYC 6×500 mg i.v., MPA, RTX 2×1 g i.v. twice within 6 months; prednisolone 80 mg/d	CYC 6×500 mg i.v., MPA, RTX 2×1 g i.v. twice within 6 months, IVIG, plasmapheresis; prednisolone 70 mg/d	CYC 6×750 mg i.v., RTX 2×1 g i.v., IVIG; repeated prednisolone pulse therapies starting with 80 mg/d	SSZ, MTX, HCQ
Characteristic findings before treatment with Obinutuzumab	Crea 3,14 mg/dL, dialysis, erythrocyturia 3327 /µL, anti-ds-DNA ab. (RIA) 15128,6 IU/mL, C3 0,28 g/L, anti-nucleos. ab. 130,9 IE/mL, nt-pro BNP 42 526 ng/L, SLEDAI-2K: 30	Crea 4,97 mg/dL, dialysis, Prot. Urine/ Crea Urine 108,27 g/molKr, anti-ds-DNA ab. (RIA) 2,5 IU/mL, SLEDAI-2K: 32	myalgia, muscle weakness, dyspnoea, CK 8946 U/L, LDH 910 U/L, CRP 13,9 mg/L	calcinosis cutis, PA 65/30/43 mmHg, PC 13 mmHg, PAR 393 dyn.sec.cmˆ–5, CI 3,3 l/min*m², VC in 75,6%, FEV1 71,0%, FVC 81,2%, TLC 85,6%, Rtot 96,4%, DLCOc SB 49,7%, DLCOc/VA 73,6%"
Characteristic findings after treatment with Obinutuzumab	Crea 1,3 mg/dL, no dialysis, erythrocyturia 258 /µL, anti-ds-DNA ab. (RIA) 224,9 IU/mL, C3 0,83 g/L, anti-nucleos. ab. 20,2 IE/mL, nt-pro BNP 520 ng/L, SLEDAI-2K: 2	Crea 1,95 mg/dL, no dialysis, Prot. Urine/Crea Urine 57,16 g/molKr, anti-ds-DNA ab. (RIA) 2,5 IU/mL, SLEDAI-2K: 17	myalgia and muscle weakness strongly diminished, no dyspnoea, CK 188 U/L, LDH 221 U/L, CRP 4,1 mg/L	calcinosis cutis disappeared, VC in 85,5%%, FEV1 82,9%, FVC 90,7%, TLC 93,5%, Rtot 84%, DLCOc SB 49,3%, DLCOc/VA 69,2%
Co-medication during treatment with OBI and dosage of prednisolone after treatment with OBI at last follow-up	MPA 360 mg 2-0-2, HCQ 200 mg 1-0-0, prednisolone 3 mg 1-0-0	MPA 360 mg 2-0-2, HCQ 200 mg 1-0-0, prednisolone 5 mg 1-0-0	AZA 50 mg 1-1/2-1, prednisolone 5 mg 1-0-0	macitentane 10 mg 1-0-0, chlorambucile, bendamustine
Global tolerance	No major side effects	No major side effects	No major side effects	No major side effects

BNP, brain natriuretic peptide; CK, creatine-kinase; CLL, chronic lymphocytic leukaemia; CREST, Calcinosis cutis - Raynaud's phenomenon - Esophageal dysmotility - Sclerodactylia - Teleangiectasia; CRP, C reactive protein; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; HCQ, hydroxychloroquine; ILD, interstitial lung disease; i.v, intravenous; IVIG, intravenous immunoglobulins; LDH, lactate dehydrogenase; MPA, mycophenolic acid; MTX, methotrexate; OBI, obinutuzumab; PAH, pulmonary arterial hypertension; RIA, radioimmunoassay ; SLE, systemic lupus erythematosus; SSZ, sulfasalazine.

developed chronic lymphocytic leukaemia requiring a B-cell depleting treatment for which obinutuzumab was chosen in accordance with current national and European guidelines.

After two cycles of obinutuzumab, the patient had a complete remission of the haematological disease and showed diminishing calcinosis cutis which gradually disappeared completely until the end of the treatment.

CONCLUSION AND PHARMACOLOGICAL CONSIDERATIONS

Obinutuzumab has recently been proven as an effective option in proliferative lupus nephritis leading to significantly better renal response compared with placebo.[1] The data presented here suggest an efficacy of obinutuzumab in different CTD even after failure of rituximab. We hypothesise that the low dependency of complement factors, the altered mechanisms of action including enhanced antibody-dependent cellular cytotoxicity (ADCC) of obinutuzumab and its presumably enhanced efficacy in inflamed tissues are factors supporting our hypothesis that obinutuzumab should be studied in various CTD after rituximab failure, but especially as first-line biologic after failure of conventional disease-modifying antirheumatic drugs (DMARDs).[2–5]

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Funding The study was supported by: Medical Faculty of University of Heidelberg, Eva Luise and Horst Köhler Foundation (to WM).

Competing interests WM, NB and H-ML have received consulting fees, speaking fees and/or honoraria from Roche (less than US$10 000 each), and unallocated funds for research from Roche (€15 000)

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 applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

To cite Kvacskay P, Merkt W, Günther J, *et al*. *Ann Rheum Dis* 2022;**81**:744–746.

Received 3 November 2021
Accepted 5 January 2022
Published Online First 13 January 2022

Ann Rheum Dis 2022;**81**:744–746. doi:10.1136/annrheumdis-2021-221756

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Increasing incidence of autoantibody-negative RA is replicated and is partly explained by an aging population

With great interest, we read the recently published report by Myasoedova *et al* in which a significant increase in incidence of rheumatoid factor (RF)-negative rheumatoid arthritis (RA) was found, in contrast to RF-positive RA.[1] Studies on trends of RA-incidence stratified for autoantibodies are scarce. Moreover, both an increase and decrease in incidence of RF-negative RA has been reported.[2 3] Because validation is important, we determined trends in incidence of RA over two decades in our region.

We defined autoantibody-positivity as autocitrullinated protein antibodies (ACPA)-positivity, since RF is less specific for RA and more often present in healthy controls, especially at older age.[4] Second, because autoantibody-negative RA has an higher age-of-onset than autoantibody positive RA,[5] we hypothesised that part of the incidence increase is explained by ageing of the population. Therefore, we also assessed the influence of the population age-distribution on the trends of incidence of RA.

Incidence rates were calculated based on the inclusion rate of patients with RA in the Leiden Early Arthritis Cohort (EAC). The Leiden University Medical Center (LUMC) is the only rheumatology referral centre within the Leiden area and inclusion in the EAC of newly presenting patients with early arthritis has been part of regular care since 1993.[6] All consecutively included patients with RA (defined as clinical diagnosis plus fulfilling the 1987 or 2010-criteria within 1 year) included in the EAC between 1994 and 2015 were studied.

First, we calculated crude incidence rates per year using the number of incident cases as the numerator and total population counts from the NUTS-3 (Nomenclature of Territorial Units for Statistics) region around Leiden as the denominator.[7] Trends over time were analysed with Poisson regression. Next, to assess the influence of age-changes in the Leiden population, a three degree of freedom spline of age was included in the Poisson models. All analyses were stratified for ACPA (anti-cyclic citrullinated peptide (CCP)2)-status, which was determined after inclusion but rarely by general practioners in line with Dutch guidelines.[8]

A total of 1697 patients with RA were included between 1994 and 2015 (mean age 57, 66% female, 48% ACPA-positive). For the total RA population, a crude incidence increase was observed (β=0.020 (95% CI 0.012 to 0.027), figure 1). This estimate approximates the proportion increase per year, where 0.02 translates to ~2% increase per year. Stratification for ACPA-status revealed that the crude incidence of ACPA-negative RA increased (0.028 (0.017 to 0.039)) while ACPA-positive RA did not significantly increase (0.009 (–0.002 to 0.021)). We thereby replicated the findings of Myasoedova *et al*. Further stratification for IgM-RF-status within ACPA-negative RA revealed no significant differences in the increase in crude incidence between RF-positive ACPA-negative and RF-negative ACPA-negative RA (0.039 (0.017 to 0.061) vs 0.023 (0.011 to 0.036); p=0.22)).

ACPA-negative RA had the peak incidence at higher age (mean age at diagnosis 59 vs 54; p<0.001; figure 2A), which is in line with previous observations.[5] We then adjusted incidence rates for the changes in age distribution in our healthcare region 1994–2015. This revealed lower estimates in both ACPA-subsets, suggesting that part of the crude incidence increase was due to ageing. After this age-correction, the incidence of ACPA-negative RA still showed some remaining increase over time (0.017 (0.006 to 0.028)). Also here, there was no increased incidence in ACPA-positive RA (0.000 (–0.011 to 0.012)).

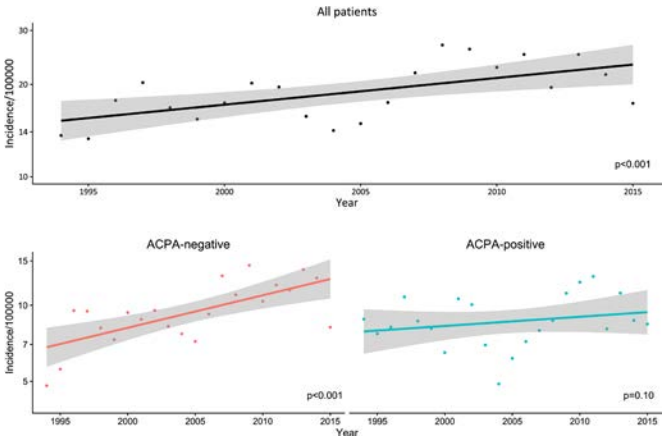

Figure 1 Crude incidence of RA in the Leiden area 1994–2015 in all patients (above) and stratified for ACPA (below): Y-axis is presented on the log-scale. Dots depict the observations per year. Fitted linear lines are depicted in bold and confidence intervals in light grey. ACPA, autocitrullinated protein antibodies; RA, rheumatoid arthritis.

Because we observed that the increase in incidence of the past decades was partly explained by ageing of the population, and it is known that the population will age even more, we estimated the further increase in ACPA-negative RA for the coming two decades based on ageing using age-specific Dutch population prognoses of Statistics Netherlands.[9] As presented in figure 2B, the estimated increase of new RA cases the next 20 years due to ageing of the population is 11% in ACPA-negative RA and 2% in ACPA-positive RA.

Our analyses are based on the assumption that all incident RA cases in the region are included in the EAC. This assumption is supported by the fact that the LUMC is the only referral centre in the region. Importantly, the referral region and strategy has not changed during the last two decennia; hence, if a proportion of patients with novel RA is not included in the cohort, this is presumably similar over time and does not affect our results on trends over time.

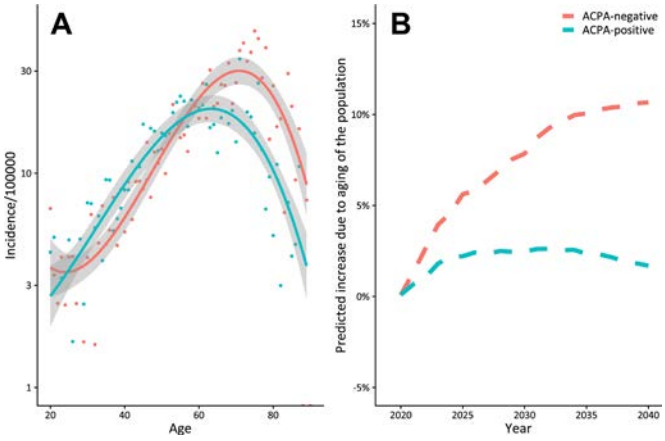

Figure 2 Crude incidence per age (A) and predicted increase in incidence due to ageing of the Dutch population (B), both for ACPA-negative and ACPA-positive RA. (A) Y-axis is presented on the log-scale. Dots depict the observations per age. Fitted lines are depicted in bold and CIs in light grey. ACPA, autocitrullinated protein antibodies; RA, rheumatoid arthritis.

In conclusion, we found an increasing incidence of ACPA-negative RA that was absent in ACPA-positive RA, which is line with the findings of Myasoedova *et al*. Moreover, we showed that the increase in ACPA-negative RA was in part explained by ageing of the population. This will make ACPA-negative RA more prevalent the coming years and promotes the need for research in this subset of RA.

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Funding The research leading to these results has received funding from the Dutch Arthritis Foundation and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (Starting grant, agreement No 714312).

Disclaimer The funding source had no role in the design and conduct of the study; collection, management, analysis and interpretation of the data; preparation, review or approval of the manuscript or decision to submit the manuscript for publication.

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 partners were involved in design of the EAC.

Patient consent for publication Not required.

Ethics approval 'Commissie Medische Ethiek' of the Leiden University Medical Centre.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Matthijssen XME, Huizinga TWJ, van der Helm-van Mil AHM. *Ann Rheum Dis* 2022;**81**:e69.

Received 14 April 2020
Accepted 4 May 2020
Published Online First 29 May 2020

Linked

► https://doi.org/10.1136/annrheumdis-2020-217900

Ann Rheum Dis 2022;**81**:e69. doi:10.1136/annrheumdis-2020-217609

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Response to: 'Increasing incidence of autoantibody-negative RA is replicated and is partly explained by an aging population' by Matthijssen *et al*

We thank Matthijssen and colleagues for their interest in our study on the epidemiology of rheumatoid arthritis (RA), where we have reported a significant increase in incidence of rheumatoid factor (RF)-negative RA and a decrease in RF-positive RA in 2005–2014 compared with previous decades.[1] Matthijssen *et al* have independently assessed the incidence of anticitrullinated peptide antibody (ACPA)-negative and ACPA-positive RA in the Leiden Early Arthritis Cohort. In concordance with our findings, they found increasing incidence of ACPA-negative RA but not ACPA-positive RA.[2] Further, Matthijssen *et al* proposed that ageing of the population can be an important contributor to these trends and estimated that the rate of increase of new ACPA-negative RA cases in the next 20 years will outpace that of ACPA-positive RA (11% vs 2% increase, respectively), thus substantially increasing the prevalence of ACPA-negative RA.

Taken together with our findings, these results strengthen the argument that the serological profile of RA is changing in recent years, and autoantibody-negative RA is becoming more common in the new millennium. These findings have broad implications for both clinical practice and research. First, autoantibody-negative RA is a more clinically challenging disease subset due to diagnostic uncertainty in early disease with multiple potential mimickers, and frequent difficulty with timely choice of effective treatment.[3] This highlights the need for increased awareness of autoantibody-negative RA among physicians, in order to facilitate timely rheumatology referral and initiation of treatment. Second, classification of RA based on RF and ACPA is conditional to the available and validated immunological assays, while the search for additional immunological and clinical subsets within autoantibody-negative RA continues.[4] Refining the understanding of pathophysiology and classification of RA disease beyond the current immunological disease markers may lead to improvement in RA diagnosis and management, opening new avenues for individualised treatment selection for different RA subtypes.

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Funding This work was funded by a grant from the National Institutes of Health, National Institute of Arthritis and Musculoskeletal and Skin Diseases (R01 AR46849). Research reported in this publication was supported by the National Institute of Aging of the National Institutes of Health under award number R01AG034676.

Disclaimer The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Competing interests None declared.

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

Patient consent for publication Not required.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Myasoedova E, Davis J, Matteson EL, *et al*. *Ann Rheum Dis* 2022;**81**:e70.

Received 7 May 2020
Accepted 12 May 2020
Published Online First 29 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217609

Ann Rheum Dis 2022;**81**:e70. doi:10.1136/annrheumdis-2020-217900

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Comment on: 'Metagenome-wide association study of gut microbiome revealed novel aetiology of rheumatoid arthritis in the Japanese population' by Kishikawa *et al*

The recent paper from Kishikawa *et al*[1] provides an extremely important new insight on the concept of oral-gut microbiome axis and rheumatoid arthritis (RA) pathogenesis.

Alterations in the gut microbiome at mucosal sites have been implicated in the pathogenesis of RA.[2–4] Increasing evidence suggests a link between RA and periodontal infections caused by *Porphyromonas gingivalis (Pg)*.[5] Oral infection by *Pg* in an animal model revealed increased serum levels of lipopolysaccharide (LPS), dysbiosis and aggravation of arthritis.[6 7] Therefore, the pathogenesis of RA is considered to be associated with the immunomodulatory activity of oral and gut microbiome.[8] However, few studies have explored the relationship between the oral-gut microbiome axis and RA pathogenesis.

We previously investigated the relationship between RA disease activity using activity indices and biomarkers; the total bacterial counts and the counts of five well-known gut bacteria species; LPS-related biomarkers and IgG and IgA anti-*Pg*-LPS antibodies in 87 patients with established RA showing inadequate responses to conventional synthetic disease-modifying antirheumatic drugs or exhibiting severe complications.[9] (online supplementary table 1)

Little significant relationship was observed between the counts of the total bacteria, five species of bacteria and activity indices and biomarkers.(table 1) The levels of LPS-related biomarkers, particularly serum LPS-binding protein (LBP), were positively correlated with activity indices and biomarkers, suggesting that bacterial LPS-LBP complexes from the gut microbiome may activate nuclear factor κB via toll-like receptor 4 and may initiate and perpetuate inflammation[10 11] in established RA.

IgA antibody responses against *Pg*-LPS, were inversely correlated with the counts of intestinal bacteria, affecting the microbiome balance, and showed a positive correlation with serum LPS and LBP levels, suggesting barrier damage due to intestinal infection with *Pg*. These results support previous findings in collagen-induced arthritis models showing that oral administration of *Pg* significantly affected the gut barrier function and the gut microbiota composition, specifically by decreasing the proportion of phylum *Bacteroidetes,* increasing the proportion of phylum *Firmicutes*.[7]

Furthermore, IgG anti-*Pg*-LPS antibody levels, which are indicative of systemic infection, are inversely correlated with RA activity indices; these results are comparable to those of our previous study of destructive RA.[12] Thus, these results demonstrate that the oral-gut microbiome axis relationship may aggravate disease activity in RA.

Kishikawa *et al* recently performed a genome-wide association study to analyse the role of the gut microbiome in RA; they compared 82 Japanese patients with early RA with 42 age-matched and sex-matched normal controls[1] (online supplementary table 1).

In this study, faecal samples were subjected to whole-genome shotgun sequencing. Case–control phylogenetic association analyses, conducted using a generalised linear regression model, showed that multiple species belonging to the *Prevotella* genus increased in the RA gut metagenome. Multiple *Prevotella spp.*, in addition to *Prevotella copri*, which was recently identified by shotgun sequencing, have been identified in the oral cavity. It has been speculated that colonisation of the intestine by oral bacteria is related to the pathogenesis of RA and other diseases suggesting existence of oral-gut microbiome axis relationship.

In Kishikawa's study, a representative finding was decreased expression of the redox reaction-related gene *R6FCZ7* which is involved in oxidative stress in the genus *Bacteroides* in RA than in healthy subjects. It was previously reported that the counts of *Prevotella* and *Bacteroides* in the gut in RA show an inverse relationship.[3] They suggested that *R6FCZ7* and the genus *Prevotella* are inversely associated via the relationship with the genus *Bacteroides*. In our study, levels of serum LPS, a potent generator of reactive oxygen species,[10] as well as IgA anti-*Pg*-LPS antibody levels, which indicate gut infection with *Pg*, are inversely associated with *Bacteroides* counts. Both studies showed that the key gene *R6FCZ7* and LPS are associated with the generation of reactive oxygen species and are regulated by the balance of gut bacteria, including oral bacteria.

From the viewpoint of clinical application, we have questions to authors after comparison with two studies discussing two oral origin microbiome; *Prevotella spp.* and *Pg*.

Prevotella copri was most abundant in patients with new-onset RA[3 4] suggesting its pathogenic role. Moreover, Maeda *et al* reported SKG mice harbouring microbiota dominated by *P. copri* from early RA patients had an increased number of intestinal Th17 cells and developed destructive arthritis when treated with zymosan.[4] Another microbiome from oral cavity, *Pg* is also considered to play a pathogenic role in RA since *Pg* peptidylarginine deiminase is implicated in the autoimmunity of RA by creating mimic antigen, circular citrullinated peptide (CCP), by autocitrullination.[5] Interestingly, anti-*Pg*-LPS antibody associated with RA clinical indices and biomarker in our study with established RA, suggesting the role for continuation of RA inflammation.[9] These data by Maeda and ours might show the possibility that multiple *Prevotella spp.* other than *P. copri* or *Pg* play not only pathogenic but prognostic role. Were there significant differences of values in prognostic factors (rheumatoid factor, anti-CCP antibody, matrix metalloproteinase 3, HLA-*DRB1* gene, bone erosion and so on) between high and low abundant groups in multiple *Prevotella spp.* or *Pg*? ; results from such analyses would have provided crucial information for understanding of RA pathogenesis.

Overall, our findings and those of others suggest that modulation of the oral-gut microbiome axis is a promising strategy for the treatment and management of RA.

Table 1 Relationships between RA activity indices, disease biomarkers, gut bacterial counts, LPS-related biomarkers and IgG and IgA anti-*Pg* LPS antibodies in 87 patients with RA.

		Activity indices											Disease biomarker								LPS-related biomarker			Anti-*Pg* -LPS	
	Top: slope values Bottom: p valu	DAS28-ESR	DAS28-CRP	SJC	TJC	pVAS	dVAS	Pain VAS	mHAQ	SDAI	CDAI	ESR	CRP	RF	Anti-CCP	Hb	MMP-3	TNF	IL-6	Faecal LPS	Serum LPS	LBP	ENC	IgG anti-*Pg*- LPS	IgA anti-*Pg*- LPS
Gut bacterial counts	Total bacteria	−0.042 0.698	−0.132 0.224	**−0.180** **0.096**	−0.058 0.591	**−0.223** **0.038**	−0.098 0.365	**−0.260** **0.015**	−0.119 0.271	−0.178 0.100	**−0.187** **0.082**	0.115 0.289	−0.032 0.768	−0.093 0.391	−0.060 0.580	0.088 0.418	**−0.179** **0.097**	−0.033 0.768	**0.230** **0.034**	−0.092 0.396	**−0.492** <0.001	**−0.242** **0.024**	**0.435** <0.001	0.078 0.471	**−0.441** <0.001
	Bifidobacterium	−0.019 0.864	0.073 0.505	0.047 0.668	0.134 0.218	−0.031 0.774	−0.015 0.889	0.025 0.816	−0.024 0.828	0.101 0.353	0.115 0.290	**−0.189** **0.080**	0.018 0.866	0.163 0.131	0.125 0.248	0.153 0.157	−0.068 0.533	0.046 0.687	0.047 0.670	**0.182** **0.092**	**0.258** **0.016**	−0.044 0.688	−0.142 0.188	−0.010 0.930	−0.160 0.139
	Lactobacillus	−0.100 0.356	−0.057 0.597	−0.172 0.112	−0.092 0.398	−0.127 0.241	−0.010 0.924	−0.085 0.433	−0.004 0.974	−0.113 0.297	−0.137 0.205	0.010 0.927	0.050 0.649	0.009 0.933	−0.051 0.640	−0.080 0.464	0.039 0.722	0.088 0.436	0.035 0.752	0.071 0.512	**−0.268** **0.012**	−0.139 0.199	**0.229** **0.033**	0.019 0.859	**−0.224** **0.037**
	Bacteroides	−0.102 0.348	−0.078 0.475	0.001 0.996	−0.047 0.667	−0.044 0.685	−0.036 0.738	0.031 0.773	−0.045 0.682	−0.061 0.573	−0.060 0.582	−0.031 0.775	−0.064 0.556	−0.024 0.829	−0.059 0.591	−0.056 0.609	−0.025 0.820	0.053 0.636	0.034 0.754	0.027 0.806	**−0.230** **0.032**	−0.122 0.259	0.125 0.250	−0.029 0.789	**−0.200** **0.064**
	Escherichia coli	−0.051 0.642	0.048 0.660	−0.070 0.519	0.041 0.709	0.004 0.968	0.163 0.132	0.018 0.869	0.026 0.815	−0.016 0.884	−0.026 0.813	−0.041 0.709	0.063 0.560	0.063 0.564	−0.060 0.583	0.044 0.688	0.000 1.000	−0.031 0.783	0.001 0.997	−0.058 0.593	−0.075 0.490	−0.033 0.759	0.050 0.648	**−0.243** **0.024**	**−0.260** **0.015**
	Staphylococcus	−0.120 0.267	−0.083 0.443	−0.156 0.150	−0.075 0.488	0.049 0.654	0.002 0.988	−0.005 0.961	0.087 0.423	−0.058 0.594	−0.058 0.594	−0.156 0.149	−0.080 0.464	0.067 0.539	−0.130 0.230	0.039 0.721	−0.087 0.424	0.108 0.335	0.020 0.853	0.075 0.492	−0.039 0.723	−0.113 0.298	−0.083 0.447	**0.219** **0.041**	−0.127 0.240
LPS-related biomarker	Faecal LPS	**0.237** **0.027**	**0.245** **0.022**	0.053 0.627	**0.203** **0.059**	0.123 0.256	**0.203** **0.059**	0.065 0.548	0.077 0.482	**0.233** **0.030**	**0.238** **0.027**	0.044 0.688	0.097 0.370	−0.029 0.790	−0.033 0.760	0.063 0.564	−0.009 0.933	0.050 0.656	−0.027 0.808		0.006 0.956	0.125 0.248	−0.029 0.793	0.006 0.953	0.037 0.736
	Serum LPS	0.032 0.766	0.053 0.626	0.112 0.303	0.090 0.405	**0.180** **0.095**	−0.026 0.814	0.155 0.152	0.112 0.303	0.115 0.289	0.159 0.141	−0.163 0.132	−0.177 0.101	−0.022 0.838	0.074 0.494	**0.220** **0.040**	−0.060 0.579	0.010 0.929	**−0.209** **0.053**	0.006 0.956		0.085 0.436	**−0.255** **0.017**	−0.075 0.491	**0.284** **0.008**
	LBP	**0.300** **0.005**	**0.244** **0.023**	0.170 0.116	−0.092 0.395	0.025 0.816	0.021 0.844	0.042 0.703	0.065 0.552	0.117 0.279	0.036 0.739	**0.497** <0.001	**0.697** <0.001	**0.234** **0.029**	**0.273** **0.010**	**−0.271** **0.011**	**0.546** <0.001	0.004 0.969	**0.348** **0.001**	0.125 0.248	0.085 0.436		0.035 0.748	0.046 0.676	**0.247** **0.021**
	ENC	0.151 0.164	0.068 0.534	**−0.229** **0.033**	−0.063 0.564	−0.037 0.732	−0.024 0.827	0.016 0.886	−0.007 0.948	−0.066 0.545	−0.126 0.245	**0.338** **0.001**	**0.289** **0.007**	−0.137 0.207	−0.012 0.910	−0.142 0.189	0.063 0.564	−0.004 0.969	**0.245** **0.023**	−0.029 0.793	**−0.255** **0.017**	0.035 0.748		0.060 0.579	**−0.321** **0.002**
Anti-*Pg* LPS	IgG anti-*Pg*-LPS	−0.148 0.170	**−0.277** **0.009**	−0.122 0.261	**−0.218** **0.043**	**−0.376** <0.001	**−0.315** **0.003**	**−0.433** <0.001	**−0.192** **0.074**	**−0.308** **0.004**	**−0.309** **0.004**	0.096 0.376	0.039 0.721	−0.016 0.885	−0.003 0.981	−0.100 0.355	**−0.199** **0.065**	0.052 0.645	0.129 0.228					
	IgA anti-*Pg*-LPS	−0.021 0.847	−0.049 0.655	−0.189 0.079	−0.170 0.117	0.071 0.511	−0.108 0.321	−0.031 0.778	−0.020 0.854	−0.013 0.907	−0.010 0.924	0.063 0.561	0.042 0.700	**0.238** **0.027**	0.157 0.146	−0.040 0.713	0.133 0.218	0.122 0.277	−0.125 0.253					

Bacterial DNA extracted from individual faecal samples was subjected to real time-PCR using the group-specific or species-specific primers. The methods used to analyse LPS-biomarkers and anti-*Pg*-LPS antibodies are described in references.[5 9 12] Patient age, sex, disease duration, treatment with MTX, drinking habits and smoking habits showed little effect on bacterial counts (data not shown).
Top values are the slopes of the correlation (correlation coefficient "ρ") and bottom values are the p values determined by Spearman's non-parametric rank correlation analysis. dark brown, bold: significant positive correlation ($p<0.05$); light brown, bold: trend to positive correlation ($0.05\leq p<0.1$); dark blue, bold: significant negative correlation ($p<0.05$); light blue, bold: trend to positive correlation ($0.05\leq p<0.1$).
Anti-CCP, anti-cyclic citrullinated peptide antibody; CDAI, Clinical Disease Activity Index; CRP, C reactive protein; DAS28-ESR, disease activity score with 28 joint counts-erythrocyte sedimentation rate; EGA, evaluator's global assessment; ENC, endotoxin neutralising capacity; Hb, haemoglobin; IL-6, interleukin-6; LBP, LPS-binding protein; LPS, lipopolysaccharide; mHAQ, modified Health-Associated Questionnaire; MMP-3, matrix metalloproteinase-3; *Pg*, *Porphyromonas gingivalis*; PGA, patient's global assessment; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; SJC, swollen joint count; TJC, tender joint count; TNF, tumour necrosis factor; VAS, Visual Analogue Scale.

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Acknowledgements The authors thank our colleagues Jessica Chang and Bonnie McKinney for reviewing and proofreading this manuscript. The authors thank Kiyomi Kamiya for assistance for patients' data collection.

Contributors HS, KoK and KT designed basic study plans, acquired, analysed and interpreted the data. KoK, KaK, HS, HB and KT drafted this manuscript. KaK, SS conducted most of the analysis in this study including faecal bacteria and LPS-related biomarker analysis. KoK, SU, CA, HT and KN conducted the clinical study with their patients. TW confirmed data by conducting related, but independent experiments, interpreted data and contributed to the preparation of this manuscript. RF conducted the statistical analysis and interpreted the data based on statistical significance. All authors have read and approved of the final manuscript.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Not commissioned; internally peer reviewed.

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2020-217808).

KK, HS, KT, SS and KK contributed equally.

To cite Kitamura K, Shionoya H, Terato K, *et al*. *Ann Rheum Dis* 2022;**81**:e71.

Received 1 May 2020
Revised 6 May 2020
Accepted 6 May 2020
Published Online First 29 May 2020

► http://dx.doi.org/10.1136/annrheumdis-2020-217897

Ann Rheum Dis 2022;**81**:e71. doi:10.1136/annrheumdis-2020-217808

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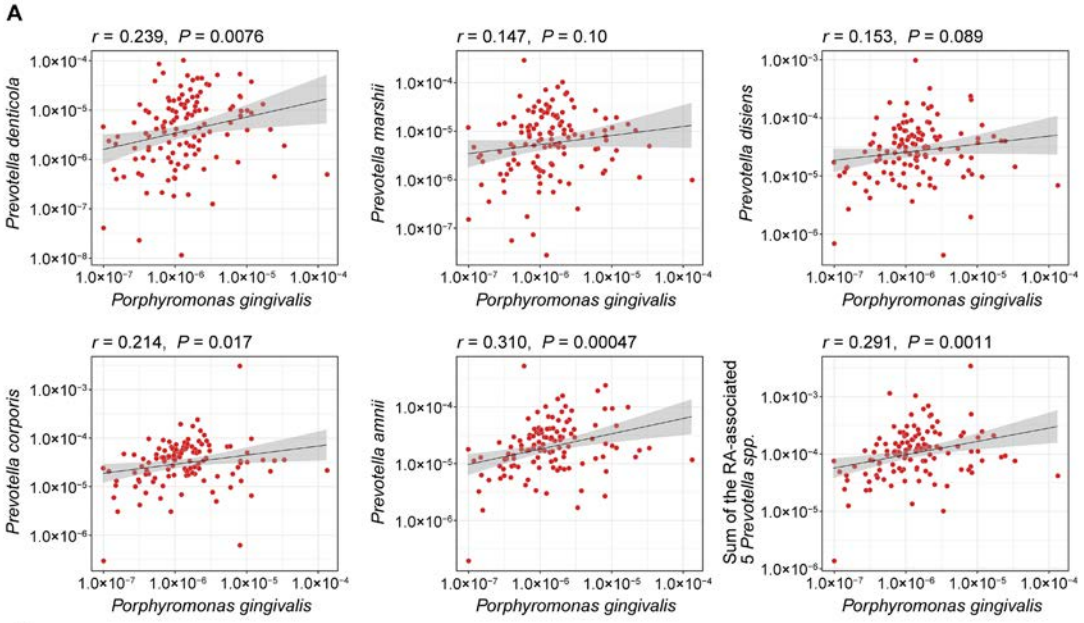
Response to: 'Comment on 'Metagenome-wide association study of gut microbiome revealed novel aetiology of rheumatoid arthritis in the Japanese population' by Kishikawa *et al.*' by Kitamura *et al*

We thank Kitamura *et al*. for their interest in our study and for providing their thoughts through correspondence.[1] They reported interesting characteristics of *Porphyromonas gingivalis* (*Pg*) regarding rheumatoid arthritis (RA) as follows: (1) oral infection of *Pg* increased serum levels of lipopolysaccharide (LPS)[2 3]; (2) oral administration of *Pg* decreased the proportion of phylum *Bacteroidetes*[3]; and (3) serum LPS levels were inversely associated with *Bacteroides* counts. In our metagenome-wide association study (MWAS) of the RA gut microbiome,[4 5] we had identified high abundances of five species belonging to the genus *Prevotella* (i.e., *P. denticola*, *P. marshii*, *P. disiens*, *P. corporis*, and *P. amnii*) in the RA metagenome. Considering that *Prevotella* in the RA gut microbiome showed an inverse relationship with *Bacteroides*,[6 7] disentanglement of gut microbiome link between *Pg* and the *Prevotella* spp. should be of interest.

In our RA MWAS, we had excluded *Pg* from the analysis because the average relative abundance of *Pg* was below the quality control threshold of 0.001%. Here, we additionally examined the case–control association test of *Pg* and found no significant association ($P = 0.78$). However, we found significant positive correlations of the relative abundance between *Pg* and three of the RA-associated five *Prevotella* species (i.e., *P. denticola*, *P. corporis* and *P. amnii*; $P < 0.017$; figure 1A).

A



B

		Prevotella denticola	*Prevotella marshii*	*Prevotella disiens*	*Prevotella corporis*	*Prevotella amnii*	Sum of five *Prevotella* spp.	*Porphyromonas gingivalis*
Porphyromonas gingivalis	Pearson's *r* *P*-value	0.239 0.0076	0.147 0.10	0.153 0.089	0.214 0.017	0.310 0.00047	0.291 0.0011	- -
RF	Fold change *P*-value	1.36 0.90	2.28 0.38	0.634 0.68	0.325 0.25	1.58 0.64	0.638 0.72	0.678 0.016
ACPA	Fold change *P*-value	1.38 0.85	2.21 0.52	0.746 0.54	3.18 0.0098	2.32 0.85	1.68 0.21	1.09 0.82
DAS28CRP	Pearson's *r* *P*-value	0.0738 0.51	0.0352 0.75	-0.0464 0.70	0.0823 0.46	0.131 0.24	0.0628 0.58	0.318 0.11
Stage	Pearson's *r* *P*-value	-0.111 0.32	-0.147 0.19	-0.143 0.20	-0.0353 0.75	-0.0152 0.89	-0.136 0.22	-0.0771 0.49

$P < 0.05$ with positive correlation $P < 0.05$ with negative correlation

Figure 1 Characteristics of *Porphyromonas gingivalis* (*Pg*) in the rheumatoid arthritis (RA) gut microbiome. (A) Correlation between the relative abundance of *Pg* and that of *Prevotella* spp. The *x*-axes indicate the relative abundance of *Pg* in a logarithmic scale. The *y*-axes indicate the relative abundance of each of the five *Prevotella* species with significant RA-control discrepancy (ie, *P. denticola*, *P. marshii*, *P. disiens*, *P. corporis*, and *P. amnii*) and the total abundance of the five *Prevotella* spp. in a logarithmic scale. (B) Correlations between the relative abundance of the taxa and RA activity indices and biomarkers. Only correlations with p values less than 0.05 were coloured (positive correlations in red and negative correlations in blue). Wilcoxon rank-sum tests were used to compare the high-level and low-level groups. Stage indicates Steinbrocker classification of the joint X-rays. RF, rheumatoid factor; ACPA, anti-citrullinated peptide antibody; DAS28CRP, Disease Activity Score 28-joint count C reactive protein.

When we focused on the total abundance of the five *Prevotella* species, significant positive correlation was also found ($r = 0.291$, $P = 0.0011$). This result suggests that *Pg* and the *Prevotella* spp. in the RA gut microbiome cooperate in the RA pathophysiology.

Another concern by Kitamura *et al*. was the distinct distributions of the RA prognostic factors between the RA case groups with high and low abundance in the *Prevotella* spp or *Pg*. They reported that serum LPS-binding protein was positively correlated with activity indices and biomarkers of RA (e.g., Disease Activity Score 28-joint count C reactive protein (DAS28CRP), CRP, rheumatoid factor (RF) and anti-citrullinated peptide antibody (ACPA)).[1] We assessed whether the relative abundance of the *Prevotella* spp and *Pg* showed the correlation with RF, ACPA, DAS28-CRP and the Steinbrocker stage. As for RF and ACPA, the RA cases were compared between the high-level and low-level groups stratified according to the threshold of 15 and 4.5 IU/mL, respectively. While *Prevotella corporis* had nominally significant positive correlation with ACPA (fold change = 3.18, $P = 0.0098$), most of the correlations were not significant (figure 1B). In our study samples, we did not observe positive correlation of the five *Prevotella* spp. and *Pg* with RA activity indices and biomarkers.

In conclusion, our study suggests that *Pg* and the *Prevotella* spp. cooperate in the RA gut microbiome. Further studies focusing on the interaction of these two taxa are warranted to elucidate RA aetiology.

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Handling editor Josef S Smolen

Contributors TK and YO designed the study, conducted the data analysis and wrote the manuscript. YM and TN conducted the experiments collected the samples. YO supervised the study.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Kishikawa T, Maeda Y, Nii T, *et al*. *Ann Rheum Dis* 2022;**81**:e72.

Received 14 May 2020
Accepted 14 May 2020
Published Online First 29 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217808

Ann Rheum Dis 2022;**81**:e72. doi:10.1136/annrheumdis-2020-217897

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Neuroinflammatory events after anti-TNFα therapy

We have read with interest the article by Kopp *et al* that has been published recently in the Annals of the Rheumatic Diseases. The article deals with the risk of neuroinflammatory events (NIEs) in patients with inflammatory arthritides (IA), receiving tumour necrosis factor alpha (TNFα) inhibitors.[1] Their cases were identified from the nationwide registries of Sweden and Denmark, in a prospective observational study. The authors found an increased risk of NIEs after anti-TNFα therapy in patients with spondyloarthropathies (SpAs) as compared with those not receiving TNF blockers, while no consistent and significant risk of NIEs after anti-TNFα treatment in rheumatoid arthritis (RA) patients. They concluded that the risk profile of NIEs in patients receiving TNFα inhibitors differs among patients with different IA which has an impact on decision-making in clinical practice.

In a prospective imaging and electrophysiological study of our clinic, patients with RA and SpAs who were eligible for anti-TNFα therapy had been investigated, during the period May 2009 to December 2011.[2] Before starting anti-TNFα therapy all patients had a full physical examination and a detailed neurological evaluation. In addition, all had brain and cervical spine MRI and neurophysiological studies with nerve conduction velocity and needle electromyography (EMG) of the upper and lower extremities. Patients with severe and uncontrolled hypertension, diabetes mellitus, dyslipidaemia, history of atherosclerotic events, heart arrhythmias, B12 and iron deficiency as well as patients with a history of head and cervical spine injury had been excluded from the study. From a cohort of 101 patients, 24 had been excluded. From the remained 77, there were 36 with RA and 41 with SpA (24 psoriatic arthritis (PsA) and 17 ankylosing spondylitis (AS)). Before the onset of therapy one patient with AS complained for numbness of the left arm and dizziness. The neurological evaluation, as well as brain and cervical spine MRI and neurophysiological studies, showed no abnormalities and the patient received anti-TNF therapy. On the other hand, two patients without any objective clinical manifestations never received anti-TNFα therapy because their brain MRI showed pathological findings compatible with multiple sclerosis (MS) (figure 1A). These two patients with brain MRI and suggestive findings of MS but without MS symptoms are classified as having radiological isolated syndrome (RIS) which is considered to be a preclinical MS syndrome.[3] Finally, 75 patients received anti-TNFα therapy. All patients were naïve to TNFα inhibitors except one patient with PsA who was switched from etanercept (ETN) to infliximab (INF) due to primary inadequate response. During follow-up (mean period 18 months) three patients manifested NIEs. More specifically: the patient with PsA who switched from ETN to INF developed clinical symptoms and signs compatible with MS after a period of 8 months. The findings were confirmed by MRI and electrophysiological studies. One patient with RA treated with adalimumab (ADA) developed optic neuritis after 9 months of treatment. Finally, another patient with AS and Crohn's disease receiving INF developed sensorimotor peripheral neuropathy after 24 months of INF treatment. The estimated rate of NIEs in our study was 4% (3/75). But, if we also calculate the incidental MRI findings of RIS in those two additional patients, the estimated rate of NIEs arises to 6.66% (5/75) leading to a p value of <0.00001 (significant at $p<0.05$). This means, that we may treat a clinically asymptomatic patient (RIS patient) with an anti-TNFα agent and as a consequence, the patient may finally develop a NIE.

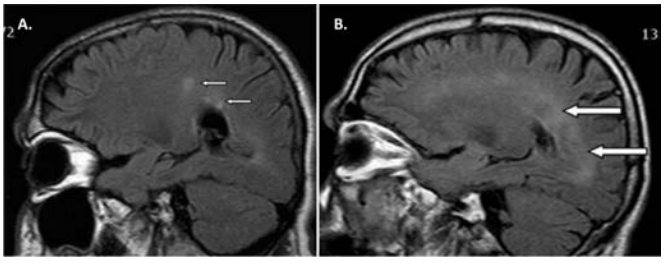

Figure 1 Sagittal fluid-attenuated inversion recovery scans demonstrating (A) ovoid hyperintense lesions in the deep periventricular white matter (thin arrows) and (B) bilateral diffuse hyperintense signal in the periventricular white matter of the parietal and occipital lobes (thick arrows).

We believe that the autoimmune phenomena like NIEs that develop during anti-TNFα therapy, are agent-depended and not disease-depended meaning that these are a class-effect phenomenon.[4 5] Indeed, new autoimmune NIEs have been described. Two patients with RA, one receiving ETN[6] and another treated with ADA developed myasthenia gravis syndrome.[7] Thus, in patients which are candidates for anti-TNFα therapy, in order to avoid NIEs a detailed neurological evaluation is mandatory. In addition, a close follow-up and an appropriate monitoring with MRI and EMG are also essential when indicated.

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Contributors All four authors have made substantial contributions to the current manuscript and more specifically: EK: acquisition of the data and analysis as well as approval of the final version. EP: drafting, data analysis and approval of the final version. AAD: conception of the work, review and approval of the final version. PVV: review and approval of the final version.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Kaltsonoudis E, Pelechas E, Voulgari PV, *et al*. *Ann Rheum Dis* 2022;**81**:e73.

Received 23 April 2020
Accepted 27 April 2020
Published Online First 20 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217802

Ann Rheum Dis 2022;**81**:e73. doi:10.1136/annrheumdis-2020-217723

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Response to: 'Neuroinflammatory events after anti-TNFα therapy' by Kaltsonoudis *et al*

First of all, we would like to thank Dr Kaltsonoudis *et al* for their interest in our study. Dr Kaltsonoudis *et al*[1] have raised an interesting suggestion in their correspondence based on their study from 2014.[2] They suggest that all patients who are candidates for tumour necrosis factor alpha inhibitor (TNFi) therapy should have a thorough neurological assessment (including brain MRI and neurophysiological tests) before commencing TNFi treatment. Moreover, they believe that the risk of neuroinflammatory events following treatment with TNFis is not disease-dependent, but agent-dependent, and that part of the observed risks following treatment may in fact be present already at treatment start.

In our large observational study,[3] we excluded all patients recorded with a hospital contact with a neuroinflammatory diagnosis prior to study entry eliminating patients with existing neuroinflammatory disease that had reached clinical attention. However, as Dr Kaltsonoudis *et al* argue, we do not know whether some of the patients had a clinically asymptomatic neuroinflammatory disease at time of entry (that is, prior to TNFi treatment start) which may have been aggravated by TNFi use.

In clinical practice, patients with rheumatological disease starting on TNFi treatment are not currently examined for a possible asymptomatic inflammatory neurological disease. In order to do so, we think that additional studies are warranted to evaluate the costs and benefits of such an intervention.

With regards to the risk of neuroinflammatory events being disease- or drug-dependent, we believe that the results from our large study, similar in the two countries under study, are robust and point to the notion that the risk is disease-dependent, although we did not specifically investigate whether there is any interaction between the treated disease and the type of TNFi used to treat it. The hypothesis of disease-specific risks is further supported by studies finding opposite risks for multiple sclerosis among patients with psoriatic arthritis[4] and rheumatoid arthritis.[5]

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Handling editor Josef S Smolen

Contributors TIK wrote the first draft of the manuscript. All authors critically reviewed the manuscript for intellectual content and approved the final version of the manuscript.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests TIK has received personal fees from Novartis. MM has received grants from Novartis and Biogen, and has received personal fees from Novartis, Biogen, Merck, Sanofi Genzyme and Teva. FTS has received grants and personal fees from Biogen, Merck, Novartis, Sanofi Genzyme and Roche. JA has received grants from AbbVie, BMS, Eli Lilly, MSD, Pfizer, Roche, Samsung Bioepis, Sanofi and UCB. LD has received grants from BMS and has received personal fees from Eli Lilly and Galderma.

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.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Kopp TI, Delcoigne B, Arkema EV, *et al*. *Ann Rheum Dis* 2022;**81**:e74.

Received 6 May 2020
Accepted 6 May 2020
Published Online First 20 May 2020

Linked

► https://doi.org/10.1136/annrheumdis-2020-217723

Ann Rheum Dis 2022;**81**:e74. doi:10.1136/annrheumdis-2020-217802

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Hydroxychloroquine is neutral in risk of chronic kidney disease in patients with systemic lupus erythematosus

With great interest, we read the lupus nephritis recommendations article by Fanouriakis *et al*.[1] The authors highlighted that hydroxychloroquine (HCQ) use is recommended for all lupus nephritis patients to reduce risk of kidney flares, end-stage kidney disease (ESKD) and death. The authors also recommended that a reduction of 50% HCQ dose in patients with glomerular filtration rate less than 30 mL/min.

We agree with the authors that HCQ is an important background therapy for all systemic lupus erythematosus (SLE) and lupus nephritis patients. However, the dose adjustment in patients with renal impairment should be more evidence-based. In the FDA (Food and Drug Administration) website (www.accessdata.fda.gov), information of 'range for renal clearance of unchanged drug was approximately 16% to 30% and did not correlate with creatinine clearance; therefore, a dosage adjustment is not required for patients with renal impairment' were disclosed.

Furthermore, previous studies had been debating on the effect of HCQ in chronic kidney diseases (CKD).[2 3] Pokroy-Shapira *et al*[4] investigated 256 lupus patients for up to 25 years and found that HCQ use was negatively associated with risk of earlier CKD. We believed that evidence of HCQ in preventing ESKD and death, or even lupus nephritis flare were limited. Therefore, we designed a retrospective cohort study from population-based data set to examine the association using HCQ and their risk of subsequent CKD in patients with SLE.

In this study, we analysed Taiwan's National Health Insurance Research Database from 1997 to 2013, which provides a strongly reliable huge database and encompasses approximately 99.9% of the Taiwan population. A total of 2050 newly diagnosed SLE patients with ICD-9 (International Classification of Diseases, Ninth Revision) codes 710.0 between 2000 to 2012 were included. After excluding patients with prior CKD and HCQ never-users, a total of 783 SLE patients who had HCQ treatment that began at −90 and +365 days from diagnosis with SLE individuals were enrolled and divided into two groups according to their prescription coverage days. Group 1 had prescription of HCQ for less than 90 days, and group 2 had HCQ prescription for more than 90 days within 1 year. The baseline characteristics of both groups were comparable after 1:2 age/sex matching and 1:1 propensity-score matching on urbanisation, hospitalisation days, comorbidities and co-medications. The cumulative incidence rate of SLE was calculated for up to 14 years with Kaplan-Meier curves. The Cox proportional regression model was used to examine HR of developing subsequent CKD among two groups.

The results revealed that the cumulative incidence of CKD showed no significant difference between two groups (figure 1). After adjusting for age, urbanisation, length of hospital stays and possible confounders, the adjusted HR of developing CKD among the >90 days HCQ group was 1.295 (95% CI 0.395 to 4.247), compared with <90 days users, indicating no statistical difference.

In conclusion, our retrospective population-based cohort study showed that HCQ use in SLE patient is neutral in subsequent risk of CKD.

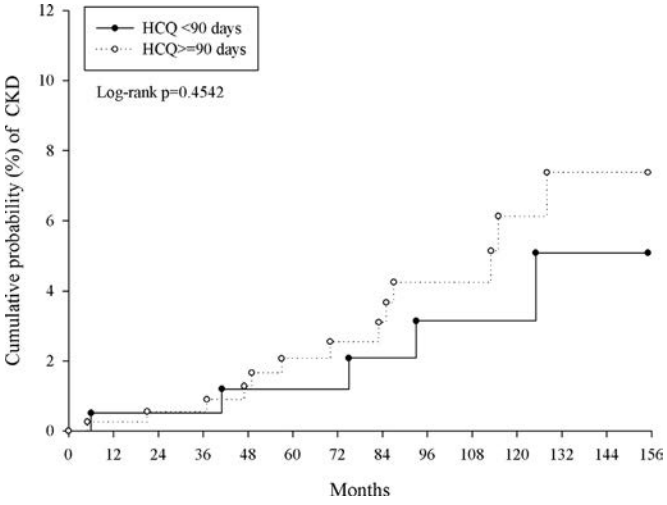

Figure 1 The comparisons of cumulative probability of CKD in systemic lupus erythematosus patients among two HCQ groups after propensity score matching. CKD, chronic kidney diseases; HCQ, hydroxychloroquine.

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Acknowledgements The authors thank all patients and staffs who made this study possible.

Contributors C-YW and MT: writing and revision of the manuscript, discussion of mechanism, data interpretation. C-YH: statistical analysis, data collection and interpretation. J-YC and JC-CW: study design and supervision, revision of the manuscript. All authors discussed the results and contributed to the final manuscript.

Funding None

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 This study was approved by Chung Shan Medical University Hospital.

Provenance and peer review Not commissioned; internally peer reviewed.

C-YW and J-YH contributed equally.

To cite Wu C-Y, Tan M, Huang J-Y, *et al*. *Ann Rheum Dis* 2022;**81**:e75.

Received 23 April 2020
Accepted 27 April 2020
Published Online First 20 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217804

Ann Rheum Dis 2022;**81**:e75. doi:10.1136/annrheumdis-2020-217728

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Response to: 'Hydroxychloroquine is neutral in risk of chronic kidney disease in patients with systemic lupus erythematosus' by Wu *et al*

We thank Drs Wu *et al* for their interest in our manuscript[1] and the stimulating data they provide regarding the value of hydroxychloroquine (HCQ) in the prevention of chronic kidney disease (CKD) in patients with systemic lupus erythematosus (SLE).[2] We agree with the authors that the evidence behind the recommendation for a 50% reduction in HCQ dose in patients with lupus nephritis (LN) and a glomerular filtration rate (GFR) less than 30% is not supported by high-level evidence (although it is known that excretion of the drug is carried out principally by direct renal clearance).[3 4] Nevertheless, *CKD is considered a risk factor for the most important side effect of HCQ, retinal toxicity*; with newer, more sensitive screening techniques, the latter is now more frequently detected than in the past, reaching 10% to 20% after 20 or more years of use.[5 6] Since HCQ is universally recommended as life-long therapy in SLE and the risk for ocular toxicity correlates with the cumulative dose (ie, daily dose and duration of intake), it was reasonable to recommend a lower dose for patients with severe CKD.

In their letter, the authors also provide data from the Taiwan National Health Insurance Research Database to question whether HCQ has an additive beneficial effect in preventing CKD in lupus patients. To this end, they analysed 783 newly diagnosed SLE patients who started HCQ treatment within 1 year from diagnosis and divided them into two groups according to their HCQ prescription coverage days for 1 year (less or more than 90 days, respectively). After propensity score matching, the authors found in their population no reduced risk of CKD in HCQ users for up to 14 years. We believe that the results of the authors' analysis should be interpreted with caution and certainly cannot be generalised—at this point—to other populations, without further confirmation. First, it is not clear what percentage of their patients had biopsy-proven LN (as opposed to only extrarenal SLE). Second, although the authors have reportedly adjusted for co-medications, it is unclear whether cumulative doses of drugs commonly used in LN, like glucocorticoids or cyclophosphamide, were comparable between the two groups. Lastly, regarding HCQ per se, the analysis has not taken into account the issue of patient adherence to treatment. Suboptimal adherence to HCQ in lupus patients has been consistently reported in several studies;[7 8] thus, conclusions based on prescription data may not accurately predict actual taking of the drug.

Unlike extrarenal SLE, where the multiple benefits of HCQ have been established,[9] data regarding the benefits of HCQ specifically in LN are less robust. As shown in our own systematic literature review informing the current recommendations for LN, most data originate from retrospective observational studies, wherein antimalarials have been reported to reduce the risk for subsequent CKD, with OR ranging from 0.18 to 0.40.[10–12] In view of current data, the European League Against Rheumatism/European Renal Association-European Dialysis and Transplant Association recommend the use of HCQ—unless contraindicated—in all patients with SLE and LN, with dose adjustments according to body weight and GFR.

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Handling editor Josef S Smolen

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Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Fanouriakis A, Bertsias G, Boumpas DT. *Ann Rheum Dis* 2022;**81**:e76.

Received 7 May 2020
Accepted 7 May 2020
Published Online First 20 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217728

Ann Rheum Dis 2022;**81**:e76. doi:10.1136/annrheumdis-2020-217804

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Physician's global assessment is often useful in SLE, but not always: the case of clinical remission

We read with interest the paper by Aranow *et al*[1] where physician's global assessment (PGA) displayed excellent inter-rater reliability, which could rely on the inclusion of highly selected lupus experts, as stated by the authors themselves. Indeed, in previous studies, PGA showed a high intra-rater and inter-rater variability,[2 3] consistently with PGA being a subjective measure.

The high inter-rater reliability observed by the authors is surprising considering that the timeframe for assessing disease activity significantly varied among respondents: 36.7% scored PGA over the previous 7–10 days, 36.7% over the previous month, the remainder over shorter or longer periods of time. Additionally, in almost one-third of respondents, lupus damage was considered when scoring PGA.

Notably, the authors suggest that PGA should be scored after considering laboratory results, owing to a better correlation with systemic lupus erythematosus (SLE) disease activity index-2000 (SLEDAI-2K) of postlaboratory versus prelaboratory PGA.

The finding that laboratory results, including serology, influence PGA performance raises some considerations.

In Aranow's study, each serological abnormality was associated with a median delta PGA prelab versus postlab of ≥0.3,[1] that is, the threshold for a *clinically* meaningful change in PGA. As PGA is incorporated in composite outcome measures including SLE-responder index and BILAG-based Combined Lupus Assessment (BICLA), the numerically significant impact of serology might reduce response rate in clinical trials, overpowering any clinical improvement.

Finally, a relevant question deals with the inclusion of PGA in a definition of *clinical* remission, which is a state of clinical quiescence, irrespective of serology.[4 5] How could PGA fit in this definition? In Aranow's study, abnormal serology alone determined a median PGA increase of 0.54 for low C4, 0.41 for elevated anti-dsDNA antibody levels, 0.41 for low C3.[1] The DORIS definition of clinical remission includes a cut-off of <0.5 for PGA; therefore, one can argue that, by affecting PGA, abnormal serology could prevent the achievement of *clinical* remission definition or could lead to a loss of *clinical* remission status.

We recently tested in a multicentre cohort of 646 patients with SLE, followed up for five consecutive years, the performance of the items included in DORIS definitions, that is, PGA <0.5 (scored prior to reviewing complement and anti-DNA antibody test), clinical (c)SLEDAI-2K=0 and prednisone ≤0.5 mg/day, alone or in combination, in defining remission and predicting damage.[6] We found that adding PGA <0.5 to cSLEDAI-2K=0 did not increase the performance of cSLEDAI-2K against damage progression while resulting in loss of remission in a relevant proportion of patients.

More recently, in the same cohort, we found that PGA≥0.5 despite cSLEDAI-2K=0, which was observed in 195 patients, was associated with (1) nonspecific patient-reported symptoms (157/195, 80.5%) and (2) objectifiable clinical manifestations not included in SLEDAI-2K or not reaching the threshold to be scored in SLEDAI-2K (38/195, 19.5%) (table 1).

As remission should identify patients with better outcome, we compared damage progression, measured by SLICC/ACR Damage Index, between patients in clinical remission according to cSLEDAI-2K=0 plus PGA<0.5 and those in conditions (1) or (2). No difference was observed in the proportion of patients accruing new damage during follow-up (cSLEDAI-2K=0 and PGA<0.5: 33.3%, condition (1): 26.8%, condition (2): 36.8%, p=0.256), suggesting that the symptoms/manifestations captured by PGA but not scored by cSLEDAI-2K could not affect damage. Thus, the inclusion of PGA in a definition of remission prevents the achievement of this status in a relevant proportion of patients without identifying a subgroup with a better prognosis.

Altogether, our data and those by Aranow *et al* highlight that inclusion of PGA in a definition of *clinical* remission gives relevance to serology, patient-reported symptoms and clinical manifestations unable to impair disease outcome while overshadowing the benefits of achieving remission according to less subjective disease activity indices.

Table 1 Symptoms and manifestations which led to PGA≥0.5* despite cSLEDAI-2K=0 in a multicentre cohort of 646 patients with lupus

Patients with PGA≥0.5 and cSLEDAI-2K=0	**195/646 (30.2%)**
1. Patients with subjective findings without objectifiable clinical manifestations likely due to SLE (condition 1)	*157/195 (80.5%)*
Musculoskeletal domain	98
Arthromyalgias	95
Low back pain	10
Morning stiffness	8
Asthenia/fatigue	66
Paraesthesia	1
Finger stings	1
Shortness of breath	1
Nausea	1
Burning mouth syndrome	1
Ocular pain	1
Dermatitis/urticaria	1
Multiple symptoms (arthromyalgias, asthenia, anxiety, panic attack, hallucinations, demoralisation, sleepiness, confusion, headache, memory deficit, dizziness, insomnia, vision loss, low-grade fever, chronic cough, paraesthesia, finger stings, chronic pharyngodynia, fast heartbeat, chronic itch, influenza syndrome, hand/foot ulcers, constipation, vaginosis and/or effluvium capillorum)	25
2. Patients with objectifiable clinical manifestations likely due to SLE (condition 2)	*38/195 (19.5%)*
Haematological involvement (i.e., lymphopenia, haemolytic anaemia)†	10
Monoarthritis	10
Proteinuria‡	6
Recurrent infections leading to withdrawal/reduction of immunosuppressive therapy	6
Chilblain lupus erythematosus	2
Lower limbs sensory neuropathy	1
Lymphadenopathy	1
Hand pitting scars	1
Impaired diffusing capacity of the lung for carbon monoxide	1
Tachyarrhythmia	1
Venous thrombosis in secondary antiphospholipid syndrome	1
Hepatic-pancreatic enzyme increase	1
Depression	1

*PGA was scored prior to reviewing complement and anti-DNA antibody test.
†not included in SLEDAI-2K.
‡Proteinuria ≤0.5 gr/day.
cSLEDAI-2K, clinical SLE disease activity index-2000; PGA, physician's global assessment; SLE, systemic lupus erythematosus.

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Contributors MZ contributed in conceiving the letter, analysed data and drafted the manuscript. FS collected and analysed data. MG analysed data and revised the letter. AD conceived and revised the letter. All the authors approved the final version.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Zen M, Saccon F, Gatto M, *et al*. *Ann Rheum Dis* 2022;**81**:e77.

Received 14 April 2020
Accepted 18 April 2020

Published Online First 20 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217687

Ann Rheum Dis 2022;**81**:e77. doi:10.1136/annrheumdis-2020-217611

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Response to: 'Phsician's global assessment is often useful in SLE, but not always: the case of clinical remission' by Zen *et al*

We thank Dr Zen and colleagues for their interest in our article on the Physician's Global Assessment (PGA)[1] in lupus, and for sharing their experience.[2] Their comment focusses on the impact of a PGA (scored prior to reviewing complement and anti-DNA antibody tests) in association with the clinical Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (cSLEDAI) on the definition of clinical remission. Of note, the cSLEDAI captures haematology and renal activity measured in the laboratory, and differs from the SLEDAI only in the omission of serology. The objective of our study was not to evaluate serology per se, but to determine the impact of knowledge of all pertinent laboratory values on physician scoring of the PGA. We found that PGA scores determined with knowledge not only of serology, but also of haematology, urinalysis, proteinuria and acute phase reactants (C-reactive protein and erythrocyte sedimentation rate), correlated significantly better with an objective measure of disease activity, the SLEDAI-2K. Our data are therefore not correctly interpreted by Zen *et al* who state that "abnormal serology alone determined a median PGA increase of 0.54…" . Of interest, Zen and colleagues report that a PGA ≥0.5 despite cSLEDAI-2K=0 associated with 'non-specific' patient-reported symptoms validating the premise that physicians do indeed consider a patient's experience when assessing disease activity.

Our data support the inclusion of laboratory evaluations, which include but are not limited to serological data, when scoring the PGA. The role of serology in a definition of remission in systemic lupus erythematosus is a separate issue, best resolved by assessing long-term patient outcomes in large cohorts. Our findings indicate that the PGA in such studies should be scored with knowledge of relevant laboratory data.

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Handling editor Josef S Smolen

Contributors All authors contributed to this reply.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Askanase A, Oon S, Huq M, *et al*. *Ann Rheum Dis* 2022;**81**:e78.

Received 5 May 2020
Accepted 5 May 2020
Published Online First 20 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217611

Ann Rheum Dis 2022;**81**:e78. doi:10.1136/annrheumdis-2020-217687

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Physician global assessment in systemic lupus erythematosus: can we rely on its reliability?

We read with great interest the recent paper by Aranow *et al*[1] about the impact of laboratory results on scoring of the Physician Global Assessment (PGA) of disease activity in systemic lupus erythematosus (SLE). PGA is an important tool for assessing disease activity, response to treatment (it is a component of the Systemic Lupus Erythematosus Responder Index (SRI)-4) and remission in SLE. Importantly, monitoring of SLE through PGA has been recommended in the recent European League against Rheumatism (EULAR) guidelines.[2]

In their paper,[1] Aranow *et al* found very high inter-rater PGA reliability values (pre-lab PGA intraclass correlation coefficient (ICC) 0.98; post-lab PGA ICC 0.99) based on 50 clinical vignettes. In our recent systematic review of the psychometric properties of the PGA,[3] we show that this instrument is valid and responsive for assessing disease activity in SLE, but has a high variability. In the paper by Aranow *et al*, the inter-rater reliability of PGA was assessed using the ICC with a two-way random-effect model based on mean scorings (ICC 2,k). This has the effect of artificially increasing reliability estimates compared with the use of single measurement models (ICC 2,1), which would also be interesting to present.

This further suggests a major need for both standardisation and training in the scoring of this increasingly used instrument, particularly among non-expert rheumatologists, as those may wish to follow the recent EULAR recommendations for SLE.[2]

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Funding The study was funded through the training Bursary Programme 2019 of the SLEuro European Lupus Society (recipient of the bursary: EC).

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.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Chessa E, Piga M, Arnaud L. *Ann Rheum Dis* 2022;**81**:e79.

Received 18 April 2020
Accepted 18 April 2020
Published Online First 27 May 2020

Linked

► https://doi.org/10.1136/annrheumdis-2020-217692

Ann Rheum Dis 2022;**81**:e79. doi:10.1136/annrheumdis-2020-217632

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Response to: 'Physician global assessment in systemic lupus erythematosus: can we rely on its reliability?' by Chessa *et al*

We thank Chessa *et al* for their interest in our article on lupus Physician's Global Assessment (PGA).[1] Their correspondence discusses the measurement used to determine PGA scoring reliability and suggests that the intraclass correlation coefficient (ICC) used, ICC2,k, with a two-way random effect model based on mean scorings may have increased the reliability estimates reported, compared with a two-way random effect models based on individual data points (ICC2,1).[2] Indeed correlation coefficients were approximately 39% lower for the pre-lab PGA and 27% lower for the post-labPGA when computed with the ICC2,1 compared with the ICC2,k. We used the ICC2,k to assess inter-rater reliability as our study was designed to evaluate and compare mean PGA scores before and after receipt of laboratory values from multiple (k=50) raters.[3] Additionally, mean-based models (ICC2,k) are conventionally used to report ICC. Which of these models is more mathematically or conceptually correct remains debated. Importantly, both models showed increased agreement when laboratory values are available (post-lab PGA) compared with the pre-lab PGA. We wholeheartedly agree that further standardisation and precise guidelines for its scoring will likely improve the performance of the PGA when used by lupus experts and practising rheumatologists.

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Handling editor Josef S Smolen

Contributors All authors contributed to this correspondence response.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Askanase A, Oon S, Huq M, *et al*. *Ann Rheum Dis* 2022;**81**:e80.

Received 5 May 2020
Accepted 5 May 2020
Published Online First 27 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217632

Ann Rheum Dis 2022;**81**:e80. doi:10.1136/annrheumdis-2020-217692

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Risk of systemic lupus erythematosus in patients with idiopathic thrombocytopaenic purpura

We read the published article by Zhu *et al*[1] with great interest. In this population-based retrospective cohort study, the authors demonstrated that the patients with idiopathic thrombocytopaenic purpura (ITP) had a 26 times higher risk of new-onset systemic lupus erythematosus (SLE) compared with the control population. However, some concerns do exist and should be addressed.

First, thrombocytopaenia is known as a common clinical manifestation of SLE and can be the initial presentation in 5% of patients with SLE.[2 3] The diagnosis of ITP is based principally on the exclusion of any known causes of thrombocytopaenia by history, clinical manifestations, physical examination, laboratory tests, bone marrow examination and so on.[4] In the study, the search of patients with ITP was according to International Classification of Diseases, Ninth Revision, Clinical Modification code 287.3. In the setting of nationwide population, obviously, most patients with thrombocytopaenia initially see haematologists, rather than rheumatologists. Under the circumstances, some early stage of SLE patients with thrombocytopaenia as the only initial manifestation may be wrongly diagnosed as ITP and were included in ITP group in the study. Therefore, serious selection bias exists, which is, at least in part, attributable to the incredibly high HR. The authors should have checked the diagnosis of ITP before these patients were included in the ITP group. A potential solution is to exclusively include the patients with negative autoantibodies at the time of ITP diagnosis. Second, only 0.19% of patients in the non-ITP group developed SLE during follow-up. In the context of extremely low incidence rates, a cohort design is deeply challenging and problematic and usually lead to poor robustness of estimates, embodied in the particularly wide 95% CI in the study (eg, 95% CI 13.7 to 46.0). Meanwhile, although the authors had controlled a range of baseline characteristics, several considerable risk factors strongly related to developing SLE still failed to be adjusted, for example, family history of SLE (or rheumatic diseases) in first-degree relatives and smoking.[5] The presence of residual factors was acceptable in some situation, but the confounding bias caused by confounding factors could be amplified in the presence of extremely low incidence rates and largely weakened the reliability of findings. In addition, we consider the time to the SLE for the two groups should be provided in the study.

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Contributors WX wrote the paper. ZZ reviewed and edited the manuscript.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Xie W, Zhang Z. *Ann Rheum Dis* 2022;**81**:e81.

Received 17 April 2020
Accepted 18 April 2020
Published Online First 24 April 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217694

Ann Rheum Dis 2022;**81**:e81. doi:10.1136/annrheumdis-2020-217659

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Response to 'Risk of systemic lupus erythematosus in patients with idiopathic thrombocytopenic purpura' by Xie and Zhang

We thank Xie *et al* for their relevant comments on our recent article in the *Annals of Rheumatic Disease* entitled 'Risk of systemic lupus erythematosus in patients with idiopathic thrombocytopenic purpura: a population-based cohort study'.[1] We are pleased that this correspondence allows us to provide additional data and further discussion to explain these comments.

Our study aimed to investigate the risk of new-onset of systemic lupus erythematosus (SLE) in patients with new diagnosis of idiopathic thrombocytopenic purpura (ITP) in the setting of claim-based National Taiwan Insurance Research Database. To ensure that we included only new cases, we excluded subjects with previous diagnosis of ITP or SLE to avoid the possibility of pre-existing SLE. All the International Classification of Diseases, Ninth Revision, Clinical Modification codings were defined by at least three ambulatory visits or one hospitalisation, as validated in previous studies.[2 3] We agree that some patients with ITP might have had subclinical or even underdiagnosed clinical SLE when ITP was diagnosed. Unfortunately, the serological data, such as antinuclear antibody, were not available in our dataset.

To further clarify this bias, we did time-varying effect to show the adjusted HR (aHR) indifferent time intervals (table 1). We agree that within 3 years of ITP diagnosis, the incredible high HR might be due to pre-existing or overlapping subclinical SLE. However, we also found that even when ITP has been diagnosed for 5–10 years, the risk of incidental SLE is still significantly high (aHR=13.0, 95% CI 3.4 to 50). For this group for 'late-onset' SLE, it is unlikely to be pre-existing SLE in patients with ITP.

For second comment about the incidence of SLE, the incidence of SLE in the non-ITP control group in our study was 2.1 (95% CI 1.4 to 3.1) per 100 000 person-months; convert to 25.2 per 100 000 person-years. It is similar to the North American study data, which was 23.2/1 00 000 person-years (95% CI 23.4 to 24.0).[4] We surely agree that there might be underdiagnosed subjects who have no diagnosis coding by rheumatologists or who are not even seeking medical visits in this claim-based dataset. However, considering the long follow-up period of our study of up to 14 years, we think this underdiagnosis rate is relatively low and should not change the results.

Finally, we agree that there are always residuals confounding in a retrospective real-world study. In this study, although smoking and family history data were unavailable, we had done extensive matching by frequency matching and propensity score on possible confounders and proxy comorbidities to improve baseline comparability of both groups.[5] Many possible confounding diseases, including rheumatoid arthritis, Sjogren's syndrome, systemic sclerosis, vasculitis, hypertension, diabetes mellitus, hyperlipidaemia, coronary artery disease, osteoporosis, cerebral vascular accident, asthma, chronic obstructive pulmonary disease, chronic kidney disease, chronic liver disease, hyperthyroidism, thyroiditis, pancreatitis and antiphospholipid antibody syndrome were thus matched or adjusted to minimise this bias. Furthermore, we also did four models of sensitivity tests to confirm the consistent results.

Table 1 Incidence of SLE in ITP and non-ITP populations

	Before PSM (1:20 age–sex matching)	
	Non-ITP n=14 460	ITP n=723
Between 1 and 6 months		
Follow-up person-months	57 677	2812
SLE/n (%)*	2/14 460 (0.01)	10/723 (1.38)
Incidence rate† (95% CI)	3.4 (0.87 to 13.9)	355.6 (191.3 to 660.9)
aHR (95% CI)	Reference	64.0 (11.2 to 366.9)
Between 6 months and 1 year		
Follow-up person-months	57 185	2673
SLE/n (%)*	2/14, 53 (0.01)	6/684 (0.88)
Incidence rate† (95% CI)	3.5 (0.9 to 14.0)	224.5 (100.9 to 499.6)
aHR (95% CI)	Reference	78.7 (13.2 to 467.1)
Between 1 and 3 years		
Follow-up person-months	286 432	12 694
SLE/n (%)*	7/14,243 (0.05)	8/657 (1.22)
Incidence rate† (95% CI)	2.4 (1.2 to 5.1)	63.0 (31.5 to 126.0)
aHR (95% CI)	Reference	33.8 (10.0 to 113.9)
Between 3 and 5 years		
Follow-up person-months	236 662	9934
SLE/n (%)*	6/11,884 (0.05)	8/514 (1.56)
Incidence rate† (95% CI)	2.5 (1.1 to 5.6)	80.5 (40.3 to 161.0)
aHR (95% CI)	Reference	22.4 (6.2 to 81.2)
Between 5 and 10 years		
Follow-up person-months	421 787	17 031
SLE/n (%)*	8/9742 (0.08)	4/399 (1.00)
Incidence rate† (95% CI)	1.9 (1.0 to 3.8)	23.5 (8.8 to 62.6)
aHR (95% CI)	Reference	13.0 (3.4 to 50.0)

Median follow-up time, ITP=67 months and non-ITP=89 months.
*n, number of individuals at risk at the beginning during the period.
†Incidence rate, per 100 000 person-months.
aHR, adjusted HR; PSM, propensity-score matching; SLE, systemic lupus erythematosus.

In conclusion, compared with previous studies that are small sample sizes or cross-sectional, our 14 years' population-based big data cohort study demonstrated that patients with ITP are at a higher risk of subsequent SLE. Clinically, patients with ITP should be educated and monitored for the risk of incidental SLE.

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Handling editor Josef S Smolen

Funding The present study was supported by the Programme of Scientific andTechnology Project (Guilin Science Research and Technology Development, grant number 2016012706–2).

Competing interests None declared.

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

Patient consent for publication Not required.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Zhu F-X, Huang J-Y, Wen Q-Q, *et al*. *Ann Rheum Dis* 2022;**81**:e82.

Received 28 April 2020
Accepted 28 April 2020
Published Online First 7 May 2020

► http://dx.doi.org/10.1136/annrheumdis-2020-217659

Ann Rheum Dis 2022;**81**:e82. doi:10.1136/annrheumdis-2020-217694

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Risk of systemic lupus erythematosus in patients with idiopathic thrombocytopenic purpura: a need for a more accurate control group?

We read with great interest the recent paper by Zhu *et al*[1] which studied the risk of developing systemic lupus erythematosus (SLE) in a population of patients with idiopathic thrombocytopenic purpura (ITP).

In their paper, Zhu *et al* performed a population-based retrospective cohort study in which they analysed the risk of SLE in a cohort of patients newly diagnosed with ITP between 2000 and 2013. Controls were selected at a 1:2 ratio through propensity score matching using the greedy algorithm. Zhu *et al* found an incidence rate of 62.0 per 100 000 person-months (95% CI 44.3 to 86.8) in the ITP group and of 2.10 per 100 000 person-months (95% CI 1.44 to 3.06) in the non-ITP group, with an average follow-up time of 80 months. The adjusted HR of incidental SLE in the ITP group was 25.1 (95% CI 13.7 to 46.0). Given that ITP is an immune-mediated disease, a control group consisting in patients with other autoimmune diseases (autoimmune haemolytic anaemia, Evans syndrome, thyroiditis…) might have been more accurate in order to compare the risk of developing SLE with other autoimmune diseases instead of using a standard control group, which could have artificially overestimated the risk of SLE.

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Contributors All authors contributed equally to the presented work.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Mertz P, Arnaud L. *Ann Rheum Dis* 2022;**81**:e83.

Received 17 April 2020
Accepted 18 April 2020
Published Online First 27 April 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217696

Ann Rheum Dis 2022;**81**:e83. doi:10.1136/annrheumdis-2020-217651

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Response to: 'Risk of systemic lupus erythematosus in patients with idiopathic thrombocytopenic purpura: a need for a more accurate control group?' by Mertz and Arnaud

We thank Dr Mertz and Arnaud[1] for their comments on our recent article in the *Annals of the Rheumatic Diseases* entitled 'Risk of systemic lupus erythematosus in patients with idiopathic thrombocytopenic purpura: a population-based cohort study'.[2] They raised the question about selection of control group and suggest a control group consisting of patients with other autoimmune diseases, such as autoimmune haemolytic anaemia (AIHA), Evans syndrome and thyroiditis, instead of using a standard control group.

We agree that many immune-mediated diseases or chronic infectious diseases might also be attributed to systemic lupus erythematosus (SLE).[3 4] As Mertz and Arnaud[1] mentioned, previous studies had reported that SLE is associated with AIHA[5] and autoimmune thyroid diseases.[6] Sometimes, AIHA and idiopathic thrombocytopenic purpura (ITP) can coexist in patients with SLE.[7] Therefore, we did not mean to compare the difference between ITP and other immune diseases, but simply ask the question: 'Is risk of SLE increased in patients with ITP, compared to non-ITP controls?'

To answer this question, which we think is more clinically relevant, we thus select the non-ITP general population as control. With regard to the control group selection, we have two strategies—negative or positive control. To compare with the normal or non-exposure group, a healthy population is the best 'negative exposure' control. In some cases, especially for drug comparative effectiveness study, an active comparator group is an example of a 'positive control'. This kind of control selection had been published in many previous studies with similar design.[8 9]

We also agree that it will be interesting to compare different immune-mediated diseases on the risk of incidental SLE. Thus, we did additional analysis to respond to this comment (table 1). In the Taiwan National Insurance Database with data on one million individuals, we retrieved data on newly diagnosed Hashimoto's disease, Graves' disease, AIHA, ITP and a general population control. The outcome is the subsequent incidence of SLE. Briefly, we found that age-adjusted and sex-adjusted HR was 25 for ITP, 19 for AIHA, 7.3 for Hashimoto's thyroiditis and 1.6 for Graves' disease, compared with the general population.

In conclusion, patients with ITP, AIHA, Hashimoto's thyroiditis or Graves' diseases are all at a higher risk for subsequent incidental SLE.

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Handling editor Josef S Smolen

Funding The present study was supported by the Programme of Scientific and Technology Project (Guilin Science Research and Technology Development; grant no 2016012706–2).

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.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Zhu FX, Huang J-Y, Qingqing W, *et al*. *Ann Rheum Dis* 2022;**81**:e84.

Received 4 May 2020
Accepted 5 May 2020
Published Online First 20 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217651

Ann Rheum Dis 2022;**81**:e84. doi:10.1136/annrheumdis-2020-217696

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Table 1 Crude and age-adjusted and sex-adjusted incidence rate of SLE in the general control, ITP, Hashimoto's disease, Graves' disease and AIHA

Group	Person-months	SLE event	Crude incidence rate*	Age-adjusted and sex-adjusted incidence rate*
General control (n=14 303)	1 273 883	26	2.04	2.04
Graves' disease (n=7345)	650 005	23	3.54	3.30
Hashimoto's thyroiditis (n=1513)	118 482	12	10.13	15.01
AIHA (n=121)	6827	7	102.54	39.43
ITP (n=697)	53 382	28	52.45	52.60

For age-adjusted and sex-adjusted incidence rate, the weighting of standardisation was the age-sex distribution in the general control.
*Rate per 100 000 person-months.
AIHA, autoimmune haemolytic anaemia; ITP, idiopathic thrombocytopenic purpura; SLE, systemic lupus erythematosus.

Associations of regular glucosamine use with all-cause and cause-specific mortality: causality assumptions need to be checked

We read with great interest the manuscript published by Li and colleagues that was published in the *Annals of the Rheumatic Diseases* in 2020.[1] They evaluated the associations of regular glucosamine use with all-cause and cause-specific mortality in a large prospective cohort. This study provides valuable and interesting results but some methodological concerns should be taken into account.

First, they presented the results in term of two models (model 1 and model 2), but it is not clear how the models were built. The rationale for the confounder selection was not provided. Causal diagrams (directed acyclic graphs) are a new approach used in the epidemiological literature to conceptualise confounding effects and to identify minimal sufficient adjustment sets.[2] Lin and colleagues have not explained these steps in their causal study and all baseline variables have been included in their multivariable models.[3]

Second, the authors constructed propensity scores using all baseline covariates,[1] but whether or not confounder variables are distributed equally between the glucosamine users and non-users appears not to have been examined. Standardised mean difference is the most commonly used statistic for examining the balance of confounding variables between groups when propensity scores are applied in a study. In fact, the success of propensity score modelling should be judged using the balance of confounders between the glucosamine user and non-users. In addition, propensity scores could be included into the model as a covariate to adjust for baseline differences. However, the assumption regarding the functional relationship between the propensity scores and outcomes (linearity, proportional hazards, etc) needs to be assessed to avoid any biases estimates.[4]

Third, they indicated that the proportional hazard assumption was evaluated in their study, but the results of the statistical test and hazard curves were not reported.

Fourth, while the baseline characteristics of glucosamine users and non-users are presented in their Table 1, p values were not reported so it is unclear whether the differences between groups are statistically significant.

Finally, the reasons for loss to follow-up are not reported and it is not clear how this important issue was handled in their longitudinal study. Both differential and non-differential reasons for loss to follow-up need to be considered, and differential reasons can lead to selection bias.[5]

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Contributors SS and MAM wrote the initial manuscript and finalised it.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Safiri S, Mansournia MA. *Ann Rheum Dis* 2022;**81**:e85.

Received 23 April 2020
Accepted 27 April 2020
Published Online First 27 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217896

Ann Rheum Dis 2022;**81**:e85. doi:10.1136/annrheumdis-2020-217732

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Response to: 'Associations of regular glucosamine use with all-cause and cause-specific mortality: causality assumptions need to be checked' by Safiri and Mansournia

We appreciated Safiri and Mansournia for their interest and comment on our recent study.[1 2] Safiri *et al*[1] mentioned that we did not explain the steps for the selection of confounders that have been included in our fully adjusted models. We acknowledged that due to the limited number of the words for the manuscript required by the journal, the content for the explanation for selection of confounders was not included. Actually, all confounders in our study were selected according to the published literature,[3–5] and these were the most important risk factors for mortality. In total, 27 confounders were included in our fully adjusted models, and the adjustment for confounding was sufficient.

As suggested by Safiri and Mansournia,[1] we examined the balance of confounding variables between the glucosamine users and non-users by using standardised mean difference. The most of confounding variables were considered balanced between the two groups (table 1), suggesting that the application of propensity scores was appropriate. We evaluated the proportional hazard assumptions for propensity scores and the outcomes, and no evidence of a violation of the assumption was observed. When we put propensity scores into the models as a covariate to adjust for baseline differences, the results were not substantially changed.

The proportional hazard assumptions for glucosamine use and outcomes were evaluated for all models, and no violation of the assumption was found. Due to 10 models for five outcomes used in our study, we did not provide the hazard curves and p values in the article.[2] However, we stated clearly that no violation of the assumption was found in the section of statistical analysis in the article.[2]

We did not provide p values for the differences in the baseline characteristics of the participants between the two groups due to a very large sample size. Even if there was a minor difference, the test also showed statistically significant results. All the p values for the differences in the baseline characteristics were less than 0.001 in this study, and all the baseline variables were included in the fully adjusted models.

Finally, in this study, we excluded participants who withdrew from the study (1299) and those with missing data on the use of glucosamine (6160). We do agree that differential reasons for loss to follow-up could lead to selection bias. However, the baseline characteristics of the participants excluded and included in this study were generally similar, and no differential reason for loss to follow-up was found.

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Contributors All authors critically reviewed the manuscript for important intellectual content.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

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

Patient consent for publication Not required.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Li Z-H, Huang Q-M, Mao C. *Ann Rheum Dis* 2022;**81**:e86.

Received 6 May 2020
Accepted 7 May 2020
Published Online First 27 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217732

Ann Rheum Dis 2022;**81**:e86. doi:10.1136/annrheumdis-2020-217896

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Table 1 Baseline characteristics of study participants by glucosamine use

Characteristics	Overall (N=495 077)	Glucosamine non-users (n=400 731)	Glucosamine users (n=94 346)	SMD
Age, mean (SD), years	56.55 (8.09)	55.95 (8.20)	59.08 (7.07)	0.409
Women	269 549 (54.4)	210 497 (52.5)	59 052 (62.6)	0.205
TDI, mean (SD)	−1.31 (3.09)	−1.20 (3.14)	−1.79 (2.79)	0.201
Education				0.017
Degree	160 288 (32.4)	129 146 (32.2)	31 142 (33.0)	
No degree	334 789 (67.6)	271 585 (67.8)	63 204 (67.0)	
Ethnicity				0.085
White	455 861 (92.1)	367 313 (91.7)	88 548 (93.9)	
Others	39 216 (7.9)	33 418 (8.3)	5798 (6.1)	
Household income (£)				
<18 000	116 815 (23.6)	95 680 (23.9)	21 135 (22.4)	0.094
18 000–30 999	127 517 (25.8)	100 419 (25.1)	27 098 (28.7)	
31 000–51 999	127 427 (25.7)	102 879 (25.7)	24 548 (26.0)	
52 000–100 000	97 565 (19.7)	80 314 (20.0)	17 251 (18.3)	
>100 000	25 753 (5.2)	21 439 (5.3)	4314 (4.6)	
BMI, mean (SD), kg/m^2	27.43 (4.80)	27.45 (4.84)	27.36 (4.65)	0.02
Smoking status				0.184
Never	271 144 (54.8)	219 107 (54.7)	52 037 (55.2)	
Former	171 668 (34.7)	135 486 (33.8)	36 182 (38.4)	
Current	52 265 (10.6)	46 138 (11.5)	6127 (6.5)	
Alcohol consumption				0.081
Never	21 931 (4.4)	18 688 (4.7)	3243 (3.4)	
Former	17 858 (3.6)	15 136 (3.8)	2722 (2.9)	
Current	455 288 (92.0)	366 907 (91.6)	88 381 (93.7)	
Physical activity (min/week)				0.137
<150	228 019 (46.1)	189 753 (47.4)	38 266 (40.6)	
≥150	267 058 (53.9)	210 978 (52.6)	56 080 (59.4)	
Vegetable consumption (servings/day)				0.167
<2.0	97 853 (19.8)	83 776 (20.9)	14 077 (14.9)	
2.0–3.9	222 743 (45.0)	179 783 (44.9)	42 960 (45.5)	
≥4.0	174 481 (35.2)	137 172 (34.2)	37 309 (39.5)	
Fruit consumption (servings/day)				0.286
<2.0	136 458 (27.6)	118 612 (29.6)	17 846 (18.9)	
2.0–3.9	201 446 (40.7)	163 136 (40.7)	38 310 (40.6)	
≥4.0	157 173 (31.7)	118 983 (29.7)	38 190 (40.5)	
Supplement or drug use				
Vitamin	157 133 (31.7)	104 719 (26.1)	52 414 (55.6)	0.627
Minerals and other dietary supplements	184 377 (37.2)	118 971 (29.7)	65 406 (69.3)	0.864
Aspirin	66 052 (13.3)	53 402 (13.3)	12 650 (13.4)	0.002
Statin	56 544 (11.4)	46 186 (11.5)	10 358 (11.0)	0.017
Non-aspirin NSAIDs	71 109 (14.4)	53 152 (13.3)	17 957 (19.0)	0.157
Chondroitin	7813 (1.6)	1581 (0.4)	6232 (6.6)	0.343
Health conditions				
Cardiovascular disease	28 709 (5.8)	24 621 (6.1)	4088 (4.3)	0.081
Cancer	39 659 (8.0)	31 506 (7.9)	8153 (8.6)	0.028
Diabetes	25 968 (5.2)	22 517 (5.6)	3451 (3.7)	0.093
Hypertension	279 956 (56.5)	225 247 (56.2)	54 709 (58.0)	0.036
Respiratory diseases	1957 (0.4)	1555 (0.4)	402 (0.4)	0.006
Digestive diseases	1417 (0.3)	1259 (0.3)	158 (0.2)	0.030
High cholesterol	86 406 (17.5)	70 408 (17.6)	15 998 (17.0)	0.016
Arthritis	23 217 (4.7)	15 440 (3.9)	7777 (8.2)	0.185
Dementia	219 (0.0)	184 (0.0)	35 (0.0)	0.004
Depression	76 642 (15.5)	53 848 (13.4)	15 133 (16.0)	0.073
Long-standing illness	162 123 (32.7)	131 024 (32.7)	31 099 (33.0)	0.006

Values are numbers (%) unless stated otherwise.
BMI, body mass index; NSAID, non-steroidal anti-inflammatory drug; SMD, standardised mean difference; TDI, Townsend Deprivation Index.

'Finding the right one'

I read with great interest the study published by Renson *et al*.[1] The authors concluded in the abstract that, '*Our data reveal a need for a waiting period of at least 6 months to perform an MRI-SIJ in postpartum women with back pain*'. But 69% of study subjects *do not* have back pain and are asymptomatic. Though the study is very systematically planned and has achieved its primary aim, the inferences drawn seem a little far-fetched and are applicable to only a limited set of individuals in real life. The fear of overdiagnosing spondyloarthritis (SpA) comes into the picture only when the dedicated sacroiliac joint (SIJ) is ordered, which may not be the case for most of the study population (69%) in real life. So, the study would have been more pragmatic if only subjects with chronic back pain 8/35 (22.85%) were included. Assessment of SpondyloArthritis international Society (ASAS) criteria mandate presence of 'Inflammatory back pain' for more than 3 months before proceeding to MRI-SIJ.[2] So, only 4/35 (11.4%) would have qualified for imaging in real life. It would have been more useful had the authors compared Spondyloarthritis Research Consortium of Canada (SPARCC) scores in individuals with/without inflammatory back pain than just back pain. The number of individuals with 'inflammatory back pain' and the number of individuals showing sacroiliitis and positive SPARCC Score at 1 year are the same (4/35). Whether these are the same subjects or different should be looked into. Also, the data related to acute phase reactants, like C reactive protein, would have added value to the results and analysis.

The study definitely emphasises the importance of avoiding overdiagnosis of SpA based on *incidental* SIJ findings. But, delaying diagnosis in 'true' SpA can lead to the progression of disease, damage accrual and increased disability duration. As evident from the recent population-based study, individuals in the 'imaging arm' (positive findings on MRI) have faster progression from non-radiographic SpA to radiographic SpA. [3] Studies have also shown more delay in diagnosis for women than males.[4] The reason for this is, in part, the classical teaching of higher male to female ratio in SpA. But a recent cohort study doesn't show any gender difference.[5] So, delaying imaging for 6 months altogether for *all* postpartum women with back pain seems unjust. Future studies comparing likelihood ratios with MRI findings alone and with the inclusion of inflammatory back pain, acute phase reactants, other SpA features, positive family history and response to non-steroidal anti-inflammatory agents would help us in finding 'The right one' who needs dedicated MRI-SIJ in the postpartum period.

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Contributors AAZ has designed and written the correspondence.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

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.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Zanwar AA. *Ann Rheum Dis* 2022;**81**:e87.

Received 27 April 2020
Revised 1 May 2020
Accepted 1 May 2020
Published Online First 28 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217856

Ann Rheum Dis 2022;**81**:e87. doi:10.1136/annrheumdis-2020-217731

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Response to: 'Finding the right one' by Zanwar

We thank Dr Zanwar for his interest in our study. We appreciate that he took the time to write down his remarks[1] regarding our work.[2] We truly believe that overdiagnosis of axial spondyloarthritis (axSpA) is an issue, since the specificity of MRI for sacroiliitis seems to be overestimated.[3–5] By including asymptomatic subjects with an uncomplicated pregnancy and childbirth, we demonstrated that pregnancy and giving birth are associated with the occurrence of spondyloarthritis (SpA)-like sacroiliac joint lesions. By using this strategy, we limited the possibility of erroneously including patients with SpA, whereas if all study subjects would have had postpartum chronic back pain, the distinction with patients with SpA would have been difficult to make. The present study design limited the possibility that the detected sacroiliac joint lesions can be attributed to a back pain-related pathology. By demonstrating important sacroiliitis-like images even in healthy, asymptomatic postpartum subjects, we showed that caution is truly warranted in interpreting MRI images of the sacroiliac joints in the postpartum period. Consequently, performing an MRI in the postpartum period should be a well-considered decision. Therefore, we want to underscore that if an MRI of the sacroiliac joints is performed during the first 6 months postpartum, the possibility of pregnancy/childbirth-associated sacroiliac joint lesions should be seriously considered, as these are difficult to distinguish from sacroiliitis in the context of SpA. When in doubt, a treatment with non-steroidal anti-inflammatory drugs can be attempted to evaluate the treatment response and to buy some time before performing an additional MRI scan.

The link of the imaging findings with the presence or absence of back pain was another point touched on. For clarity, Assessment of Spondyloarthritis International Society classification criteria for axSpA do not mandate presence of inflammatory back pain (IBP) for more than 3 months before proceeding to MRI of the sacroiliac joints.[6 7] Furthermore, IBP has been shown to have high sensitivity but low specificity.[8] As in our study only four subjects had IBP, it was not possible to make hard statements regarding this matter. Three out of four subjects with IBP had sacroiliitis at baseline, persisting in two out of four at month 6. The residual sacroiliitis at month 12 was limited in both subjects. The decline in Spondyloarthritis Research Consortium of Canada (SPARCC) scores over time in these subjects suggests that these lesions are pregnancy/childbirth-related and are not attributed to SpA. Inflammatory serum markers were not regarded in this study since they were not available for all subjects and they are heavily influenced by the process of childbirth itself and are therefore presumably not directly linked to the presence of sacroiliac joint MRI lesions.

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Handling editor Josef S Smolen

Acknowledgements We thank Dr Zanwar for his comments regarding our study.

Contributors All authors contributed equally to this letter.

Funding This study was funded by Assessment of SpondyloArthritis International Society research grant.

Competing interests None declared.

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

Patient consent for publication Not required.

Provenance and peer review Commissioned; internally peer reviewed.

To cite Renson T, Van den Bosch FE, Elewaut D. *Ann Rheum Dis* 2022;**81**:e88.

Received 11 May 2020
Revised 12 May 2020
Accepted 12 May 2020
Published Online First 28 May 2020

Linked

► http://dx.doi.org/10.1136/annrheumdis-2020-217731

Ann Rheum Dis 2022;**81**:e88. doi:10.1136/annrheumdis-2020-217856

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