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

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ARD is published by BMJ Publishing Group Ltd, London WC1H 9JR, UK. T: +44 (0)20 3556 5889 E: ard@bmj.com

Twitter: @ARD_BMJ

ISSN: 1468-2060 (online)

Impact Factor: 12.350

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Greetings from the editor 2019

First of all, let me wish you a healthy, happy and prosperous New Year—may your personal and professional wishes come true. Second, let me thank you for your continued support of the Annals of the Rheumatic Diseases (ARD)—as readers, as authors, as reviewers or as members of our team. Finally, please allow me to look back at the past year from the perspective of the Editor.

As I had promised in my previous greetings note, we started three new sections in 2018: ‘Views on News’, ‘Heroes and Pillars of Rheumatology’ and ‘Thinking the Unthinkable’. Three ‘Views on News’ were published last year and conveyed what the authors thought were publications in other journals with important implications for our field. A first publication on a hero in the ‘Heroes and Pillars of Rheumatology’ part has also appeared, a section intended to tell the younger rheumatologists about people (or publications) that paved the roads to modern rheumatology—more will follow. Finally, ARD also made an initial blink into the ‘unthinkable’ future, which already elicited an interesting online discussion.

ARD has also promised to provide readers with actual data for all data points in all figures in articles on clinical trials. This is in the process of implementation, as can be seen already in some trial papers published in recent months; the data are shown either directly in the publication (such as in ref 9) or in supplementary materials (such as in ref 10). We sincerely hope that in the future—thinking the thinkable—no publication on clinical trials in any journal will show figures without revealing the data for all data points.

But where are we heading to in 2019? Well, first we have noticed that supplementary materials are often difficult to access in our journal and others. We do not want frustrated readers and, therefore, from 2019 on we will provide the supplementary materials downloadable with the pdf files of the respective articles. This will enable our readers to have access to important information without having to search in the web—sometimes in vain. Second, we realised recently that our instructions to authors may benefit from incorporating some additional aspects of transparency in the context of the submission, decision and publication processes. These have to be aligned with other BMJ journals, and soon our instructions to authors will provide unambiguous information in this respect, developed in collaboration with the chair of our advisory board which serves as an ethical guidance committee for the journal. Third, we will expand our editorial board by including patients; this will not only allow us to follow BMJ’s Patient and Public Partnership strategy, but patients are part of European League Against Rheumatism’s (EULAR) constituency and we appreciate their input in all areas of the organisation. Patients will serve as reviewers of research papers and, thus, their participation in shaping the content of the journal will go beyond their contributions to recommendations. We will also continue developing lay versions of research that is published in ARD and deems to be especially important to patients.

Needless to say that ARD also welcomes your suggestions on contents and concepts so that they can contribute to the development of the journal. Please send your thoughts to the editorial office at ard@bmj.com.

There will also be some small but visible structural changes in ARD. From now on, the page headings will refer to the topics covered by the respective articles rather than focusing on whether a paper deals with a clinical or basic research question. This will enable us to serve you even better in our continued efforts to focus on the newest developments in clinical, epidemiological and translational research, attempting to meet the quality standards that you expect and which this journal stands for.

When speaking about serving you, the readers of this journal, it is a pleasure to acknowledge. Thus, already 80 years ago the journal was dedicated to advance the field, a dedication that we have maintained until today and are determined to further expand over the next years.

But 2019 also marks another anniversary: 20 years ago EULAR decided to acquire a rheumatology journal and you hold the result of these deliberations in your hands—the EULAR Journal, a journal that is co-owned and commanaged by both BMJ and EULAR in the best collegial way, devoted to serving the field of rheumatology. The journey that ARD has taken from being a relatively small journal to one that is used by almost 15 000 subscribers and tens of thousands other readers is exemplary for what can be achieved even in the days of digital publishing. ARD serves both, those who wish to hold a print issue in their hands as well as those who wish to read papers online. And the ‘online first’ publications of EMUNET and the ‘online first’ publications of EULAR offer already the same typeset and quality as the final print version will have—for your reading pleasure.

However, there is yet another anniversary that I would like to make you aware of: in 2009 EULAR founded the ‘Emerging EULAR NETwork’ (EMEUNET), to provide high-quality, young-generation rheumatologists with an opportunity to contribute to all EULAR activities. All EULAR activities? Of course: all activities, including the EULAR Journal. Members of EMEUNET are on the editorial board of ARD as well as of our sister journal RMD Open, and both journals have established a peer review mentoring programme to train EMEUNET members as reviewers. Congratulations to EMEUNET on the occasion of its 10th anniversary and many thanks to the many reviewers who have served and serve as mentors for EMEUNET; most, if not all, of their mentees have developed excellent skills and need to be applauded. EMEUNET is also supporting ARD with respect to social network activities, which is gratefully acknowledged.

But now back to the future, back to this very first issue in 2019. As mentioned above, ARD will continue along the paths set forth during this decade and also continue providing you with the newest recommendations and criteria, primarily based on EULAR activities or combined efforts of the American College of Rheumatology and EULAR. And a first such recommendation paper for 2019 is brought to you in this very issue of ARD.
Editorial

Enjoy reading it and all the other papers that meet your interests in this and the subsequent issues.

Again a happy 2019.

Josef S Smolen

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To cite Smolen JS. Ann Rheum Dis 2019;78:1–2.

REFERENCES
Deep phenotyping of osteoarthritis: a step forward

Francis Berenbaum

Is osteoarthritis (OA) only one and the same disease or is it actually a nebula of several diseases for which we still lack markers allowing to differentiate them? This issue is currently the subject of much debate in the medical and scientific community and remains unresolved. It is not a trivial question because deciphering disease phenotypes could be used to select a group of patients for whom a particular treatment will be effective or for whom the prognosis will be better or worse.

According to the Osteoarthritis Research Society International (OARSI), OA is a disorder involving movable joints characterised by cell stress and extracellular matrix degradation initiated by microinjury and macroinjury that activate maladaptive repair responses, including proinflammatory pathways of innate immunity. The disease manifests first as a molecular derangement (abnormal joint tissue metabolism), followed by anatomical and/or physiological derangements (characterised by cartilage degradation, bone remodelling, osteophyte formation, joint inflammation and loss of normal joint function), which can culminate in illness. It is clear that this complex definition reveals, on the one hand, a poor understanding of the underlying mechanisms, and on the other hand the potential extent of heterogeneity of the disease. With such a definition, it is easy to imagine the theoretical possibility of many different disease subgroups, depending on clinical, biological, genetics and/or imaging variables.

Some believe that the successive failures of the many therapeutic trials carried out in recent years to delay OA are due to the phenotypic heterogeneity of the patients included in these trials, masking the possible efficacy of the drug X reserved for the only phenotype Y. This reasoning is analogous to what is now consensually accepted in cancer, where drugs used to be poorly effective in clinical trials before patient selection have become remarkably effective in specific phenotypes based on tumour gene signatures. Thus, recognising OA like a spectrum of diseases covering several phenotypes would make it possible to envisage a much more individualised medicine. It must be said that so far the vision of drug developers in this field is closer to ‘one size fits all’, which may explain the fact that there is no disease-modifying drugs on the market yet and that the quality of life of patients with OA remains poor.

A better knowledge of OA phenotypes could also be useful in the prognostic evaluation of the disease. Only a small percentage of patients with early knee OA will progress up to total joint space narrowing on standard radiographs even after decades. A better identification of fast versus slow progressors could accelerate therapeutic research by optimising the selection of patients to be included in trials by reducing the number of patients to include and/or by reducing the duration of the trials. The broadest approach to identifying such OA phenotypes is taken by the APPROACH (Applied Public-Private Research enabling Osteo-Arthritis Clinical Headway) consortium, a large public (European Union)-private partnership project initiated 3 years ago with funding from the Innovative Medicines Initiative.

There are several possibilities to phenotype OA. Some, which could be described as a ‘top-down’ phenotyping approach, rely on an a priori rationale, the one that the different phenotypes result from what is already known about the disease in a hypothesis-driven manner (figure 1). Thus, this approach is mainly based on the known risk factors (obesity-associated OA, trauma-associated, ageing-associated OA and so on) and on imaging results (bone marrow lesions-associated OA, inflammatory OA and so on). This kind of ‘top-down’ phenotyping has already improved our knowledge on the pathophysiology of the disease (eg, the discovery of an increased prevalence of hand OA in obese patients has paved the way to the hypothesis of close links between obesity and hand OA). Is OA only one and the same disease or is it actually a nebula of several diseases? The question is currently the subject of much debate in the medical and scientific community.

Figure 1 How to phenotype osteoarthritis? On the left side, phenotypes result from what is already known about the disease in a hypothesis-driven manner. On the right side, the phenotypes come from unsupervised clustering computational methods. The granularity of the phenotype precision depends on the number of data collected and on the quality of data integration. BML, bone marrow lesions; MetS, metabolic syndrome; OA, osteoarthritis.
between adipokines, low-grade inflammation, metabolic syndrome and OA) or to test new molecules (for instance, anti-interleukin-1 antibodies and synovitis-associated OA, biphosphonates and bone marrow lesion-associated OA). Another way to phenotype the disease, which could be described as a ‘step-up’ approach, is to look for these phenotypes without any a priori, relying solely on sophisticated statistical methods developed quite recently to find unsupervised clusters. Because of the absence of any hypothesis that drives the research, you consider that the more data you get (at the individual level) the more precise phenotypes you expect (defined as ‘deep phenotyping’). The work of Ji and collaborators7 published in Annals of the Rheumatic Diseases is a new step to a future deep-phenotyping approach in OA. It opens a very innovative, original and pioneering path in the field by taking advantage of the considerable technological progress of the last few years to study the extended expression of the transcriptome at a single cell level. The authors performed an RNA sequencing analysis on 1464 chondrocytes from 10 patients with OA undergoing total knee replacement at different stages of severity coupled to a computational analysis and histological assays of the cartilage explants. By gathering the different populations of chondrocytes according to their transcriptome, they identified seven molecularly defined clusters of chondrocytes, including three novel phenotypes with distinct functions. So far, chondrocyte subtypes were divided into proliferative chondrocytes (which are found in the proliferative zone of growth plates), prehypertrophic chondrocytes (which modulate the onset of hypertrophic chondrocytes), hypertrophic chondrocytes (which regulate the mineralisation of the surrounding cartilage matrix) and fibrocartilage chondrocytes. In this work, three unknown clusters were found. The authors called these new chondrocyte populations effector chondrocytes (enriched in genes involved in metabolism), regulatory chondrocytes (enriched in genes involved in signalling pathways) and homeostatic chondrocytes (enriched in circadian clock rhythm marker genes). It is noteworthy that there were also two subpopulations within hypertrophic chondrocytes (called HTC-A and HTC-B) which may have distinct roles in OA pathophysiology. Then, the authors investigate the relationships between these seven endotypes of OA cartilage chondrocytes and the OA severity based on a knee scoring system and the OA stage of the samples. In parallel, they assessed the expression level of 19366 protein-coding genes across the seven OA chondrocyte endotypes. By stratifying the patient samples into two groups (expression levels above or below the determined cut-off values based on each individual gene profile), they identified 336 predictive genes, with 199 related to favourable outcomes and 137 related to unfavourable outcomes. Interestingly, favourable genes were mainly expressed in the three newly described chondrocyte populations, while unfavourable genes were expressed mainly in proliferative chondrocytes, prehypertrophic chondrocytes and fibrocartilage chondrocytes. Finally, based on a correspondence analysis (a multivariate statistical technique similar to principal component analysis but applies to categorical rather than continuous data), they demonstrated that proliferative chondrocytes, prehypertrophic chondrocytes and fibrocartilage chondrocytes were correlated with worse clinical outcomes. This work sheds new light on the pathophysiology of the disease by triggering our curiosity on the lineage of these new chondrocyte phenotypes and their expression in joint development, in OA initiation and in cartilage regeneration. It also opens up our research agenda in the field of OA pathophysiology by restoring its nobility to cartilage, a tissue rather neglected in recent years for the benefit of the subchondral bone and synovial tissue.

Attempts to find molecular players in OA progression based on -omics technologies have already been published. The study published in this issue is the first one that looks at such molecular players at the single cell level, and also that delineates how the repartition of different cell populations could discriminate patient phenotypes according to clinical outcomes. There are, however, a number of limitations to this study, especially if the ultimate goal is to improve the management of patients. First, this study is based on a limited number of samples since only 10 patients were the subject of this experimental study. It cannot be held to the authors because the current technology makes it challenging to perform such studies on hundreds of samples. There is no doubt that the rapid progress in big data management, bioinformatics and molecular biology technologies should solve this problem quickly. Second, it is obvious that in real life such a phenotypic approach based on the molecular analysis of cartilage cells makes it almost impossible today to apply in current practice because of the aggressiveness of the procedure in obtaining cartilage, even if cartilage samples are already being taken today to treat traumatic cartilage defects in non-bearing areas. Third, we lack longitudinal data to know whether these chondrocyte phenotypes are fixed or whether there may be a change from one phenotype to another over time according to OA progression. Moreover, cells present in the other joint tissues such as synoviocytes, osteoblasts and osteoclasts (to name only three) could also play critical roles in the outcome, justifying to realise this same kind of analysis in other joint tissues in the future.

Altogether, this work shows us brilliantly how new technologies in the molecular biology field coupled with the considerable progress of these last years in bioinformatics can help us to better understand the pathophysiology of the disease, but also how concretely we can hope to improve the prognosis and the treatment. The new challenge in the next years will be to integrate these huge amounts of clinical, biological, genetics, imaging and -omics data sets in order to eventually delineate useful phenotypes for predicting outcomes and treatment responses (figure 1). This new step of data integration for future deep phenotyping is just starting in our field,11 and the tools for integrating these numerous layers of data sets should be operational very soon. Precision medicine can now be reasonably considered as an achievable option in OA in the next decade.

Handling editor: Josef S Smolen
Contributors: FB is design and writing.
Funding: FB is supported by ROAD–Fondation Arthritis Networking and Société Française de Rhumatologie.
Competing interests: None declared.
Patient consent: Not required.
Provenance and peer review: Commissioned; internally peer reviewed.
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http://dx.doi.org/10.1136/annrheumdis-2017-212863

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Editorial


Gene editing for inflammatory disorders

David T Ewart, Erik J Peterson, Clifford J Steer

ABSTRACT
Technology for precise and efficient genetic editing is constantly evolving and is now capable of human clinical applications. Autoimmune and inflammatory diseases are chronic, disabling, sometimes life-threatening, conditions that feature heritable components. Both primary genetic lesions and the inflammatory pathobiology underlying these diseases represent fertile soil for new therapies based on the capabilities of gene editing. The ability to orchestrate precise targeted modifications to the genome will likely enable cell-based therapies for inflammatory diseases such as monogenic autoinflammatory disease, acquired autoimmune disease and for regenerative medicine in the setting of an inflammatory environment. Here, we discuss recent advances in genome editing and their evolving applications in immunoinflammatory diseases. Strengths and limitations of older genetic modification tools are compared with CRISPR/Cas9, base editing, RNA editing, targeted activators and repressors of transcription and targeted epigenetic modifiers. Commonly employed delivery vehicles to target cells or tissues of interest with genetic modification machinery, including viral, non-viral and cellular vectors, are described. Finally, applications in animal and human models of inflammatory diseases are discussed. Use of chimeric autoantigen receptor T cells, correction of monogenic diseases with genetically edited haematopoietic stem and progenitor cells, engineering of induced pluripotent stem cells and ex vivo expansion and modification of regulatory T cells for a range of chronic inflammatory diseases are reviewed.

INTRODUCTION
Many chronic human autoimmune and autoinflammatory diseases require indefinite therapy. Despite major developments in molecular mechanism-focused therapy for rheumatic conditions in the past several decades, substantial morbidity and mortality still attends these diseases. Emerging gene therapy approaches offer the promise of more specific and durable treatments that may circumvent toxicities of traditional medical management for inflammatory disease. Rare periodic fever syndromes with monogenic causes are clear candidates for potential application of genetic modification therapy. Of course, the aetiology of most chronic inflammatory disorders is complex, involving contributions of multiple genetic and environmental factors. Nonetheless, the explosive growth in technology for precise and robust genetic editing over the past decade has generated interest in using targeted genetic modifications for the treatment of these acquired polygenic inflammatory diseases as well.

The principle of gene editing involves deliberate and specific modifications to genomic DNA or mRNA with the intent to modify gene product expression, structure or function. Chromosomal DNA modifications rely primarily on the ability to generate double-stranded breaks (DSB) that serve to localise targeted changes to the effected DNA. Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (CRISPR/Cas9) has rapidly become the tool of choice to this end, but older techniques such as zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) have been used extensively. New applications of the nucleases Cas9 have the potential to greatly expand our ability to manipulate nucleic acids and protein expression, structure and function. Emerging technologies such as base editing, activation or repression of transcription via Cas9-targeted transcription factors or Cas9-targeted epigenetic modifiers and RNA editing are novel technologies that could greatly expand our ability to discretely control pathobiology. Viral and non-viral vectors can be employed to deliver effectors of genome editing to the cell or tissue of interest, including T cells, haematopoietic stem cells (HSC), induced pluripotent stem cells (iPSC) and organ parenchymal cells.

GENOME EDITING
The overall goal of genome editing is to alter effector protein expression, structure or function. Gene editing has traditionally been used to this end and facilitates efficient and accurate modification of DNA at a specific locus or loci by generation of a DSB. DSBs are then modified by endogenous cellular machinery that mediates either homologous recombination (also termed ‘homology-directed repair [HDR]’), or non-homologous end joining (NHEJ). HDR repairs DSBs by transfer of the modified or wild-type DNA into its homologous target location in the genome. Despite prior extensive use, targeted modification of genomic DNA by HDR has been limited by relative inefficiency, high rates of random template DNA insertion, off-target insertion, and function only during S and G2 cell cycle phases.

NHEJ, in contrast, is the most common manner of DSB repair in mammalian cells, is active during all phases of the cell cycle and does not require a homologous template for repair. Due to the latter, NHEJ can create insertions and deletions of varying sizes (termed ‘indels’) at the repair site, which can mutate the protein coding sequence in a negative fashion. Thus, the method of DSB repair is an important consideration when crafting experimental techniques for genome editing.

Investigation of inflammatory disease pathobiology has flourished with the modern capabilities of genome editing. Using conventional and conditional targeted murine alleles, as well as reporter genes, and mice humanised to express human genes and gene variants, the biology of major inflammatory states, such as rheumatoid arthritis and...
systemic lupus erythematosus, has been rigorously addressed both systemically and at the tissue level. In some instances, this has led to the development of highly potent targeted therapies. Modern techniques for manipulation of the genome are increasingly precise, efficient and facile. The rapid development and capability of the technology will undoubtedly foster increasingly insightful biologic discovery and efficacious therapies for both immune and inflammatory disorders. In this section, we provide a brief history of older tools of genome editing, many of which are still used today, and review state-of-the-art technologies for genetic manipulation of human inflammatory disorders (table 1).

Older tools for genetic editing

Single-stranded oligonucleotides and meganucleases were initial forays into gene editing. Both are inefficient, difficult to use and have variable specificity.6–11 ZFNs are artificially engineered endonucleases that represented a significant step forward in efficiency and capability.12 13 They have been used in multiple applications in human and animal models.14–16 However, there is lingering concern over ZFN off-target effects,17 and the time, difficulty and sophisticated laboratory methods required for their design and construction.

TALENs are an evolution of ZFNs18 and are notable for their high rates of DNA cleavage and limitless target range.19 They too have been used in a wide variety of organisms.19 However, TALENs share many drawbacks with other endonucleases, including the capacity for off-target effects,19 inefficient delivery to target cells due to large size and epigenetic modifications on the target chromosome interfering with successful HDR.20

CRISPR/Cas9

The CRISPR/Cas9 system is a defence mechanism found in bacteria and archaea.21 22 CRISPR sequences consist of small unique ‘spacer’ sequences, which are interspersed in stretches of highly conserved repetitive DNA sequences, termed ‘CRISPR repeats’, typically located next to groups of highly conserved protein-coding genes called CRISPR-associated (cas) genes that often carry domains similar to nucleases, helicases, polymerases and nucleotide-binding proteins.21 22 The combination of spacer sequences complementary to intracellular but foreign nucleic acids and the Cas9 nuclease fosters degradation of the foreign genetic material and protection from pathogens.

The CRISPR/Cas9 system employs two components for creating DSBs—a customisable single-stranded guide RNA (sgRNA) and an endonuclease (figure 1). The sgRNA is made up of a precursor CRISPR RNA (crRNA) that contains the full length of CRISPR repeats and embedded spacer sequences23 that hybridise to a separately transcribed complementary trans-activating crRNA. The sgRNA guides the Cas9 enzyme, a double-stranded RNA-specific ribonuclease, to any target site.24–27 The RNA-guided Cas9 enzyme surveys the genome, recognising conserved three-nucleotide species-specific protodiradical motifs (PAM) in the target genome. On binding, the Cas9-gRNA complex detects the DNA complementarity with the guide RNA and creates site-specific DSBs to generate a blunt end usually at

![Figure 1](https://example.com/figure1.png)
CRISPR/Cas9 is versatile, efficient, simple to design and use, increasingly specific and is rapidly supplanting other modalities of gene editing.26 Moreover, its functionality is also being broadened. Using multiple gRNAs with the same Cas9 nuclease allows for targeting multiple genes simultaneously.30 31 A single amino acid substitution in either of the two-nuclease domains of Cas9 results in a ‘nickase’ that cleaves only one strand of DNA,32 potentially reducing off-target effects. The refinements of CRISPR/Cas9 and other microbial endonucleases33 34 have resulted in CRISPR/Cas9 rapidly becoming the workhorse for a range of applications including gene therapy, functional genomic screening, transcriptional modulation and synthetic biology.

Base editing with Cas9

Historically, genetic modification has relied on the ability to create a DSB at a specific site of interest and to use the endogenous cell repair machinery for HDR or NHEJ (figure 2A). Methods that induce DSBs have been plagued by ‘off-target’ random insertions or deletions (indels) of variable predictability, and by low efficiency under circumstances practical for therapeutic development, including modification of non-dividing cells.30 32 Komor et al designed and tested a novel method of generating single base pair changes without first generating a DSB.35 The group recognised the capacity of ‘dead’, enzymatically inactive, Cas9 to retain its ability to target and bind DNA in a guide RNA-directed manner. They sought to couple this function with the ability to make a base substitution without a DSB (figure 2B). They then used cytidine deaminases to catalyse the deamination of cytosine (C) to uracil (U), which binds complementary bases like thymine (T). This method is synergistic with the effects of Cas9 in that on binding of the sgRNA/Cas9, nine nucleotides of DNA are unpaired to a single strand,36 with single strandedness being a requirement for cytidine deaminase activity.37 Using a chimeric fusion protein comprising rat-derived cytidine deaminase APOBEC1 fused with nuclease-deficient Cas9, the group observed base editing efficiency rates as high as 37% with a 1.1% indel formation rate.35 These rates compared favourably with standard wild-type Cas9 and sgRNA, reagents which averaged 0.5% efficiency with a 4.3% indel formation rate. A second form of fusion protein was slightly less efficient.
but with even lower indel formation rate. Proof of principle experiments showed that human disease-associated mutations could be corrected in mouse astrocytes and human breast cancer cell lines using base editing techniques.

RNA editing

There is considerable interest in using the specificity of Cas9 and related nucleases in conjunction with sgRNAs for transient, targeted modification of gene expression, structure or function. Post-transcriptional modification of mRNA is natural biologic process and synthetic modifications would be limited by the degradation of the mRNA and subsequently translated proteins. Cox et al described a method harnessing the specificity of CRISPR-associated RNA-guided ribonuclease Cas13 fused to adenosine deaminase acting on RNA 2 (ADAR2) for targeted modification of specific RNAs by deamination of adenosine to inosine, which is equivalent to guanosine in translation and splicing. The investigators created a mutated ADAR2 deaminase domain with relaxed sequence constraints to increase editing efficiency and fused it to catalytically inactive Cas13 (figure 2C). They were able to demonstrate RNA editing for programmable A to I (G) replacement of reporter, endogenous and disease-associated transcripts. However, many off-target events were observed adjacent to the guide RNAs. The investigators then determined that the ADAR2 motif of their fusion protein was responsible for the off-target mutations and performed rationale mutagenesis to enhance specificity to the intended target site. Editing nucleic acid sequence with this tool offers several advantages including the lack of a prerequisite sequence constraint such as a PAM, activity only on transcribed sequences, direct deamination without requiring endogenous repair pathways and, lastly, the transient nature of RNA rather than DNA modification.

Activation and repression of transcription with Cas9

The Cas9 capacity to specifically target certain loci in the genome can also be employed to modify determinants of protein expression without changing nucleotide sequence. In this application, Cas9 acts as a chaperone for effector proteins, such as transcription activators, repressors or epigenetic modifiers, and guides them to specific locations. Mali et al used a nuclease-null Cas9 protein, the VP64 activation domain and promoter-specific sgRNAs to stimulate transcription. To maintain the targeting guides them to specific locations. To achieve successful targeted gene therapy, efficient delivery vehicles and vectors are essential. Plasmids encoding proteins responsible for genome editing must successfully enter the nucleus of targeted cells in order to facilitate transcription and eventually translation. The cell and nuclear membrane are physical barriers to the passage of large hydrophilic molecules like DNA, RNA and proteins. Furthermore, DNA, RNA and protein are all subject to intracellular and extracellular degradation by nucleases and proteases. Methods for circumventing the physical and degradative barriers to delivery can generally be divided into viral vectors, non-viral vectors, non-vector agents and cellular delivery vehicles.

Viral vectors

Retroviruses, adenoviruses and adeno-associated viruses (AAV) are the three primary classes of viruses that have been used to deliver genetic material and can potentially be used in vitro, ex vivo and in vivo. Retroviral vectors use reverse transcription for replication and a subtype, lentiviruses, can integrate viral DNA into cells without the need for replication. Retroviruses have successfully edited DNA in gene therapy trials; however, the risk of oncogenesis cannot be understated. More recently lentiviruses have been modified to contain weaker cellular promoters, termed self-inactivating, and carry much reduced risk of mutagenesis. Integrate-defective lentiviral vectors do not integrate into the host genome, remain episomal and gradually dilute via cell division, and have been used for transient expression of ZFNs and donor templates in vitro.

Adenoviral vectors deliver viral double-stranded DNA to the nucleus, allowing transient expression of the desired proteins such as nucleases. AAV vectors are smaller viral particles that rarely integrate into the host genome. They have garnered considerable attention for their ability to deliver small nucleases, such as ZFNs or Cas9, to sites of interest. An AAV vector was used to correct murine models of haemophilia A and B via a donor template and ZFN to induce liver-specific human factor VIII and IX within the albumin gene. Despite numerous preclinical and clinical successes, there is still a considerable lack of knowledge about the long-term efficacy and safety of AAVs as it pertains to gene editing for human disease. AAVs also suffer from size limits for the expressed transgenes, pre-existing immunity against AAV vectors as well as humoral responses, potential for limited durability in dividing cells, genomic integration’s association with mutagenesis and lack of precise control of the therapeutic gene. Indeed, Cox et al had to modify and reduce their Cas13-ADAR2 fusion construct to fit within the 4.7 kb size limit of an AAV for delivery to HEK293T.

Non-viral vectors

In general, non-viral delivery vectors include nanoparticles and cationic carriers. Nanoparticles may comprise genomic application of fusing the specificity of nuclease-deficient Cas9 with sgRNA to a protein effector, in this instance the catalytic core of human acetyltransferase p300 to catalyse the acetylation of histone H3 lysine 37. They observed transcriptional activation associated with targeted p300 epigenetic modification of enhancers both proximal and distal to the intended gene. Similar fusions of other epigenetic modifiers including demethylases, methyltransferases and deacetylases have been generated and are likely to greatly expand the capability to perform complex and precise epigenetic modification.

DELIVERY OF EFFECTORS FOR GENOME EDITING

To achieve successful targeted gene therapy, efficient delivery vehicles and vectors are essential. Plasmids encoding proteins responsible for genome editing must successfully enter the nucleus of targeted cells in order to facilitate transcription and eventually translation. The cell and nuclear membrane are physical barriers to the passage of large hydrophilic molecules like DNA, RNA and proteins. Furthermore, DNA, RNA and protein are all subject to intracellular and extracellular degradation by nucleases and proteases. Methods for circumventing the physical and degradative barriers to delivery can generally be divided into viral vectors, non-viral vectors, non-vector agents and cellular delivery vehicles.
material complexed with cations, allowing endocytosis and cell membrane transfer of negatively charged DNA. Cationic nucleic acid carriers can be divided into lipid-based agents, such as lipofectamine, and polymeric.59 These non-viral vectors offer several advantages including transient expression, the capacity for repeated administration, potential for larger strand genomic delivery and possibly improved efficacy.60 Lipofectamine has been used for successful delivery both in vitro and in vivo, as well as for CRISPR/Cas9-mediated modification of murine iPSCs.61 However, in general, these agents have to date not been used extensively in clinical application.

Cellular vectors
Gene editing can also be performed on cells ex vivo. After return to the host, gene-edited cells may elicit long-lasting biologic effects. Chimeric antigen receptor (CAR) T cells have had remarkable success in treatment of otherwise lethal haematological malignancies and have been shown to expand and persist during and after treatment.62 Briefly, T cells are isolated from human subjects by leukapheresis and gene editing performed ex vivo to induce expression of an extracellular single-chain antigen-binding domain (scFv) fused to CD137-CD3z signalling domains.63 The resultant modified CAR T cells are then reinfused to the host for targeted effector function. T lymphocytes have tremendous capacity to drive immune response—both adaptive and pathologic—in an antigen-specific manner. Gene therapies that seek to capitalise on the narrow specificity and cytotoxic potential of T cells are under study in a number of autoimmune diseases.

Non-vector delivery methods
Electroporation is historically the most widely recognised and used method of facilitating the delivery of genomic material into cells and subsequently the nucleus. It has been used for ex vivo genome editing by introduction of Cas9 and sgRNA-encoding plasmids into haematopoietic stem and progenitor cells as well as T cells.64 Mechanical deformation of the cell with microfluidic devices has also been used for efficient CRISPR-mediated gene editing.65 Finally, direct injection of targeted cells or tissues has been used to bypass the cell and nuclear membranes. Hydrodynamic injection induces tissue damage but has been shown to be effective.66 Generation of experimental animals is also accomplished in a one-step fashion with injection of genome-editing machinery into embryos or zygotes. Microinjection of Cas9 mRNA and sgRNAs into single-cell mouse embryos can effectively target multiple genes.6768

Future directions for delivery methods
Viral vector, non-viral vector and non-vector approaches for delivery of the molecular machinery responsible for genetic editing all remain foci of active research, especially in regard to increasing cell or tissue-specific delivery. One specificity-enhancing strategy is exemplified by modification of the CRISPR-Cas endonuclease to include asialoglycoprotein receptor ligands that are preferentially internalised into hepatocytes.69

TARGETS FOR GENE EDITING IN INFLAMMATORY DISEASES
Chimeric autoantibody receptor T cells
Due to the non-specific nature of current immunosuppressive treatment protocols, there is considerable interest in disrupting pathologic components of the immune system while otherwise preserving its natural function (figure 3).

Ellebrecht et al reported a murine proof of concept using modified T cells for the treatment of the autoimmune skin disease pemphigus vulgaris (PV).70 The authors reasoned that T cells could be genetically modified to specifically recognise and eliminate pathogenic B cell clones that express autoreactive antigen receptors while maintaining the rest of the B cell compartment. The approach of targeting individual B cell clones differs from conventional anti-CD20 therapies, which deplete the entire CD20 B cell compartment. They studied PV because epitopes targeted
by pathologic antibodies are well described. To specifically target the pathologic autoantibody-producing B cells, they used a lentiviral vector to genetically modify T cells to express autoantigen desmoglein (Dsg) 3 fused to CD137–CD3ζ signalling domains. Resulting chimeric autoantibody receptor (CAAR) T cells were then infused to treat animal models of PV, including a humanised mouse model of disease. CAAR T cells were found to expand, persist and exhibit cytotoxicity against cells expressing anti-Dsg3 B cell receptors in diseased recipients in vivo. Histopathologically, the CAAR T cell–treated mice showed an absence of IgG deposition in mucosal samples and no histologic blister formation. CAAR T cells were able to infiltrate the epidermis in a human xenograft model and similarly ameliorated the disease process. Importantly, cytotoxicity occurred without apparent off-target toxic effects.

Treatment of monogenic inflammatory diseases

The genetic characterisation of inherited periodic fever syndromes has progressed remarkably in the era of rapid, inexpensive genome sequencing. Causative single mutations have been identified in a number of heritable syndromes, raising the possibility of corrective genetic therapy for these disorders. Familial Mediterranean fever (FMF) is caused by mutations in the MEVF gene.91 Cryopyrin-associated periodic syndrome (CAPS), which encompasses familial cold autoinflammatory syndrome, Muckle-Wells syndrome and neonatal-onset multisystem inflammatory disease, is associated with mutations in the NLRP3 gene, which encodes cryopyrin, a component of the inflammasome, on chromosome 1q44.92 93 Several other examples of hereditary autoinflammatory diseases include: hyperimmunoglobulinaemia D with recurrent fevers,94 tumour necrosis factor (TNF) receptor-associated periodic syndrome (TRAPS),95 Blau syndrome,96 pyogenic arthritis, pyoderma gangrenosum and acne syndrome (PAPA),97 and chronic recurrent multifocal osteomyelitis.98 There are also increasingly well-described examples of monogenic diseases that, among other pathologies, display a phenotype of severe derangement of immunity leading to autoimmunity: Aicardi-Goutières syndrome,99 STING-associated vasculopathy with onset in infancy (SAVI), chronic atypical neutrophilic dermatosis with lipidodystrophy and elevated temperature (CANDLE)100 and many other immunodeficient conditions.101

Wiskott-Aldrich syndrome (WAS) is an example of monogenic autoimmunity-associated disorder that has been approached with gene therapy. WAS is a rare X linked immunodeficiency caused by coding variants in the WAS gene, the protein product of which regulates the actin cytoskeleton in haematopoietic lineages. Patients have thrombocytopenia, recurrent infections, eczema, an increased incidence of autoimmunity, higher risk of lymphoproliferative disorders and lymphoid malignancies, and frequently die during the third decade of life.102 103 HLA-matched allogenic haematopoietic stem cell transplantation (HSCT) is curative; however, significant risks of procedure-related morbidity and mortality persist.104 In an early attempt at gene therapy for WAS using γ-retroviral vectors, 7/9 trial subjects developed acute leukaemia secondary to viral enhancer-mediated insertional mutagenesis.105 Subsequent attempts using a self-inactivating lentiviral vector for genetic modification of autologous HSCs prior to auto–HSCT have been successful, and no patients have shown leukaemic transformation to date.106 107

WAS is not the only human condition in which enthusiasm for genetic modification approaches has been tempered by safety concerns. Attempted treatment of severe-combined immunodeficiency was marred by leukaemogenic transformation secondary to retroviral insertion of enhancers of oncogenic genes.108 More recently, use of an AAV for in vivo transduction of spinal alpha motor neurons in non-human primates and piglets with a human SMN transgene resulted in severe hepatitis and degeneration of the targeted tissues.109 These outcomes suggested an ongoing need for careful preclinical and clinical safety studies when manipulating the genome for therapies of human disease. This is especially true for many of the monogenic autoinflammatory diseases which pose lesser morbidity and mortality threats compared with severe syndromes such as WAS.

In some autoinflammatory syndromes, such as FMF, CAPS and TRAPS, the pathogenic cell type is likely to be bone marrow derived and radio and/or chemotherapy sensitive. These conditions thus represent ideal first targets or ‘low-hanging fruit’ for cure or amelioration with genetic modification of haematopoietic cells. In contrast, tissue injury from interferonopathies such as SAVI and CANDLE is likely to be caused by complex multicellular gene-phenotype interactions, and thus strategies targeting multiple cell types and tissues will be required. While still their infancy, highly efficient base editing techniques (see section above) may make genetic editing for both lethal and non-lethal monogenic disease a tenable strategy.

Stem cell modification

There is considerable interest in genetically modifying iPSCs for the treatment of human disease. Advantages of iPSCs include their potential durability in vivo, their ability to differentiate into tissues affected by chronic inflammatory diseases and the ability to create abundant quantities without the use of embryos or other products of conception.

Controlled delivery of anticytokine therapy

One approach for the use of genetically modified iPSCs is known as closed-loop biologic drug delivery system. Anticytokine therapies with monoclonal antibodies and decoy receptors have revolutionised the treatment of chronic inflammatory diseases in the last two decades.110 However, targeting key proinflammatory molecules such as TNF-α or interleukin (IL) entails a risk of side effects, including infections, and requires constant dosing and patient exposure to the drug which may interfere with the pleiotropic roles of the targeted cytokine.111 To address the problem of non-specific loss of cytokine function in all tissues created by such therapies, Brunger et al sought to engineer resistance to cytokine function only among cells of interest. They modified murine iPSCs using CRISPR/Cas9 to insert anti-inflammatory molecules (eg, IL-1Ra or chimeric human sTNFR1-murine IgG) in the Ccl2 locus.112 The Ccl2 gene product regulates trafficking of inflammatory monocytes/macrophages, basophils and T lymphocytes in response to inflammatory cues such as TNF-α and IL-1.113 The inserted naturally occurring cytokine antagonists within the Ccl2 locus mitigated the inflammatory effects of physiologic concentrations of IL-1 and TNF-α when the iPSCs were cultured in monolayer as well as after differentiation into engineered cartilage tissues. The engineered cartilage tissues were observed to be resistant to the pathophysiologic effects of IL-1 and TNF-α. The authors proposed that this approach could be used for a targeted cell-based anticytokine vaccine.

Modification of stem cells for tissue regeneration

Approaches utilising genetic modifications have found recent application in the field of regenerative medicine in attempts to restore function of damaged and diseased tissues.114 115

Osteoarthritis (OA) is a highly prevalent disabling disease for which disease-modifying therapy (other than joint replacement) has not been identified. However, OA joints are characterised by the presence of proinflammatory cytokines,116 which could hinder the therapeutic effect, engraftment and/or longevity of stem

Figure 4  Schematic illustration of the strategy for generating iPSCs resistant to IL-1-mediated signalling for tissue engineering applications. (A) Binding of IL-1 ligand to the IL-1RI results in activation of a proinflammatory transcription programme involving the transcription factors NF-κB, JNK and MSK-1. (B) gRNAs target the genome-editing nuclease Cas9 to two sites flanking exon 2 of IL-1RI, which encodes the signal peptide sequence. (C) Cas9 induces DNA DSBs, which may be repaired via NHEJ. (D) NHEJ leads to a subset of alleles with fully intact IL-1RI, while others may have genomic disruptions at the IL-1RI locus, including excision of the signal peptide sequence, resulting in loss of signalling through IL-1RI. DSB, double-stranded break; gRNA, guide RNA; IL-1, interleukin-1; IL-1R1, IL-1 receptor type 1; iPSC, induced pluripotent stem cell; NHEJ, non-homologous end joining. (Reproduced from Brunger et al61 with permission; © Arthritis and Rheumatology)

cell-based therapy in that milieu. Rather than utilising genetically engineered iPSCs to modify inflammatory signalling at the local tissue level, Brunger et al used CRISPR/Cas9 to engineer murine iPSCs to be inflammation resistant61 with the aim of utilising the engineered cells for regenerative medicine in a hostile, inflammatory microenvironment. They deleted the IL-1 receptor type 1 gene, selected clones with homozygous deletion and used specific differentiating factors to generate cartilage from the edited iPSC clones (figure 4). The resulting genetically modified cartilage was resistant to cytokine-mediated tissue degradation relative to wild-type and heterozygous clone cartilage.

Webber et al also used iPSCs to explore the possibility of tissue regeneration in recessive dystrophic epidermolysis bullosa, a severe disorder caused by mutations to the COL7A1 gene that causes life-threatening derangement of skin integrity.99 Patient-derived primary fibroblasts were isolated and the COL7A1 gene defect restored to wild-type status using CRISPR/Cas9. Genetic modification was assessed and found to be specific to the intended locus apart from a single, predicted off-target modification in a clone treated with Cas9 nuclease rather than the nickase. iPSCs were generated from the modified fibroblasts and showed ability to differentiate into keratinocyte, mesenchymal stem cells and HSCs.

T regulatory cells

T regulatory cells (Tregs), defined classically as CD4+CD25high FOXP3+, are a naturally occurring subset of helper T cells that have immunoregulatory functions including suppression of antigen-specific T cells and maintenance of peripheral tolerance. The observed frequency in suppressive function of Tregs in association with autoimmune disease100,101 has led to a growing interest in their potential therapeutic use. Effectiveness of Treg cellular therapy depends on availability of large numbers of Tregs—many times greater than the quantities of naturally occurring Tregs available for harvest. In vitro expansion protocols are therefore essential to Treg cellular therapeutic approaches. However, there are barriers to efficient in vitro enrichment of antigen-specific T cells,102 such as loss of regulatory phenotype and insufficient in vitro expansion. These obstacles may be overcome with gene editing techniques. There is also interest in using gene editing to induce in vivo production of antigen-specific Tregs. The use of gene editing of Tregs has recently been comprehensively reviewed.103

Genetically modified Treg cell therapy

Isolating sufficient numbers of Tregs with or without a rare antigen specificity from the natural T cell population is a major challenge for Treg-focused therapy. Several recent strategies for producing large quantities of clinical grade Tregs and for generating antigen-specific Tregs in vitro have employed genome engineering. CD4+ T cells have been modified ex vivo to express the ‘master’ transcription factor FOXP3 in a fashion that allows them to differentiate into a large population of Tregs. Treg populations expanded by inducible FoxP3 have been used to treat immunodysregulation polyendocrinopathy enteropathy X linked syndrome, caused by a dysfunction in the FoxP3 gene, in human patients as well as animal models of autoimmunity.104–106

Another approach is the use of TCR gene transfer to direct polyclonal Tregs to express a specific TCR gene and thus redirect specificity towards a single antigenic epitope. This technique has
been demonstrated to be effective in several murine models of transplant tolerance, inflammatory arthritis and in human T cells for islet cell targeting.107–110

Finally, in a manner analogous to the generation of CAR T cells for therapy of malignancy, Tregs can be engineered to express extracellular scFv or antigenic domains fused to downstream intracellular signalling molecules. Antigen-specific chimeric receptors have been used in murine experimental autoimmune encephalitis (EAE)111 112 and a murine model of inflammatory bowel disease.113 114 There are ongoing efforts to examine the utility of citrullinated-peptide-specific CAR T cells for the treatment of rheumatoid arthritis.115 Chimeric autoantigen receptor Tregs have been studied in a murine model of haemophilia A with autoantobody formation to factor VIII.116 Autoantibody-producing B cells were able to ‘tolerized’ and autoantibody production halted.

Induction of antigen-specific Tregs in vivo

The liver is a robust reticuloendothelial organ and contains resident cells that support a tolerogenic effect on self and foreign antigens by expression of surface ligand inhibitors for T cell activation and production of inflammatory mediators.117–119 As such it is uniquely positioned as a target for genetic therapies for induction of antigen-specific tolerance. Keeler et al used an AAV8 vector with the cDNA for the neuroprotein, myelin oligodendrocyte glycoprotein (MOG), under the control of a liver-specific promoter to induce hepatic expression of MOG in a murine model of multiple sclerosis, EAE. Hepatic expression of MOG used the tolerogenic properties of the liver to produce MOG-specific FOXP3+ Tregs in vivo. Mice treated prophylactically were protected from developing EAE and in mice already experiencing mild to moderate neurological deficits the vector alone was effective at reversing clinical and pathological signs of disease. When combined with immunosuppression, AAV immunotherapy rescued mice from fatal end-stage EAE and severe paralysis.120 Using a similar strategy, a lentiviral vector was used to induce insulin B chain 9-23, the immunodominant T cell epitope in non-obese diabetic (NOD) mice, in NOD hepatocytes. The lentiviral treatment induced insulin B chain 9-23-specific effector T cells but also FoxP3+ Tregs, which halted islet immune cell infiltration and protected from T1D. When combined with anti-CD3 monoclonal antibody, T1D was reversed, and shown to be dependent on Tregs.121

RNA editing and transcriptomic modification

The technology for and application of RNA editing is still in its infancy relative to the technologies for making permanent changes to the genome. However, there are speculative applications of RNA editing for inflammatory diseases. Cox et al were able to repair transcripts of the 878G>A (AVPR2 W293X) mutation associated with X linked nephrogenic diabetes insipidus and the 1517G>A (FANCC W506X) mutation associated with Fanconi anaemia.122 They also suggested the possibility of utilising similar methods for correction of any of the thousands of G to A mutations associated with pathologic states, potentially offering the ability to mimic protective alleles for at-risk individuals during times of immunologic and inflammatory stress.

CONCLUSIONS

The technology to manipulate genomic material has allowed tremendous discovery in the biology of inflammatory disease. While still in its infancy, genetic editing is now, decades after the discovery of the nature and structure of DNA, entering the realm of therapy (table 2). This is particularly true for severe monogenic disease. There are several apparent niches for gene editing in the treatment of inflammatory diseases, including correction of monogenic autoinflammatory syndromes, CAAR T cell therapy for auto-antigen-specific targeting of pathologic B cell clones, modification of iPSCs for controlled cytokine delivery and tissue regeneration and Treg-based therapies. As technology matures, cell therapies based in genome editing will likely strive towards the ‘holy grail’ of autoimmune disease therapy, that is, targeted inhibition of the pathologic components of the immune system without necessitating generalised immunosuppression. New tools for transient modification of protein structure, function and expression will allow us to fine-tune the delicate balance the immune system maintains between defence and tolerance to self. Harnessing the inherent specificity of our own immune system to target pathologic B or T cell clones, particular cellular subsets necessary for disease or disrupt trafficking to affected tissues will likely become feasible in the near future. Specific therapy will more potently inhibit the inflammatory processes yet spare the remainder of the immune system. But therapies utilising genetic editing will likely expand beyond ex vivo modification of cells of the immune system when we can reliably target certain cell types or tissues in vivo. One can imagine stepwise approaches for targeted genetic honing to redirect from inflammation to homeostasis in chronic inflammatory diseases affecting a particular organ or cell type. Addition to or antagonism of various growth factors targeted to organ parenchymal or interstitial cells could potentially be applied to fibrotic inflammatory diseases, which at present have a paucity of treatment options. Furthermore, more elegant and coordinated control of gene expression could allow the capability to generate replacements for organs or tissues damaged by inflammation, such as a biologic rather than plastic and metallic replacement for a joint with end-stage OA. The increasing capability, ease of use and reliability of modern tools for gene editing will undoubtedly lead

Table 2  Human inflammatory disease states for which gene editing approaches and therapies have been considered, and for which studies in animal models or primary human cells have been reported

<table>
<thead>
<tr>
<th>Inflammatory disease</th>
<th>Model</th>
<th>Delivery vector/method</th>
<th>Reference</th>
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<td>Lentivirus</td>
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CRISPR, clustered regularly interspaced short palindromic repeat; HSC, haematopoietic stem cell; IPEX, immunodysregulation polyendocrinopathy enteropathy X linked; iPSC, induced pluripotent stem cell.
to new pathophysiologic insights and therapies for immune and inflammatory diseases.

Contributors All authors contributed to the conception and design as well as the literature review for this work. Each participated in drafting and revising the important intellectual content and approved the final version to be published. All are accountable for all aspects of this 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 consent Not required.

Provenance and peer review Commissioned; externally peer reviewed.

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Review

First published as 10.1136/annrheumdis-2018-213454 on 4 August 2018. Downloaded from http://ard.bmj.com/ on 10 January 2019 by guest. Protected by copyright.
2018 update of the EULAR recommendations for the management of hand osteoarthritis

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ABSTRACT
Since publication of the European League Against Rheumatism (EULAR) recommendations for management of hand osteoarthritis (OA) in 2007 new evidence has emerged. The aim was to update these recommendations. EULAR standardised operating procedures were followed. A systematic literature review was performed, collecting the evidence regarding all non-pharmacological, pharmacological and surgical treatment options for hand OA published to date. Based on the evidence and expert opinion from an international task force of 19 physicians, healthcare professionals and patients from 10 European countries formulated overarching principles and recommendations. Level of evidence, grade of recommendation and level of agreement were allocated to each statement. Five overarching principles and 10 recommendations were agreed on. The overarching principles cover treatment goals, information provision, individualisation of treatment, shared decision-making and the need to consider multidisciplinary and multimodal (non-pharmacological, pharmacological, surgical) treatment approaches. Recommendations 1–3 cover different non-pharmacological treatment options (education, assistive devices, exercises and orthoses). Recommendations 4–8 describe the role of different pharmacological treatments, including topical treatments (preferred over systemic treatments, topical non-steroidal anti-inflammatory drugs (NSAIDs) being first-line choice), oral analgesics (particularly NSAIDs to be considered for symptom relief for a limited duration), chondroitin sulfate (for symptom relief), intra-articular glucocorticoids (generally not recommended, consider for painful interphalangeal OA) and conventional/biological disease-modifying antirheumatic drugs (discouraged). Considerations for surgery are described in recommendation 9. The last recommendation relates to follow-up. The presented EULAR recommendations provide up-to-date guidance on the management of hand OA, based on expert opinion and research evidence.

INTRODUCTION
Hand osteoarthritis (OA) is a common musculoskeletal disease, with prevalence rising steeply with increasing age.1–3 The disease is associated with hand pain, stiffness, functional limitation, decreased grip strength and reduced quality of life.4–6 Clinical hallmarks of the disease include bony enlargement and deformities of the hand joints, at times accompanied by soft tissue swelling.7 Hand OA has a variable disease course.8 The first European League Against Rheumatism (EULAR) recommendations for the management of hand OA were published in 2007.9 The American College of Rheumatology (ACR) published management recommendations for hand, hip and knee OA in 2012, including evidence available to the end of 2010, and other societies, including an expert group of occupational therapists and the Italian Society for Rheumatology, formulated treatment recommendations in 2011 and 2013, respectively.10–12

For a long time, hand OA was a ‘forgotten disease’, resulting in a paucity of clinical trials to guide recommendations, and therefore many of the propositions of previous recommendations were based mainly on expert opinion.13 However, in recent years, hand OA has attracted more attention, and new data have become available on several pharmacological and non-pharmacological treatments, including but not limited to: self-management, application of thumb base orthoses, topical non-steroidal anti-inflammatory drugs (NSAIDs), oral corticosteroids, various intra-articular therapies and treatment with conventional synthetic and biological disease-modifying antirheumatic drugs (cs/bDMARDs), for example, hydroxychloroquine and tumour necrosis factor (TNF) inhibitors. These more recent data have given new insights into treatment options. It was therefore timely to update the 2007 management recommendations. In this paper, we present the 2018 update of the EULAR recommendations for the management of hand OA.

METHODS
The development of the update was performed according to the 2014 EULAR Standard Operating Procedure (SOP).14 As prescribed by the SOP, the process set out in Appraisal of Guidelines for Research & Evaluation II (AGREE II) was followed.15 The convener (MK), methodologist (LC) and fellow (FK) defined research questions for the systematic literature review (SLR) and prepared a 1-day task force meeting. The task force further comprised 10 rheumatologists, 1 plastic surgeon (MR), 3 healthcare professionals in the field of physiotherapy and occupational therapy (KD, IK, TS) and 2 patient research partners (EG, WS). Two task force members were Emerging EULAR NETwork members (IKH, FK). The task force represented 10 countries across Europe. Under guidance of the methodologist, the fellows performed an SLR on the efficacy and safety of all pharmacological and surgical treatment options.

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Handling editor Francis Berenbaum

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Received 25 May 2018
Revised 28 June 2018
Accepted 1 August 2018
Published Online First 28 August 2018
Recommendation

non-pharmacological, pharmacological and surgical therapies available for hand OA. Although published separately, the SLR and the current updated management recommendations are complementary and should be considered together.

To explore current clinical practice in hand OA treatment and which topics healthcare professionals and patients felt should be covered in the update of the recommendations, members of the task force completed an online survey prior to the 1 day meeting.

Using the previous recommendations as a basis, together with the data obtained from the survey and the SLR, the convenor, methodologist and fellow prepared a proposal for wording for the update of the recommendations.

The results of the survey and the SLR were sent to the task force members in advance of a 1 day meeting where they were again presented. Through group discussion, overarching principles were formulated and the recommendations were updated. For every proposed overarching principle and recommendation, the results from the survey, evidence from the SLR and a proposed formulation were presented. Following discussion and rewording of the statement, voting was undertaken. A 75% majority was required to approve the statement. In case of disagreement, discussion was resumed and changes to the statement were made. The second voting round required a 67% majority, and if the formulation remained unagreed, an additional round of discussion followed. The third voting round required only 50% support for approval of the statement. The wording of the statements was considered final after the 1 day meeting.

After the meeting, the level of evidence (LoE) and grade of recommendation (GoR) were added to each recommendation, derived from the evidence from the SLR and according to the Oxford Centre for Evidence-Based Medicine standards. Finally, the overarching principles and recommendations (including LoE and GoR, and rationale for each statement based on the survey data, evidence from the SLR and discussion during the 1 day meeting) were sent to all task force members, who were asked to add their level of agreement (LoA) to each of the statements. The vote for the LoA was carried out anonymously on a numerical rating scale of 0–10 (0: do not agree at all, 10: fully agree). The mean and SD were calculated.

The final manuscript was reviewed, revised and approved by all task force members, followed by a final review by the EULAR Executive Committee.

RESULTS

Overarching principles

Overarching principles were not stated in the 2007 recommendations and were a new inclusion in the 2018 update. Overarching principles are generic statements, serving as the basis for management of patients with hand OA. Some of the 2007 recommendations were included in the 2018 update in the form of an overarching principle. The LoA of each overarching principle is presented in table 1.

The primary goal of managing hand OA is to control symptoms, such as pain and stiffness, and to optimise hand function, in order to maximise activity, participation and quality of life. Management should aim to achieve the best possible activity performance, participation and quality of life. Studies have shown that patients with hand OA have a decreased health-related

<table>
<thead>
<tr>
<th>Table 1</th>
<th>2018 Update of the EULAR recommendations for the management of hand OA</th>
<th>LoE*</th>
<th>GoR†</th>
<th>LoA (0–10)</th>
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<td><strong>Overarching principles</strong></td>
<td></td>
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<tr>
<td>A.</td>
<td>The primary goal of managing hand OA is to control symptoms, such as pain and stiffness, and to optimise hand function, in order to maximise activity, participation and quality of life.</td>
<td>9.7 (0.7)</td>
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<td>B.</td>
<td>All patients should be offered information on the nature and course of the disease, as well as education on self-management principles and treatment options.</td>
<td>9.8 (0.8)</td>
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<td>C.</td>
<td>Management of hand OA should be individualised taking into account its localisation and severity, as well as comorbidities.</td>
<td>9.9 (0.2)</td>
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<td>D.</td>
<td>Management of hand OA should be based on a shared decision between the patient and the health professional.</td>
<td>9.6 (1.1)</td>
<td></td>
<td></td>
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<td>E.</td>
<td>Optimal management of hand OA usually requires a multidisciplinary approach. In addition to non-pharmacological modalities, pharmacological options and surgery should be considered.</td>
<td>9.3 (1.2)</td>
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<td><strong>Recommendations</strong></td>
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<tr>
<td>1.</td>
<td>Education and training in ergonomic principles, pacing of activity and use of assistive devices should be offered to every patient.</td>
<td>9.3 (1.1)</td>
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<td>2.</td>
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<td>9.1 (1.6)</td>
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<td>3.</td>
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<td>9.3 (1.0)</td>
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<td>4.</td>
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<td>8.6 (1.8)</td>
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<td>5.</td>
<td>Oral analgesics, particularly NSAIDs, should be considered for a limited duration for relief of symptoms.</td>
<td>9.4 (0.9)</td>
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<td>6.</td>
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<td>7.3 (2.7)</td>
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<td>7.</td>
<td>Intra-articular injections of glucocorticoids should not generally be used in patients with hand OA; however, may be considered in patients with painful interphalangeal joints.</td>
<td>7.9 (2.4)</td>
<td></td>
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<td>8.</td>
<td>Patients with hand OA should not be treated with conventional or biological disease-modifying antirheumatic drugs.</td>
<td>8.8 (1.8)</td>
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<td>9.</td>
<td>Surgery should be considered for patients with structural abnormalities when other treatment modalities have not been sufficiently effective in relieving pain. Tendon transfer should be considered in patients with thumb base OA and arthroplasty or arthroplasty in patients with interphalangeal OA.</td>
<td>9.4 (1.4)</td>
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<td>10.</td>
<td>Long-term follow-up of patients with hand OA should be adapted to the patient’s individual needs.</td>
<td>9.5 (1.7)</td>
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*1a: systematic review of 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’.15

†1: based on consistent level 1 evidence; 2: based on consistent level 2 or 3 evidence or extrapolations from level 1 evidence; 3: based on level 4 evidence or extrapolations from level 2 or 3 evidence; D: based on level 5 evidence or on troublingly inconsistent or inconclusive studies of any level.17

EULAR, European League Against Rheumatism; GoR, grade of recommendation; LoA, level of agreement; LoE, level of evidence; NSAIDs, non-steroidal anti-inflammatory drugs; OA, osteoarthritis; RCT, randomised clinical trial.


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quality of life. Symptoms such as pain, stiffness and decreased hand function are hallmarks of the disease, and contribute to altered quality of life. This overarching principle was based on the International Classification of Functioning, Disability and Health framework. The wording ‘optimise’ and ‘maximise’ were chosen to reflect that management of hand OA should be more ambitious than merely aiming for a patient-acceptable symptom state.

All patients should be offered information on the nature and course of the disease, as well as education on self-management principles and treatment options Education is considered a core treatment in the management of patients with hand OA, and should be offered to all patients. This overarching principle is an additional, more generic statement on education, besides the first recommendation concerning specific education and training. In patients with chronic complaints returning for follow-up, information and education provision should be an ongoing process involving reinforcement and expansion. Explicit evidence supporting the efficacy and content of provision of information and education in hand OA is lacking. Trained health professionals other than the physician can play an important role in the provision of information and education.

Management of hand OA should be individualised taking into account its localisation and severity, as well as comorbidities This overarching principle was modified from the 2007 recommendation about individualisation of treatment. In the premeeting survey, >75% of health professionals indicated that patient characteristics that are considered important include: age, type of complaint (eg, pain or disability), mechanical factors, patient’s wishes and expectations, presence of inflammation, severity of structural damage and presence of erosions. In the survey, most health professionals also supported different treatment approaches according to disease location (especially thumb base OA) or OA subset (especially erosive or ‘inflammatory’ OA). The 2007 recommendation included consideration of many of these individual factors. Yet although many of these factors are known to be determinants of worse outcome (eg, presence of inflammation is known to be associated with disease progression), evidence of effect modification is lacking for most of these factors. Moreover, it is unknown whether treatment of modifiable factors will in turn change disease outcomes (eg, there is no evidence that treatment of inflammation reduces disease progression). OA localisation (most importantly finger vs thumb base OA), OA severity and presence of comorbidities were thought to be the only aspects that may currently influence treatment decisions. This is also reflected in the recommendations. ‘Severity’ can encompass several features, including a high number of hand joints with OA, one or two severely affected joints or acute joint inflammation due to OA. The patient’s wishes and expectations were not mentioned separately in this overarching principle, since this concept is incorporated in the overarching principle concerning shared decision-making.

Management of hand OA should be based on a shared decision between the patient and the health professional Shared decision-making, an approach to healthcare in which health professionals and patients mutually share information to reach consensus about the preferred management strategy, should be the basis of management in hand OA. This overarching principle implies that not only the best available evidence, but also the patients’ wishes and expectations are important to be considered when making decisions on managing the disease. Achieving shared decision-making depends on building and maintaining a good relationship between patient and health professional, and sharing the best evidence, in order to be able to make an informed decision. It pertains to all stages of management, including, for example, setting a treatment goal, choosing the best strategy to achieve it or considering other strategies when the treatment goal is not reached.

Optimal management of hand OA usually requires a multidisciplinary approach. In addition to non-pharmacological modalities, pharmacological options and surgery should be considered Hand OA is both a heterogeneous disease, leading to a variety of signs and symptoms, and a chronic disease. Over the course of the disease, patients with hand OA therefore often require multidisciplinary care. Health professionals involved in care for patients with hand OA, may include, for example, the general practitioner, rheumatologist, occupational or physical therapist, orthopaedic or plastic surgeon and the rehabilitation specialist. Which care is delivered by each health professional differs by country, depending for example on local preferences or customs and social security systems. In some clinics, structured multidisciplinary care programmes or integrated care pathways are provided. However, it is unclear whether such programmes providing a structured combination of different non-pharmacological therapies are efficacious. For example, no consistent beneficial effect of combination programmes including education, joint protection and exercises over education alone has been determined.

The second part of this overarching principle, that different treatment modalities should be considered, was modified from the first 2007 recommendation, and initially discussed as a separate overarching principle (LoA: 100%). Later, the concept ‘multidisciplinary care’ was added, since it was recognised that different modalities may be provided by different health professionals. By modifying the 2007 recommendation, this overarching principle now also reflects that the first step in hand OA management should focus on non-pharmacological therapies, which may be complemented by pharmacological and/or surgical options, although not necessarily for all patients with hand OA, depending on the level of symptoms.

Recommendations

In total, 10 recommendations were formulated (table 1). Table 1 also presents the LoE, GoR and LoA for each recommendation. Many of the 2007 recommendations were modified because new evidence has emerged since the previous SLR, and were formulated as recommendations rather than ‘statements’ reflecting the state of the evidence and/or expert opinion. Two recommendations are new (#8, #10), one recommendation was split into two (old #3 into new #1 and #2), two recommendations were combined into one (old #7 and #8 into new #5) and one recommendation was deleted (old #4). The recommendation that was deleted concerned the use of heat and ultrasound, which was based on expert opinion and extrapolation from hip or knee OA studies.

Education and training in ergonomic principles, pacing of activity and use of assistive devices should be offered to every patient Education and training in ergonomic principles and pacing of activity, formerly included in the recommendations under the
Exercises to improve function and muscle strength, as well as to reduce pain, should be considered for every patient

Although exercise was endorsed in the 2007 recommendations, no supporting evidence was available at that time. Since then, multiple trials (n=7) have been performed, and their results were summarised in a Cochrane review.32 It was shown that hand exercises have small beneficial effects on self-reported pain and function, joint stiffness and grip strength, while resulting in few and non-severe adverse effects. However, the interventions studied were heterogeneous, varying from home-based exercises after a single instruction session to multiple supervised sessions per week for several weeks, and also the frequency of exercising, number of repetitions per exercise and type of exercises (eg, strengthening or stretching) were variable. Furthermore, the review authors debated whether the effects that were found constituted a clinically relevant improvement, and the beneficial effects were not sustained when patients stopped exercising. Exercises should aim at improving joint mobility, muscle strength and thumb base stability. Exercise regimens aimed at the first carpometacarpal (CMC-1) joint differ from those for interphalangeal joints.

Orthoses should be considered for symptom relief in patients with thumb base OA. Long-term use is advocated

Since the 2007 recommendations many orthosis trials have been performed, of which five compared orthoses to usual care or a non-pharmacological intervention.33–37 These trials provide evidence for beneficial effects of a thumb base orthosis, especially on pain and to a lesser extent on function, but not on grip strength, when used for a prolonged period (at least 3 months). No improvements were evident when used for shorter periods. Long-term use is thus advocated. The 2007 recommendations advised the use of orthoses to ‘prevent/correct lateral angulation and flexion deformity’ in patients with thumb base OA, yet no evidence to date supports an effect of orthoses on angulation or deformity, and therefore the statement was reworded.

No straightforward advice can be given for the type of orthosis (short or long, custom-made or prefabricated, neoprene, thermoplast or other material) or instructions for use (eg, during activities of daily living, at night, constantly), as studies are heterogeneous and no consistent benefit of one type of orthosis over the other could be identified. Trials showing a long-term beneficial effect of orthosis use investigated a custom-made thermoplast long orthosis to be worn during activities of daily living,35 and a custom-made neoprene long orthosis to be worn at night.37

It is important to pay attention to prescribing a well-fitted orthosis, preferably custom-made by a specialised health professional. This will likely improve patients’ compliance and increase long-term use.

Most trials were performed in patients with thumb base OA, and only one trial investigated night-time distal interphalangeal joint (DIP) orthoses, which did not prove to be efficacious, and is therefore not specifically recommended.38

Topical treatments are preferred over systemic treatments because of safety reasons. Topical NSAIDs are the first pharmacological topical treatment of choice

Topical NSAIDs are recommended as a first-line pharmacological treatment, due to their favourable safety profile compared with oral analgesics and beneficial effects on pain and function.39–41 Topical diclofenac gel showed small improvements in pain and function after 8 weeks compared with placebo in one high-quality study.41 Moreover, topical NSAIDs can show similar pain relief as oral NSAIDs.39 40 Pooled safety data from randomised clinical trials comparing topical diclofenac gel with placebo in patients with hand and knee OA also showed similar low rates of adverse effects in subgroups of low-risk versus high-risk patients (ie, age ≥65 years, and with comorbid hypertension, type 2 diabetes or cerebrovascular or cardiovascular disease).42 When a large number of joints are affected, systemic pharmacological treatment may be preferred. At present, no data are available on long-term effects of topical NSAIDs.43

Capsaicin is another topical treatment, which is however known to be associated with frequent local adverse effects (burning and stinging sensation), and therefore success of blinding of the (positive) placebo-controlled trial investigating its efficacy is questionable.44

Topical application of heat was regarded by the task force as a self-management strategy that patients can apply at home, with weak and conflicting evidence for a possible beneficial effect.45–47 It was therefore not included as a separate recommendation in this update. Cold packs, in case of inflammation during an OA flare, may also give symptomatic relief, though studies in hand OA have not been performed, and a single knee OA study comparing hot and cold application with usual care found no between-group differences.48

Oral analgesics, particularly NSAIDs, should be considered for a limited duration for relief of symptoms

This recommendation is a combination of the 2007 recommendations concerning paracetamol and oral NSAIDs.

Oral NSAIDs effectively improved pain and function after 2–4 weeks in three high-quality studies.49–51 However, adverse effects are well-known, especially in the elderly. No new evidence was identified compared with the 2007 recommendations. The advice to prescribe NSAIDs at the lowest effective dose, for a limited duration (preferably on-demand), with attention for the risk-benefit ratio, especially in patients at high risk of gastrointestinal, cardiovascular or renal adverse effects, remains unchanged.

Paracetamol is prescribed by many health professionals, and also in the premeeting survey the vast majority of health professionals indicated that they prescribed paracetamol to their patients with hand OA. Patients’ experience with paracetamol is known to be variable. It has generally been regarded as a safe treatment option, although lately its risk-benefit profile has been a topic of debate, even leading to controversy about including it in the National Institute for Health and Care Excellence (NICE) guidelines on OA.52 Three small trials, two only published as conference abstracts, have studied paracetamol (1000–3900mg
daily) in hand OA. In these trials, paracetamol was not superior to placebo or an active comparator. Two large meta-analyses of trials in patients with knee and hip OA found small effects on pain, with doubtful clinical significance. Evidence from these trials showed that paracetamol was associated with an increased risk of liver test abnormalities, although the clinical relevance of this finding is unknown, but not with increased risk of any other safety parameter. A narrative review of long-term observational studies in the general adult population found a dose-response increased risk of mortality (n=2 trials), cardiovascular (n=4), gastrointestinal (n=1) and renal adverse effects (n=4). This should, however, be interpreted with caution, as these observational studies were associated with a large risk of bias (most importantly confounding by indication) and imprecision of measurement of paracetamol exposure (eg, reliance on self-reported medication use or prescription databases). In conclusion, the efficacy of paracetamol in hand OA is still uncertain and likely to be small, and this drug is also not free from adverse effects, although for now there is no reason to refrain from prescribing paracetamol, preferably for a limited duration, in selected patients (eg, when oral NSAIDs are contraindicated). Tramadol (with or without paracetamol), was also regarded by the task force as an alternative oral analgesic, although currently no evidence in patients with hand OA is available to support its use.

Chondroitin sulfate may be used in patients with hand OA for pain relief and improvement in functioning. Chondroitin sulfate and glucosamine are among the most widely used over-the-counter nutraceutical products for OA. Chondroitin sulfate was shown to be effective for relief of hand OA symptoms in one well-performed trial, although in patients with knee and hip OA a clinically meaningful effect of glucosamine and chondroitin preparations has not been proven. A single report of two independent placebo-controlled trials reported structure-modifying effects of chondroitin polysulfate (a preparation that is not commercially available), but not of chondroitin sulfate. However, this evidence was judged unconvincing to promote chondroitin sulfate for structure modification. No placebo-controlled trials of glucosamine have been performed in patients with hand OA. Owing to the limited evidence available to support this recommendation, and even less convincing data from trials in knee and hip OA which led to discouragement of chondroitin sulfate and glucosamine use by NICE, this recommendation was formulated more as a suggestion than a recommendation to use.

In addition to the nutraceuticals discussed here, other so-called Symptomatic Slow Acting Drugs for Osteoarthritis (‘SYSADOA’) were included in the 2007 recommendation, namely avocado soybean unsaponifiables, diacerein and intra-articular hyaluronan. Currently, however, there is no evidence for clinical efficacy of these preparations. The task force further agreed that at this moment in OA no drugs are available with disease-modifying properties, and therefore these substances should also not be advocated as such.

Intra-articular injections of glucocorticoids should not generally be used in patients with hand OA, but may be considered in patients with painful interphalangeal joints. This recommendation was completely revised, since the previous recommendation was largely based on expert opinion and new evidence could not confirm a beneficial effect of intra-articular glucocorticoids over placebo in patients with thumb base OA. In contrast, in one trial of patients with painful interphalangeal OA, intra-articular glucocorticoid injections were more effective than placebo for pain during joint movement and joint swelling. The formulation ‘should not generally be used’ was chosen, since the task force recognised that in specific cases where, for example, clear joint inflammation is present, injection with glucocorticoids may still be a therapeutic option. Evidence pertaining specific subgroups that could benefit from intra-articular glucocorticoids, for example, patients with active joint inflammation due to a flare of the disease, is lacking. It is also unknown whether image-guided injections are more beneficial or safer than blind injections, although a Cochrane review of shoulder injections could not establish clinical advantages of guided injection. Injections in small finger joints are preferably performed by a rheumatologist.

Patients with hand OA should not be treated with conventional or biological disease-modifying anti-rheumatic drugs. This recommendation was newly added, after several studies have emerged demonstrating the lack of efficacy of csDMARD/bDMARD. In clinical practice, severe cases of inflammatory, often erosive, hand OA are occasionally prescribed csDMARDs or even bDMARDs. However, the 2007 recommendations did not include advice on the use of these drugs, and no evidence was available at that time. Trials investigating the efficacy of hydroxychloroquine, different TNF-inhibitors and anti-interleukin-1 could not demonstrate efficacy of these anti-rheumatic drugs in patients with hand OA. Trials investigating methotrexate, sulphasalazine or colchicine have not been performed. Two trials investigated low-dose oral glucocorticoids (3–5 mg daily), one in combination with dipyridamole, yet reached conflicting conclusions. Evidence for short-term use of oral glucocorticoids is therefore still equivocal; at this moment, there is no reason to prescribe glucocorticoids for prolonged periods of time in patients with hand OA.

Surgery should be considered for patients with structural abnormalities when other treatment modalities have not been sufficiently effective in relieving pain. Trapeziectomy should be considered in patients with thumb base OA and arthrodesis or arthroplasty in patients with interphalangeal OA. This recommendation was slightly modified compared with the 2007 recommendation on surgery. Trials with a placebo-controlled or sham-controlled group have not been performed, and so this recommendation remains mostly based on expert opinion. In the first part of the updated recommendation, treatment failure has now been defined more specifically as ‘not sufficiently effective in relieving pain’, since surgical interventions are mostly effective to relieve pain, and are less effective in improving function (expert opinion). Surgery should only be considered in persistently symptomatic patients with structural abnormalities despite conventional treatments, including both non-pharmacological and pharmacological therapies. Second, the recommendation does not solely focus on the thumb base joint as before, since surgery can be a viable treatment option in cases with severe painful interphalangeal OA as well.

Surgical interventions vary for the different hand joints. In the CMC-1 joint, trapeziectomy is generally the surgical technique of choice. An updated Cochrane review of the evidence of surgery for thumb base OA found no consistent benefit of one surgical technique over the other, although in general more complicated interventions than simple trapeziectomy led to more adverse effects and were not more effective. Complications reported in the studies included pain, instability, nerve dysfunction, superficial...
wound infections, tendon pulling sensation and chronic regional pain syndrome. Arthroplasty (typically silicone implants) is the preferred surgical technique for the proximal interphalangeal (PIP) joints, with the exception of PIP-2, for which arthrodesis may be considered. Arthrodesis is the recommended approach for the distal interphalangeal joints. No controlled trials of surgery for interphalangeal OA have been published so far.

It is important that patients receive rehabilitation postoperatively. Osteotomy was deleted from the recommendation, as it is an obsolete technique for treating hand OA.

Long-term follow-up of patients with hand OA should be adapted to the patient’s individual needs

A recommendation on follow-up was not included in the previous recommendations. Due to the lack of evidence for the cost-effectiveness of long-term follow-up, an evidence-based statement could not be made. Hand OA is a heterogeneous disease, and the spectrum of patients seen with hand OA is diverse, which resulted in a general recommendation. ‘Individual needs’ that may be taken into consideration when assessing the need for follow-up include severity of symptoms, presence of erosive disease, use of a pharmacological therapy that needs re-evaluation and patient’s wishes and expectations.

It was discussed whether long-term follow-up is always indicated for patients with erosive OA. In spite of evidence that these patients have more clinical and structural progression, the task force perceived that currently follow-up does not add a benefit. In the absence of a disease-modifying treatment, the goal of follow-up differs from the situation in many other rheumatic diseases. Follow-up will likely increase adherence to non-pharmacological therapies like exercise or orthoses, and provides an opportunity for re-evaluation of treatment (eg, revision of orthoses, or adjustment of pharmacological treatment). For most patients, standard radiographic follow-up is not useful at this moment. Follow-up does not necessarily have to be performed by the rheumatologist. At what moment other health professionals should refer a patient back to the rheumatologist, should be considered at an individual patient level.

Research agenda

A research agenda was developed (table 2).

DISCUSSION

This is the first update of the EULAR recommendations for the management of hand OA, containing five overarching principles and 10 recommendations. After a decade, it was timely to update the recommendations, as many new studies had emerged during this period. In light of this new evidence, many of the 2007 recommendations were modified and new recommendations were added. Furthermore, recommendations were formulated as recommendations rather than ‘statements’ reflecting the state of the evidence and/or expert opinion.

In this update, two patient research partners with hand OA were included as active members of the task force, while the 2007 task force did not include patient research partners. This is an important improvement, since patients are one of the important target-users of these recommendations, and in evidence-based clinical decision making, the patient perspective is valued as equally important to research evidence and clinical expertise.

New in the 2018 update is also the use of overarching principles. This is in line with other EULAR sets of management recommendations. Some of the 2007 recommendations were in retrospect already more an overarching principle, and were (modified and) included in the 2018 update as such, for example, statements regarding individualised treatment, and combination of non-pharmacological and pharmacological treatment modalities.

Moreover, the 2018 update of the SLR summarising the evidence for the recommendations, is published as a separate manuscript. As pointed out in their discussion, Zhang et al did perform a systematic search of the literature to underpin the recommendations, but rather than reviewing all possible treatments, a limited number of key propositions were highlighted. The publication of the complete SLR, including a detailed description of its methodology and results, provides the interested reader with a full update of the currently available evidence concerning the management of hand OA and provides

<table>
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<tr>
<th>Theme</th>
<th>Research questions</th>
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<tr>
<td>Pathophysiology</td>
<td>Does treatment of inflammation lead to a decrease in structural progression?</td>
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<tr>
<td>Treatment strategy</td>
<td>Which contextual factors influence treatment effects?</td>
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<tr>
<td>Trial methodology</td>
<td>Clear definition of study population to accommodate later subgroup analyses or stratification based on patient characteristics.</td>
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<tr>
<td>Outcomes</td>
<td>Evaluation of outcome measures in hand OA, and use of existing outcome core sets for future hand OA trials.</td>
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<td>Education</td>
<td>Evaluation of efficacy of education without concomitant exercise.</td>
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<tr>
<td>Exercise</td>
<td>Assessment of most effective type of hand exercises, most optimal method of delivery and most optimal frequency.</td>
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<td>Orthoses</td>
<td>Assessment of methods to increase adherence to exercise.</td>
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<tr>
<td>Topical treatments</td>
<td>Another placebo-controlled trial of topical NSAID.</td>
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<tr>
<td>Oral analgesics</td>
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<td>Intra-articular therapies</td>
<td>Placebo-controlled trial of intra-articular glucocorticoids specifically in CMC-1 joints with OA inflammation.</td>
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<td>DMARDs</td>
<td>Placebo-controlled trial of methotrexate.</td>
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<td>Surgery</td>
<td>Randomised controlled trial of most commonly used surgical interventions.</td>
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<tr>
<td>Follow-up</td>
<td>Investigation of trajectories in hand OA to define subgroups.</td>
</tr>
<tr>
<td>Implementation</td>
<td>Determination of optimal implementation of the guidelines in people with hand OA.</td>
</tr>
</tbody>
</table>

CMC-1, first carpometacarpal; DMARDs, disease-modifying antirheumatic drugs; OA, osteoarthritis.
more insight in the size of the effects of different interventions compared with placebo or control treatment. It is important to note that the recommendations as presented in table 1 cannot be read and interpreted without the accompanying text, and this manuscript and the separately published SLR form an integral part, and should be considered together.

Guidelines for the management of OA from other large (international) societies, including the 2012 ACR recommendations and the NICE guidelines, mainly focus on large joint OA (ie, knee and hip). However, these recommendations cannot readily be extrapolated to the situation of OA in the hand because of the unique functionality of the hands compared with large joints, and emerging evidence for different risk factors and possibly even pathophysiological mechanisms of OA at different joint sites.

These recommendations are targeted at all health professionals who care for patients with hand OA. Since hand OA is a prevalent disease encountered by a variety of healthcare providers in primary and secondary care, this not only includes rheumatologists, but also for example general practitioners, orthopaedic and plastic surgeons, occupational and physical therapists and rehabilitation physicians. Furthermore, these recommendations aim to inform patients about their disease to support shared decision-making, as well as students. Other targeted stakeholders include pharmaceutical industry, policy makers and health insurance companies.

Efforts to implement these recommendations will be made by dissemination across national societies, online and by presentations in (inter)national congresses and educational sessions for healthcare providers. A slide deck to facilitate dissemination will be provided on the EULAR website. Evidence of optimal systematic implementation is lacking and this was highlighted in the research agenda.

Although a relatively long period passed between the first set of recommendations and the current update, it is expected that the next update of the recommendations may be needed sooner, as the field of hand OA is growing. Advances in research of OA pathophysiology as well as outcome measurement, increase the likelihood of finding new therapeutic options. The next update should be undertaken when sufficient new data are available, either on the current treatment options, or on new therapies.

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FPBK performed the systematic literature review, supervised by MK and LC. All authors were part of the Task Force, completed an online survey prior to the face-to-face meeting and voted on the level of agreement. MK, FBPK, FJB, KSD, EG, IKH, GH-B, HI, IK, EM, MJFPR, WS, JS, TAS, RW and LC attended the face-to-face meeting. FBPK and MK wrote the manuscript, with contribution and approval of all coauthors.

Funding EULAR

Disclaimer
The views expressed in this paper are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health and Social Care.

Competing interests
The individual declaration of conflicts of interest is available on demand at the EULAR secretariat and is summarised below: MK has received consultancy fees/fee as local investigator of industry driven trials from AbbVie, GlaxoSmithKline, Merck, Levicept (all through institution) and has received research funding (through the institution) from Pfizer and APPROACH-M. FJB has received honoraria from Boehringer Ingelheim España, SA, Boehringer Ingelheim International GmbH, Fundación Española de Reumatología (FER), Janssen Cilag International NV, Pfizer Inc, Sanofi-Aventis Recherche & Development, Bristol-Myers Squibb International Corporation, Bristol Myers Squibb Research and Development, Hospira Inc., Grendenthal GmbH, Bloiberica, UCB, Gebro and research funding (all through institution) from Novartis Farmacéutica, SA, Bristol, Menarini International Operations Luxembourg SA, AbbVie Deutschland GmbH & Co KG, Boehringer Ingelheim España, SA, Boehringer Ingelheim International GmbH, Fundación Española de Reumatología (FER), Janssen Cilag International NV, Gedeon Richter Plc, Pfizer Inc, GlaxoSmithKline Research & Development Limited, YL Biologies Limited, Amgen, Inc, Sanofi-Aventis Recherche & Development, Gilead Sciences, Inc, Eli Lilly and Company, Ablynx NV, Bristol-Myers Squibb International Corporation, Bristol-Myers Squibb Research and Development, Hospira Inc, Astellas Pharma Europe BV, Theodora Medica International SA, Archigen Biotech Limited, ONO Pharma UK Ltd, UCB Biosciences GMBH, Nichi-iko Pharmaceutical Co, Ltd, Genentech Inc Grendenthal GmbH, Celpgene Corporation. MD has received research funding from AstraZeneca for a PI-led ‘sous de gout’ study and honoraria for advisory boards on osteoarthritis and gout from AstraZeneca, Grendenthal, Mullinckrodt and Roche. KSD is part-funded by a Knowledge Mobilisation Research Fellowship (KMRF-2014-03-002) from the NIHR Collaborations for Leadership in Applied Health Research and Care-West Midlands. GBH has received honoraria from Pfizer AstraZeneca, Roche, Glaxo, Expanscience and research funding (all through institution) from Pfizer and Roche. EM has received honoraria from Celgene, Expanscience, Fidia, Genervier, Ibisa, LCA, Rottapharm-Meda-Mylan-France, and Rottapharm Biotech-I Italy, TRB Chemedica. RR has received honoraria from AbbVie, MSD, Celgene, Janssen, Pfizer, UCB and research funding from HORIZON 2020 (going through the institution). JS has received honoraria from AbbVie, Amgen, AstraZeneca, Astro, BMS, Celgene, Celltrion, Chugui, Gilead, Glaxo, ILTOO, Janssen, Lilly, Medimmune, MSD, Novartis-Sandoz, Pfizer, Roche, Samsung, Sanofi, UCB and research funding from AbbVie, AstraZeneca, Janssen, Lilly, MSD, Pfizer, Roche. JSS is Editor-in-Chief of ARD and Editor of Rheumatology (Textbook). TS has received honoraria from AbbVie, Janssen, MSD, Novartis and Roche and grant support from AbbVie (going through the institution). ZS has received honoraria from AbbVie, Roche, Pfizer, Le zinc, Chemie, UCB, Bristol-Myers. RW has received honoraria from AbbVie, UCB, Bristol-Myers Squibb, MSD, Janssen-Cilag, Menarini. LC has received research funding (through the institution) from Pharmaceutical laboratories (AbbVie Spain, Bristol-Myers Squibb, Celgene, Eisai Farmacéutica, Gb Pharma, Grünenthal Pharma, LEO Pharma, Merck Sharp & Dohme España, Novartis Farmacéutica, Pfizer, Roche Farma, Sanofi Aventis, UCB Pharma), Scientific societies (Academia de Dermatología y Venerología, Asociación Emeritense de Reumatología, Eular, Italian Society of Rheumatology, Sociedad Castellano-Manchega, SORDOM, SEDISA, SEIO, Sociedad Española de Neumología y Cirugía Torácica, SERPE, Societat Catalana de Reumatologia), Contract Research organisations (Società Salus, Continuando Medica, Cochere, Congressos e Eventos e Azafatas, Med Comunicación, Proyectos Incentivos y Congresos), Research groups and Foundations (AIRE-MB, FISABIO, Fundación Parc Taulí, Fundación Asturcor, Fundación Clínica, Fundación de Investigación Sanitaria de Baleares, Fundación de Investigación Sanitaria de Reumatología, Fundación para la Investigación Biomédica del Hospital Universitario de La Princesa, Fundación para la Investigación Biomédica del Hospital Universitario 12 de Octubre, Fundación Pública Andaluza para la Investigación de Málaga en Medicina y Salud, Hospital Universitario Fundación Alcorcón, Reumacare), Individual researchers (Dr Ramón Mazucchelli, Dr Xavier Juanola, Dr Aíñar Abdelkader) and is director of Instituto de Salud Musculosquelética.

Patient consent
Not required.

Provenance and peer review
Not commissioned; externally peer reviewed.

REFERENCES
Recommendation


Recommendation


How to treat patients with rheumatoid arthritis when methotrexate has failed? The use of a multiple propensity score to adjust for confounding by indication in observational studies

Sytseke Anne Bergstra,1 Lai-Ling Winchow,2 Elizabeth Murphy,3 Arvind Chopra,4 Karen Salomon-Escoto,5 João Euroco Fonseca,6 Cornelia F Allaart,1 Robert B M Landewe7,8

ABSTRACT

Objectives To compare consecutive disease modifying antirheumatic drugs (DMARD)-treatment regimes in daily practice in patients with rheumatoid arthritis (RA) who failed on initial methotrexate, while using a multiple propensity score (PS) method to control for the spurious effects of confounding by indication.

Methods Patients with newly diagnosed RA who had failed initial treatment with methotrexate were selected from METEOR, an international, observational registry. Subsequent DMARD-treatment regimens were categorised as: (1) conventional synthetic DMARD(s) (csDMARD(s)) only (143 patients), (2) csDMARD(s)+glucocorticoid (278 patients) and (3) biological DMARD (bDMARD)±csDMARD(s) (89 patients). Multiple PS that reflect the likelihood of treatment with each treatment-regime were estimated per patient using multinomial regression. Linear mixed model analyses were performed to analyse treatment responses per category (Disease Activity Score (DAS)) after a maximum follow-up duration of 6 and 12 months, and results were presented with adjustment for the multiple PS.

Results After 6 months, follow-up PS-adjusted treatment responses yielded a change in DAS per year (95% CI) of −2.00 (−2.65 to −1.36) if patients received a bDMARD; of −0.96 (−1.33 to −0.59) if patients received csDMARD(s)+glucocorticoids and of −0.73 (−1.21 to −0.25) if patients received csDMARDs only. These changes were −0.91 (−1.23 to −0.60); −0.43 (−0.62 to −0.23) and −0.39 (−0.66 to −0.13), respectively after 1 year of follow-up.

Conclusions In this analysis of worldwide common practice data with adjustment for multiple PS, patients with RA who had failed initial treatment with methotrexate monotherapy had a better DAS-response after a subsequent switch to a bDMARD-containing treatment regimen than to a regimen with csDMARD(s) only, with or without glucocorticoids.

INTRODUCTION

Methotrexate should be (part of) the initial treatment for patients with rheumatoid arthritis (RA). If the desired treatment target is not met, various other treatment options can be considered. These include switching to—or adding—a different conventional synthetic disease modifying antirheumatic drug (csDMARD) and/or a b(iological) DMARD or a glucocorticoid. To date, evidence about the preferred follow-up strategy in terms of early as well as sustained response is sparse.

Previous trials with static treatment or with a treat-to-target design that aimed at long-term outcomes have shown mixed results. The BeSt study showed no difference in early or sustained clinical response between step-up combination therapy versus sequential monotherapy with csDMARDs. Three other studies showed no clear benefits of adding a bDMARD versus escalating to triple csDMARD therapy after 4–6 months. However, one study suggested that when escalating to bDMARD therapy, more patients achieved a EULAR good response after 1 year.

Key messages

What is already known about this subject?
- Evidence about the preferred follow-up strategy after methotrexate failure is sparse.
- There are concerns about the risk of bias when using routinely collected data to solve clinical research questions.

What does this study add?
- Patients who fail initial treatment with methotrexate monotherapy experience more decrease in disease activity and better treatment survival after switching to treatment with a biological DMARD (disease modifying antirheumatic drug) than to treatment with conventional synthetic DMARD(s) with or without a glucocorticoid.
- A multiple propensity score is demonstrated that can be used to control for bias when comparing routinely collected data in multiple non-randomised treatment groups.

How might this impact on clinical practice or future developments?
- The results of this study could impact the choice among treatment strategies in patients who fail initial methotrexate treatment.
In trials, random allocation of patients to different treatments provides prognostic similarity, so that differences in treatment effects can only be attributed to differences in treatment. But stringent inclusion and exclusion criteria make patients in clinical trials being different from patients in daily practice. Increasingly, real world data of patients with RA are routinely collected and captured in large observational databases. The possibility to use these data to solve clinical questions has increased. But unlike randomised controlled trials, observational studies (registries), in which physicians (rather than a trial protocol) determine treatment, lack the benefit of prognostic similarity. Thus, differences in treatment responses are due to differences in treatments and due to differences in disease severity (confounding by indication). Therefore, crude comparisons across treatments do not suffice due to potential biases that may spuriously affect the conclusions.

Classic binomial propensity scores (PS) are now rapidly becoming popular in rheumatology to adjust for this bias, but they often provide a simplification of the truth. A multinomial propensity adjustment may allow to some extent for a better comparison of multiple non-randomised treatment groups, but this technique is still underused and not widely known. Propensity adjustment may allow to some extent for a better comparison of multiple non-randomised treatment groups, but this technique is still underused and not widely known.

In this study, we have compared the 1-year efficacy of several treatment-strategy options for patients with RA who have failed initial methotrexate. We used data from a worldwide observational database of patients with RA and will introduce the ‘multiple PS’ as a method to adjust for confounding by indication and to better allow the comparison of multiple treatment regimens.

**METHODS**

**Data selection**

Data were extracted from METEOR, an international, observational registry including patients with a rheumatologist diagnosis of RA. Data in METEOR were anonymised and reflected daily clinical practice, therefore medical ethics approval was not required. An extensive description of METEOR has been previously published.

Only patients with RA who had a treatment failure on initial treatment with methotrexate monotherapy (also excluding systemic glucocorticoids) were selected, if they had a symptom duration <5 years, had newly diagnosed RA (defined as a DMARD start within 3 months after diagnosis), age at first visit ≥16, at least one visit with available composite disease activity measure (Disease Activity Score (DAS), DAS28, Simplified Disease Activity Index, Composite Disease Activity Index or RAPID3) and at least two available visits after start of the second treatment strategy.

All available follow-up visits were selected from start of the second treatment strategy (after methotrexate), until a maximum follow-up duration of 1 year. Follow-up could be shorter if the end of available follow-up was reached or if a treatment failure of the second treatment strategy occurred before 1-year follow-up was achieved.

Treatment failure (including initial failure to methotrexate monotherapy) was defined as a change in treatment strategy (either a change in type of drug or the addition of a new drug). Stepdown strategies (eg, from combination therapy of methotrexate+prednisone to methotrexate monotherapy) or strategies with changes in medication dose were excluded.

**Treatment groups and outcome measures**

The treatment strategies chosen after failure of the initial methotrexate treatment were divided into three categories: (1) one or more csDMARDs (excluding bDMARDs and glucocorticoids), (2) combination treatment with csDMARD(s) plus a glucocorticoid and (3) treatment including a bDMARD (with or without csDMARDs). Response to these second treatment strategies was measured over time by DAS.

**Statistical analyses**

**Multiple imputation**

Missing data were imputed using multivariate normal imputation (30 imputed datasets). Analyses were subsequently performed in the imputed datasets.

**Multiple PS**

To allow a comparison of multiple non-randomised treatment strategies, it is possible to adjust for spurious effects of confounding by indication by estimating a multiple PS. This score (between 0 and 1) indicates the likelihood per patient of being treated with one out of several (more than two) treatment categories. This likelihood is conditional on a selection of pretreatment variables that together reflect to some extent the patient’s disease severity and perceived prognosis. Since treatment category is a nominal variable, the multiple PSs were estimated using multinomial regression analysis, with treatment category as dependent variable. Linear regression analyses, with DAS as dependent variable, were performed to identify all available pretreatment variables related to the outcome of the study (in this study DAS). Variables with correlations at p<0.10 were selected for inclusion in the multiple PS. Furthermore, it was checked whether adding interaction terms would further improve balance of the model. Since three treatment groups were compared, three PSs were estimated per patient. Since these three scores add up to 1, only two out of three scores are needed to adjust for in further analyses. After estimating the multiple PSs, it was checked by visual analysis of a density plot whether the distributions of the multiple PSs overlapped, since ‘perfect predictability of treatment category’ is not allowed. Patients who did not have a probability of being indicated for each treatment category were disregarded. Then, it was tested whether balance in the distribution of all included variables between the three treatment groups had been achieved, which is a requirement for a successful propensity model. For continuous variables, this was assessed using ANCOVA with treatment group as fixed factor. For dichotomous variables, logistic regression analysis was used and for nominal variables multinomial logistic regression analysis was used, both with treatment group as independent variable. These analyses were first performed without adjustment and then after adjustment for two of the three multiple PSs as well as their interactions. If the analyses were non-significant (p>0.05) after adjustment, balance was considered present. An extended, stepwise description of the multiple PS estimation has been provided in online supplementary file 2.

**Estimating the treatment effect with multiple PS adjustment**

Finally, the treatment effect over time was analysed, first with a maximum follow-up duration of 6 months and next of 1 year. Only visits when patients were on the medication of interest were selected.

First, the treatment effect was analysed by linear mixed modeling with DAS as dependent variable, without adjusting for the multiple PS. Treatment group, follow-up time and the interaction between treatment group and follow-up time served as independent variables, the latter providing the parameter estimates.
Table 1  Baseline characteristics per treatment group, non-imputed data

<table>
<thead>
<tr>
<th></th>
<th>csDMARD(s) (n=142)</th>
<th>csDMARD(s)+glucocorticoids (n=278)</th>
<th>bDMARD+csDMARD(s) (n=89)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>142</td>
<td>51.2 (15.5)</td>
<td>277</td>
<td>49.8 (14.2)</td>
</tr>
<tr>
<td>Gender (% female)</td>
<td>142</td>
<td>71.1</td>
<td>278</td>
<td>85.6</td>
</tr>
<tr>
<td>BMI</td>
<td>98</td>
<td>26.7 (5.2)</td>
<td>184</td>
<td>27.0 (7.0)</td>
</tr>
<tr>
<td>Symptom duration at diagnosis (months) median (IQR)</td>
<td>142</td>
<td>7.54 (3.02–23.9)</td>
<td>278</td>
<td>12.0 (5.64–35.4)</td>
</tr>
<tr>
<td>RF (% positive)</td>
<td>138</td>
<td>72.5</td>
<td>275</td>
<td>78.2</td>
</tr>
<tr>
<td>ACRA (% positive)</td>
<td>97</td>
<td>72.2</td>
<td>112</td>
<td>69.6</td>
</tr>
<tr>
<td>Smoking (% never)</td>
<td>100</td>
<td>67.0</td>
<td>250</td>
<td>91.2</td>
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<tr>
<td>Current</td>
<td>19.0</td>
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<td></td>
<td>3.6</td>
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<tr>
<td>Stopped</td>
<td>14.0</td>
<td></td>
<td></td>
<td>5.2</td>
</tr>
<tr>
<td>HAQ</td>
<td>72</td>
<td>0.80 (0.72)</td>
<td>216</td>
<td>0.76 (0.65)</td>
</tr>
<tr>
<td>DAS</td>
<td>91</td>
<td>2.50 (1.02)</td>
<td>179</td>
<td>2.89 (1.17)</td>
</tr>
<tr>
<td>ESR</td>
<td>124</td>
<td>29.9 (25.2)</td>
<td>244</td>
<td>43.9 (28.8)</td>
</tr>
<tr>
<td>CRP median (IQR)</td>
<td>65</td>
<td>6 (3–13)</td>
<td>71</td>
<td>5 (1.9–12)</td>
</tr>
<tr>
<td>VAS pt gbl (mm)</td>
<td>116</td>
<td>44.9 (25.6)</td>
<td>219</td>
<td>46.5 (22.2)</td>
</tr>
<tr>
<td>RAI median (IQR)</td>
<td>116</td>
<td>3 (1–6)</td>
<td>256</td>
<td>4.5 (1.5–11)</td>
</tr>
<tr>
<td>SJC median (IQR)</td>
<td>116</td>
<td>2 (0–5)</td>
<td>256</td>
<td>2 (0–5)</td>
</tr>
</tbody>
</table>

Mean (SD) reported if not described otherwise. Continuous, normally distributed variables were analysed using one-way ANOVAs, continuous, non-normally distributed variables were analysed using non-parametric tests, categorical variables were analysed using χ² tests.

ACPA, anticyclic citrullinated protein antibodies; ANOVA, analysis of variance; bDMARD, biological DMARD; BMI, body mass index; CRP, C reactive protein; csDMARD, conventional synthetic DMARD; DAS, Disease Activity Score; DMARD, disease modifying antirheumatic drug; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; RAJ, Ritchie Articular Index; RF, rheumatoid factor; SJC, swollen joint count; VAS pt gbl, visual analogue scale patient global assessment.

for changes in DAS over time. Random intercept and random slope were added to each model to account for irregular time intervals between visits, assuming an ‘exchangeable’ covariance matrix.

Subsequently, a final model was estimated, by adding two of the three PSs and their interaction to the linear mixed models. If the interaction term between treatment group and follow-up time proved statistically significant (p<0.10), models were stratified by medication group and reanalysed.

As a secondary analysis, we compared differences in time-to-stop treatment between treatment groups after a maximum follow-up duration of 1 year, using Cox proportional hazards regression and adjusted the analysis for the multiple PSs. All analyses were performed using STATA SE14.

RESULTS

Data of 509 patients from METEOR were selected for inclusion in this analysis (online supplementary figure 1). Included patients had slightly shorter symptom duration and higher disease activity and Health Assessment Questionnaire (HAQ) than non-included patients (online supplementary table 1).

Baseline characteristics per treatment group at the start of the second treatment strategy are described in table 1. Patients proceeding to csDMARD(s)+glucocorticoid included more often females, smoked less often, had longest symptom duration and had a higher DAS than patients in the other two treatment groups. Patients proceeding to bDMARDs had the shortest symptom duration and most swollen joints. Median follow-up duration on studied treatment was 6.9 (IQR 4.1; 9.4) months for patients receiving csDMARD(s), 7.8 (IQR 3.0; 10.2) months for patients receiving csDMARD(s)+glucocorticoid and 9.0 (IQR 6.2; 10.9) months for patients receiving treatment including a bDMARD. When limiting follow-up duration to a maximum of 6 months on studied treatment, median follow-up duration was 3.9 (IQR 0.92; 5.0) months for patients receiving csDMARD(s), 3.6 (IQR 2.3; 5.0) months for patients receiving csDMARD(s)+glucocorticoid and 3.7 (IQR 2.2; 5.1) months for patients receiving treatment including a bDMARD. Furthermore, patients proceeding to csDMARDs had been longer on methotrexate monotherapy before changing treatment (median (IQR) 303 (125–481) days) compared with patients proceeding to csDMARD(s)+glucocorticoid (156 (68–397) days) or to treatment including a bDMARD (190 (91–411) days). Also, at baseline, methotrexate dose was lower for patients proceeding to csDMARD(s)+glucocorticoid (median (IQR) 7.5 (7.5–15) mg) than for patients proceeding to csDMARD(s) (15 (10–20) mg) or to treatment including a bDMARD (15 (15–20) mg).

The specific medication combinations per treatment group are provided in online supplementary table 2.

Since the METEOR registry captures daily practice data, rheumatologists were free to choose their own disease activity measure. Consequently, DAS (based on erythrocyte sedimentation rate (ESR)) was missing in 33% of all visits. However, in only 7% of all visits, no composite disease activity measure was available and in only 3% of all visits, no component of these measures was available.

Pretreatment variables that were associated with the outcome DAS (p<0.10) were included in the multiple PS. These included the variables age, gender, weight, symptom duration, rheumatoid factor, anticyclic citrullinated protein antibodies, ESR, visual analogue scale patient global, Ritchie Articular Index, swollen joint count, HAQ, smoking and country of residence. In addition, the interaction between symptom duration and country was added to improve the model. Body mass index and presence of erosions were not associated with DAS and were therefore not included. C reactive protein and ESR were both associated with DAS, but for reasons of multicollinearity only ESR was included.

The final multiple propensity model had an adjusted R² of 0.34 (95% CI 0.29 to 0.40). Assessment of the overlap of the distributions of the multiple PSs identified five patients with a multiple PS >0.95, who did not have a probability of receiving treatment with csDMARD(s) and were therefore disregarded from further analyses (online supplementary figure 2).
Then, it was assessed whether balance had been achieved in the distribution of all included variables between the three treatment groups. While most variables were unbalanced before adjustment, after multiple PS adjustment, balance was achieved in the distribution of all included variables (p≥0.05), indicating that the multiple PS could be used for further analyses (online supplementary table 3). After multiple PS adjustment, we found statistically significant interactions between treatment group and follow-up time, both after 6 months (p=0.001) and after 1 year (p=0.029), indicating significant differences in treatment response between the three treatment groups. The adjusted treatment effect over time stratified for the different treatment groups is shown in table 2A. Both after 6 months and after 1 year, patients receiving a bDMARD experienced most decrease in DAS per year (6 months: −2.00 (−2.65 to −1.36), 1 year: −0.91 (−1.23 to −0.60)), followed by patients receiving csDMARD(s)+glucocorticoid (6 months: −0.96 (−1.33 to −0.59), 1 year: −0.43 (−0.62 to −0.23)) and by patients receiving treatment with csDMARD(s) alone (6 months: −0.73 (−1.21 to −0.25), 1 year: −0.39 (−0.66 to −0.13)). When comparing the adjusted (table 2A) and unadjusted (table 2B) treatment effects, the unadjusted model showed slightly larger treatment-effects, indicating that the multiple PS (at least partly) adjusted for confounding by indication.

Results of the Cox regression showed that patients receiving treatment including a bDMARD had a lower hazard for discontinuing treatment compared with patients receiving csDMARD(s) alone (HR (95% CI) 0.38 (0.24 to 0.60)), but there were no differences between csDMARD treatment with or without a glucocorticoid (HR (95% CI 0.89 (0.66 to 1.20), figure 1). These results again slightly differed between adjusted and unadjusted models (online supplementary table 4).

**DISCUSSION**

In this study of a large observational database capturing daily clinical practice, we have compared several treatment strategies in patients with RA who had failed initial treatment with methotrexate. Furthermore, we have illustrated the use of the multiple PS as a method to control for bias when comparing multiple non-randomised treatment groups. After adjustment, we found that patients who switched to a bDMARD had more decrease in DAS than patients receiving csDMARD(s) therapy or combination therapy including a glucocorticoid, either after a maximum follow-up duration of 6 months or of 1 year.

Most randomised trials have not shown important differences in disease activity between adding a bDMARD and escalating to triple csDMARD therapy after 4–6 months follow-up.4 5 Our results were more in line with randomised trials that showed superiority of bDMARD treatment compared with triple csDMARD therapy after 12 months follow-up.6 Differences between our findings and previous trials may be explained by

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**Table 2** Change in DAS over time for each medication group (n=509)*

<table>
<thead>
<tr>
<th>Maximum follow-up duration</th>
<th>6 months</th>
<th>1 year</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Results adjusted for the multiple propensity scores</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>csDMARD(s)</td>
<td>−0.73</td>
<td>−0.39</td>
</tr>
<tr>
<td>csDMARD(s)+glucocorticoid</td>
<td>−0.96</td>
<td>−0.43</td>
</tr>
<tr>
<td>bDMARD (acsDMARD(s))</td>
<td>−2.00</td>
<td>−0.91</td>
</tr>
</tbody>
</table>

| **B. Unadjusted results** |          |        |
| csDMARD(s)                | −0.73    | −0.43  |
| csDMARD(s)+glucocorticoid | −1.05    | −0.48  |
| bDMARD (acsDMARD(s))      | −2.03    | −0.98  |

*Results stem from linear mixed model analyses. Parameter estimates represent the unit of change in DAS per year.

bDMARD, biological DMARD; csDMARD, conventional synthetic DMARD; DAS, Disease Activity Score; DMARD, disease modifying antirheumatic drug.
differences in baseline characteristics between studies. Patients in trials had far higher disease activity at the start of treatment than patients in our registry had. They also importantly differed in symptom duration.

While in most previous studies only two strategies were compared, we could compare three strategies simultaneously. We also found that combination therapy including a glucocorticoid did not necessarily result in more DAS improvement than csDMARD(s) therapy without glucocorticoids, in spite of a numerical difference in DAS after 6 months. These two treatment strategies had not formally been compared in randomised clinical trials before.

One problem inherent to immediately escalating from methotrexate monotherapy to a bDMARD-strategy is cost-effectiveness. Ideally, a positive treatment response to bDMARDs should be predicted upfront, but this is currently impossible. From a societal perspective and depending on the exact medication costs, escalating to a bDMARD, resulting in a rapid clinical improvement, may be cost efficient, but further research into this topic is needed.

We found that patients who switched to a bDMARD had a lower hazard to switch treatment again in the subsequent follow-up period (indicating sustained response) than patients who switched to csDMARD therapy, with or without glucocorticoids. With longer follow-up, more patients who switched to a bDMARD continued to have a low DAS, whereas more patients in the other groups failed with a high DAS. This explains why the estimated decrease in DAS was less in the maximum 1-year follow-up analysis than in the maximum 6 months follow-up analysis. Stable treatment follow-up was more than a month longer in the bDMARD group than in the csDMARD plus glucocorticoid group and more than 2 months longer than in the csDMARD without glucocorticoid group. One limitation of the current study is that several treatments were clustered into three subgroups, which is definitely a simplification of the truth. Although there is no consistent evidence that there are, for example, differences in the efficacy of different bDMARDs and medication doses for many drugs are often fixed, it is still possible that differences in medication strategies within subgroups could influence treatment outcomes.

In conclusion, in this analysis with real life clinical data, we have shown that after multiple PS adjustment patients with RA who had failed initial treatment with methotrexate monotherapy experienced more decrease in disease activity after switching to treatment with a bDMARD than to treatment with csDMARD(s) plus glucocorticoid or to csDMARD(s) alone. Furthermore, treatment-survival was better in patients receiving treatment with a bDMARD. This could have important consequences for clinical practice, when choosing among treatment strategies in patients who fail initial methotrexate treatment.
Rheumatoid arthritis

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CLINICAL SCIENCE

Ultra-low-dose CT detects synovitis in patients with suspected rheumatoid arthritis

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ABSTRACT

Purpose To prove the feasibility and measure the diagnostic accuracy of contrast-enhanced ultra-low-dose CT (ULD-CT) for the detection of inflammatory soft-tissue changes (synovitis, tenosynovitis and peritendonitis) in patients with arthritis of the hand.

Materials and methods In this institutional review board–approved study, 36 consecutive patients over the age of 50 with suspected rheumatoid arthritis underwent ULD-CT (estimated radiation exposure <0.01 mSv) and MRI of the hand with weight-adapted intravenous contrast administration. ULD-CT subtraction and MR images were assessed for synovitis, tenosynovitis and peritendonitis by three readers using a modified Rheumatoid Arthritis MRI Score (RAMRIS). Patients were asked which modality they would prefer for future examinations. Sensitivity and specificity of ULD-CT for detection of inflammatory changes were calculated using MRI as standard of reference. The sum scores were correlated using Pearson’s r.

Results All 36 patients showed synovitis in MRI. ULD-CT had 69% sensitivity on the patient level and 65% on the joint level with 87% specificity. Sensitivity was higher in patients with more severe inflammation (80% for MRI RAMRIS >1). There was almost perfect correlation between the modified RAMRIS sum scores of ULD-CT and MRI (Pearson’s r=0.94). Regarding preferences for future examinations, 85% preferred ULD-CT over MRI. ULD-CT detected more differential diagnoses than MRI (8 vs 2/12).

Conclusion Contrast-enhanced ULD-CT of the hand allows for depiction of soft-tissue inflammation at the hand and can be achieved using very low radiation exposure (<0.01 mSv). ULD-CT may evolve to a fast and comfortable alternative to MRI, although it is not as sensitive as MRI for detecting mild disease.

Key messages

What is already known about this subject?

► CT can be used as standard of reference for bone destruction in inflammatory diseases; however, it is not able to distinguish between inactive and active disease.

What does this study add?

► Contrast-enhanced ultra-low-dose CT using subtraction allows for a depiction of active soft-tissue inflammation of the wrist and finger joints in patients with suspected rheumatoid arthritis and can be achieved with similar radiation exposure than digital radiography.

► Ultra-low-dose CT showed better accuracy for differential diagnoses; however, it was not as sensitive as MRI for detecting mild disease.

► Despite exhibiting radiation exposure, ultra-low-dose CT was preferred over MRI by the patients due to a shorter examination time.

How might this impact on clinical practice or future developments?

► Ultra-low-dose CT may develop to an alternative imaging technique for patients unwilling or unable to undergo MRI or when arthrosonography is not available.

Recent technical advances such as ultra-low-dose CT (ULD-CT)9 can reduce CT radiation exposure to that of conventional radiographs. Furthermore, preliminary attempts to detect active inflammation using contrast-enhanced CT have been reported.10 11

The aim of our study was to prove the feasibility of contrast-enhanced ULD-CT of the hand and wrist and determine its diagnostic accuracy in arthritis of the hand compared with MRI.

INTRODUCTION

Synovitis, tenosynovitis and peritendonitis are key features of active inflammatory arthritis in patients with peripheral rheumatic disorders.1 They can be detected using contrast-enhanced MRI or ultrasonography.2 3 CT is to date not recommended by imaging guidelines for rheumatoid arthritis.4 With its high resolution and bone contrast, CT may be considered a gold standard for bone destruction,5 and dual-energy CT depicts gouty depositions and bone marrow oedema.6 8 However, CT cannot distinguish between inactive and active disease, and there are concerns about the radiation exposure.

METHODS

Patients

Thirty-seven consecutive patients presenting to the rheumatology department with joint pain and swelling of the wrist and/or finger joints and suspected rheumatoid arthritis between September 2016 and October 2017 were prospectively enrolled. All patients had to be over 50 as requested by the local ethics board. Exclusion criteria were contraindications to MRI and intravenous contrast medium, for example, kidney dysfunction with
a glomerular filtration rate <60 mL/min, and inability to give informed consent. A final diagnosis was established by the expert rheumatologist based on all available data (eg, clinical information, laboratory tests and imaging results including X-ray).

**Imaging**

All patients underwent a contrast-enhanced ULD-CT and MRI of the same hand in superman position with a maximum interval of 1 hour between the two examinations. The patients were randomised to ULD-CT or MRI first. The ULD-CT protocol included a scanogram and two 16 cm scans on a 320-row detector scanner (Canon Aquilion One Vision; Canon Medical Systems, Japan) without table movement before and 3 min after intravenous injection of iodinated contrast agent. Both scans were performed at 80 kVp to maximise the sensitivity for contrast media. A rotation time of 0.275 s and a tube current of 8.25 mAs was applied to reach a ULD-CT level of radiation exposure. The resulting total dose–length–product was 48 mGy cm and the estimated effective dose <0.01 mSv. The MRI protocol included clinical standard sequences with coronal T1 and short-tau inversion recovery and a two-plane (coronal and axial) fat-saturated T1-weighted sequence 3 min after contrast agent administration. The total MRI scan time was 25 min. Doses were adjusted to body weight: 1 mL/kg Ultravist 370 (Bayer, Germany) for ULD-CT and 1 mL/kg of a 1:4 mixture of gadolinium–DOTA (Dotarem, Guerbet, France) and isotonic saline for MRI, both at a flow rate of 3 mL/s, respectively. The maximum injected volume was 100 mL.

Precontrast and postcontrast ULD-CT images in soft-tissue kernel were postprocessed using a special software (SureSubtraction Ortho V5; Canon Medical Systems, Japan) for the reconstruction of colour-coded subtraction images with 3 mm slice thickness.

**Image reading**

Three readers scored the images independently for synovitis, tenosynovitis and peritendonitis blinded to all identifying or clinical information and the results of the other modality. A modified Rheumatoid Arthritis MRI Score (mRAMRIS) including a 0 to 3 rating of flexor and extensor tendons was used.\(^6\) In two separate reading sessions, the readers evaluated MRI and ULD-CT images in consensus for an imaging diagnosis using contrast enhancement for active inflammation and morphological information provided by the respective modality.

**Patient comfort**

The patients were asked to complete a short questionnaire to assess their concerns regarding radiation exposure, the duration of the examination and contrast agent injection. Specifically, they had to rate the following questions on a 1-to-5 scale: (1) How were your concerns before the (modality) examination (1: no concerns, 5: severe concerns)? (2) How was your comfort during the (modality) examination (1: very good, 5: very poor)? (3) How was your anxiety during the (modality) examination (1: no anxiety, 5: severe anxiety)? (4) Assuming medical equivalence, which examination would you prefer for future examinations?

**Statistical analysis**

For the comparison of CT and MRI, a location (joint or tendon) was considered positive if two of three readers agreed on the presence of inflammation. For statistical purposes, the scores of joints were grouped, resulting in five groups per patient: (1) wrist, (2) metacarpophalangeal joints, (3) proximal interphalangeal joints, (4) extensor and (5) flexor tendons. Sensitivity and specificity of ULD-CT for detection of synovitis (score >0) on the patient and joint group level were calculated using MRI as standard of reference. A sensitivity analysis on the joint group level was performed defining MRI scores higher than 1 as positive. A Pearson test was applied for significant correlations of MRI and ULD-CT sum scores. Inter-rater reliability was calculated using Fleiss’s kappa. The imaging diagnoses derived from the consensus reading were compared descriptively with the final diagnosis established by the rheumatologist. The questionnaire results were compared using Wilcoxon’s matched-pairs signed-rank test and McNemar test where appropriate. A p value smaller than 0.05 was considered significant.

**RESULTS**

**Patients**

One patient did not undergo contrast-enhanced MRI and was excluded from analysis. Thus, 36 patients (10 men and 26 women) were included. They had a mean age of 60.1 (SD 7.2; range 50–77) years, a mean weight of 77.3 (SD 14.3) kg and a mean C reactive protein of 18 (SD 42.6) mg/L. Twenty-four patients were finally diagnosed and classified with rheumatoid arthritis according to the American College of Rheumatology/European League Against Rheumatism criteria (16 seronegative and 8 seropositive), six with inflammatory osteoarthritis of the hand, three with psoriatic arthritis/peripheral spondyloarthritis, two with calcium pyrophosphate dihydrate deposition disease (CPPD) and one with undifferentiated arthritis.

**Image reading**

Sixteen patients underwent MRI following ULD-CT, 20 ULD-CT first. All 36 patients had synovitis, tenosynovitis or peritendonitis on MRI (mean sum score 9.9±8.7). ULD-CT revealed inflammation in 69.4% (25/36) of the patients with a mean sum score of 7.5±9.6 (see table 1). Among the false-negative patients, there were six with the diagnosis of RA (one of them seropositive), three with osteoarthritis, one with undifferentiated arthritis and one with CPPD. Imaging examples are presented in figure 1. The results of the consensus reading for the final diagnosis are shown in figure 2.

The specificity on the patient level could not be calculated due to missing true-negative samples. The analysis on the joint group level yielded a combined sensitivity of 65% (95%
Rheumatoid arthritis

Figure 1 Imaging examples. (1) 68-year-old female patient with seronegative rheumatoid arthritis. (A, C) Coronal (A) and axial (C) T1 with fat saturation shows normal findings at the metacarpophalangeal (MCP) joints and the wrist. There is also no enhancement in ultra-low-dose CT (ULD-CT) subtraction with colour coding (1B, D) in corresponding slice orientation. For better anatomical orientation, the subtraction images were fused with the conventional ULD-CT. Therefore, the bone is faintly visible. (2) A 62-year-old male patient with severe active rheumatoid arthritis. (A) Coronal T1 with fat saturation shows synovitis of the MCP joints and the wrist (white arrowhead). (B) ULD-CT subtraction shows enhancement of the MCP joints and the wrist (white arrowhead) correlating well with MRI. (C and D) Axial MRI (C) and CT subtraction (D) show severe synovitis of the MCP joints and carpus and tenosynovitis of the flexor tendons (white arrowheads). There is also contrast medium in the veins (white arrows). (3) A 67-year-old female patient with calcium pyrophosphate dihydrate deposition disease (CPPD). (A) CT shows calcifications in the scapholunate and lunotriquetral ligament indicating CPPD, which is not visualised by T1-weighted MRI (B) and was occult in radiography (black arrowheads). (C, D) Contrast-enhanced MRI shows tenosynovitis of the second and third flexor tendons and synovitis of the wrist, which was also detected with ULD-CT (white arrowheads). However, the mild synovitis of the second MCP joint was not visualised by ULD-CT (white arrows).

CI 56% to 73%), specificity of 88% (95% CI 78% to 94%), positive predictive value of 90% (95% CI 82% to 95%) and negative predictive value of 59% (95% CI 49% to 68%). Sensitivity for the individual readers ranged from 54% to 84% and specificity from 82% to 94%. Sensitivity increased markedly to 80% for MRI scores >1. A detailed analysis of patients with and without final diagnosis of RA is shown in online supplementary table 1. There was an almost perfect correlation of the ULD-CT sum scores with MRI with a Pearson’s r of 0.94. Inter-rater reliability (Fleiss kappa) was 0.55 for MRI and 0.65 for ULD-CT.

Patient comfort
Thirty-four patients completed the questionnaire. The results are presented in online supplementary figure 2. Interestingly, the patients seemed to be more worried about the MRI examination when asked about their concerns. They felt more comfortable during the ULD-CT examination than during MRI and felt less anxiety during the CT scan despite the warm sensation caused by the CT contrast medium. Most patients appreciated the short CT examination time and that there was less noise during the scan. Moreover, 85% (29/34) preferred ULD-CT for future examinations, 3% (1/34) MRI and 12% (4/34) were undecided.

**DISCUSSION**

To the best of our knowledge, this is the first study to describe ULD-CT subtraction reconstruction for the detection of synovitis. The radiation exposure of ULD-CT was less than 0.01 mSv (10 times less than a chest radiograph and comparable to an X-ray of hands and feet). ULD-CT had limited sensitivity for mild inflammation but reliably detected severe synovitis and tenosynovitis. Specificity was very high and correlation with MRI excellent ($r=0.94$). ULD-CT yielded a superior inter-rater reliability and capability to detect differential diagnoses. Most patients preferred ULD-CT over MRI due to the short scanning time.

CT is a fast, standardised technique with safe contrast administration in patients without renal dysfunction or hyperthyroidism. State-of-the-art reconstruction algorithms enable low-dose scanning with radiation exposure similar to a radiograph. In view of patient preference and recent concerns about gadolinium-based MRI contrast agents, CT may become a suitable alternative, especially for patients with contraindications to MRI or unable to tolerate the rather long examination times. Subtraction after contrast medium may also give additional information of active inflammation in patients who undergo CT for other indications, for example, dual-energy CT for gout. The reconstruction of the subtraction images takes only 2 min and can be done by a technician. One should also keep in mind that the CT source images offer additional information on bone erosion, new bone formation and soft-tissue calcification that MRI is not able to provide. In our study, 6% of the patients (2/36) were diagnosed with CPPD based on the presence of crystals in typical localisation in CT (and not in radiography). This diagnosis altered treatment, and these patients directly benefited from study participation. Furthermore, some authors suggest that the superior spatial resolution of CT allows for the depiction of enhancement patterns that are not visualised by MRI.

Only a few studies investigated contrast-enhanced CT for the evaluation of active arthritis. Polster et al used postcontrast CT with digital bone subtraction in four patients to delineate synovitis. They also reported that the patients preferred CT over MRI. Fukuda et al used dual-energy CT to generate iodine contrast maps to detect synovitis. With 16 patients suffering from psoriatic arthritis, they found 78% sensitivity and 87% specificity.

Despite a well-planned design, our study has some limitations. Our patient cohort is small, but it is the largest number of patients undergoing contrast-enhanced CT in arthritis published to date, and we obtained meaningful statistical results. We obtained a
rather low sensitivity because we used very low radiation exposure. However, sensitivity can be improved by applying higher radiation to reduce image noise. The final diagnosis might be biased by the imaging results because the rheumatologists were not blinded. We did neither compare CT subtraction with other postprocessing techniques (eg, dual-energy iodine map or digital bone masking) nor with sonography. Sonography has proven high diagnostic accuracy in patients with rheumatoid arthritis; however, it has disadvantages in standardisation. We included patients with suspected and not proven rheumatoid arthritis. This leads to a rather inhomogeneous collective. However, we believe that the imaging findings (synovitis) are comparable and our results better reflect daily clinical practice. Finally, our collective was limited to patients over 50 due to requirements of the Federal Office for Radiation Protection. Nonetheless, we do not see technical reasons for ULD-CT losing diagnostic accuracy in younger patients.

In conclusion, our study proves the feasibility of ULD-CT in suspected rheumatoid arthritis. The method is preferred by patients. As such, ULD-CT may be a suitable alternative for patients unable or unwilling to undergo MRI or if arthrosonography is unavailable. Future studies should compare the different techniques in larger patient populations and investigate how image quality can be improved.

Acknowledgements The authors thank Bettina Herwig for language editing.

Contributors TD: conception and design of the study, design of scoring system, image scoring, data evaluation, statistical calculations; article draft, critical revision of the manuscript for important intellectual content. STU: patient acquisition, data management, image scoring, critical revision of the manuscript for important intellectual content. DP: patient acquisition, conception and design of the study with critical revision of the manuscript for important intellectual content. US: patient acquisition, critical revision of the manuscript for important intellectual content. SH: patient acquisition, critical revision of the manuscript for important intellectual content. RB: patient acquisition, critical revision of the manuscript for important intellectual content. KH: conception and design of the study, image scoring, data evaluation, with critical revision of the manuscript for important intellectual content and final approval of the version to be published.

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 consent Obtained.

Ethics approval The study was approved by the local ethics committee (EA1/259/15) and the German Federal Office for Radiation Protection (BFS) (25-22462/2-2016-008).

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement Imaging and scoring source data are available per request from the first author.

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Rheumatoid arthritis–associated DNA methylation sites in peripheral blood mononuclear cells

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ABSTRACT

Objectives To identify novel DNA methylation sites significant for rheumatoid arthritis (RA) and comprehensively understand their underlying pathological mechanism.

Methods We performed (1) genome-wide DNA methylation and mRNA expression profiling in peripheral blood mononuclear cells from RA patients and health controls; (2) correlation analysis and causal inference tests for DNA methylation and mRNA expression data; (3) differential methylation genes regulatory network construction; (4) validation tests of 10 differential methylation positions (DMPs) of interest and corresponding gene expressions; (5) correlation between PARP9 methylation and its mRNA expression level in Jurkat cells and T cells from patients with RA; (6) testing the pathological functions of PARP9 in Jurkat cells.

Results A total of 1046 DNA methylation positions were associated with RA. The identified DMPs have regulatory effects on mRNA expressions. Causal inference tests identified six DNA methylation–mRNA–RA regulatory chains (eg, cg00959259-PARP9-RA). The identified DMPs and genes formed an interferon-inducible gene interaction network (eg, MX1, IFI44L, DTX3L and PARP9). Key DMPs and corresponding genes were validated their differences in additional samples. Methylation of PARP9 was correlated with mRNA level in Jurkat cells and T lymphocytes isolated from patients with RA. The PARP9 gene exerted significant effects on Jurkat cells (eg, cell cycle, cell proliferation, cell activation and expression of inflammatory factor IL-2).

Conclusions This multistage study identified an interferon-inducible gene interaction network associated with RA and highlighted the importance of PARP9 gene in RA pathogenesis. The results enhanced our understanding of the important role of DNA methylation in pathology of RA.

Rheumatoid arthritis (RA) is a complex autoimmune disease with characteristic chronic articular synovial inflammation.1 The pathogenesis of RA so far is largely unclear yet. Epigenetic factors have recently emerged as potential elements in explaining and redefining diseases. Among a variety of epigenetic regulatory mechanisms, DNA methylation is the most frequently studied factor because of relatively more mature detection technology. The best-known function of DNA methylation is to regulate nearby gene expression. DNA methylation

Key messages

What is already known about this subject?
► DNA methylation is a frequently studied epigenetic factor that plays an important role in the pathogenesis of RA.
► Most previous epigenome-wide association studies have focussed on methylation level alone, and lacked the integration of DNA methylation with mRNA expression data to fully reveal the pathophysiological contributions and the functional roles of DNA methylation in RA.

What does this study add?
► An interferon-inducible gene interaction network was associated with RA by integrating DNA methylation with mRNA expression data.
► The importance of PARP9 gene was highlighted in RA pathogenesis.

How might this impact on clinical practice or future developments?
► As DNA methylation plays the important roles in pathogenesis of RA, it may serve as potential biomarker and provide helpful clues for developing diagnostics, classification and therapy for RA.

are most commonly observed in promoter regions where they control transcription of the nearby target genes, often in a cell-type-specific manner.

Growing evidence has suggested that DNA methylation plays an important role in the pathogenesis of RA. Early candidate gene methylation studies identified aberrant methylation changes in some specific genes, such as IL6, IL10 and CXCL12 in RA (reviewed in Klein and Gay). Further epigenome-wide association studies (EWASs) by using modern high-throughput microarray technology, such as Illumina 450K, have been performed to systematically identify methylation markers associated with RA in various cells. These EWASs for RA have identified a large number of robust and novel candidate differentially methylated genes (DMGs), which vary widely across studies. Such inconsistency may be partially due to the great difference in study design (eg, coverage of methylation microarrays, case diagnosis, sample age,
control selection, definition of differential methylation) as well as cell/tissue specificity.

6 Most previous EWASs focused on methylation level alone, which may lead to incomplete interpretation of the results regarding the functional mechanism underlying the associations between the DMGs and RA. Considering that DNA methylation normally regulates nearby gene expression, integration of DNA methylation with mRNA expression data is essential to fully reveal the pathophysiological contributions and the functional roles of DNA methylation in RA.

7 To identify more novel RA-associated DNA methylation sites and fully understand their underlying pathological mechanism, we generated and integrated two omics datasets (methylole and transcriptome) from the same subjects and constructed an integrative regulatory network of RA-related DMGs. We validated the differential methylation and differential expression in two additional sample sets, and performed in-depth causal inference test (CIT) to evaluate the potential DNA methylation–mRNA expression–RA regulatory effect. Furthermore, we explored the potential pathogenic mechanisms of an important gene involved by conducting a series of molecular functional tests. The flow chart of the analytical pipeline and procedures is shown in online supplementary figure S1.

MATERIAL AND METHODS

Please see online supplementary methods for full details.

Study subjects

A total of 43 subjects (RA:healthy control=25:18) at discovery stage, 52 subjects (RA:healthy control=25:27) for DNA methylation testing and 70 subjects (RA:healthy control=35:35) for gene expression testing at validation stage were recruited respectively. All patients with RA met the 1987 criteria of the American College of Rheumatology. The basic characteristics of all subjects are presented in online supplementary table S1.

RESULTS

1046 DNA methylation positions were associated with RA

We measured DNA methylation levels at 485,577 methylation sites in peripheral blood mononuclear cells (PBMCs) from 25 patients with RA and 18 healthy controls using Illumina Infinium HumanMethylation450K BeadChip. After quality control and screening procedure, 473,368 methylation positions were subject to differential analysis. In total, 1046 differentially methylated positions (DMPs) (∣Δβ∣ >0.05 and detection p < 0.05) including 574 hypermethylated and 472 hypomethylated DMPs (online supplementary data S1, online supplementary figure S2) were identified, which correctly separated most of RA cases and controls in the clustering analysis (online supplementary figure S3). According to the annotation, 730 DMPs were physically located within 598 unique genes. Functional enrichment analyses showed that the 598 genes were significantly enriched in some biological processes related to RA (online supplementary figure S4), for example, GO:0031295/T cell co-stimulation, GO:0006955/immune response, and GO:0030852/T-cell receptor signalling pathway. The enrichment was also found in Kyoto Encyclopedia of Genes and Genomes pathways that were confirmed related to RA (eg, hsa04672/Intestinal immune network for IgA production and hsa04514/Cell adhesion molecules) and several autoimmune diseases (eg, hsa05322/Systemic lupus erythematosus and hsa05320/Autoimmune thyroid disease) (online supplementary table S2). The above observations suggested that the DMGs play a critical role in the pathogenesis of RA.

Identified DMPs have regulatory effects on mRNA expressions

We examined mRNA expressions in PBMCs from the same sample set (25 patients with RA and 18 healthy controls) by using the LncRNA&mRNA Human Gene Expression Microarray V4.0 (CapitalBio, Beijing, China). Among the 598 DMP-located genes, only 445 genes (covering 540 DMPs) showed expression data in the mRNA expression microarray. Pearson correlation analyses showed significant associations between DNA methylation levels of 107 DMPs and mRNA expressions of 91 unique genes (p<0.05) corresponding to the DMPs (positive correlation: negative correlation=44:63). Among the 91 genes, 67 genes (covering 81 DMPs) were differentially expressed between RA cases and healthy controls (p<0.05). Subsequent in-depth analyses were focused on the 67 differentially expressed genes (DEGs, also DMGs) and the 81 corresponding DMPs. Of note, Poly(ADP-Ribose) Polymerase Family Member 9 (PARP9) had three DMPs located in 5′UTR (Chr3: 122281975, cg00959259; Chr3: 122281939, cg08122652; and Chr3: 122281881, cg22930808), and its mRNA expression level also differed significantly between RA cases and healthy controls (p=3.83E−07 after Bonferroni correction (p value less than 0.05/445=1.12E−04) (online supplementary table S3).

Causal inference tests identified six DNA methylation–mRNA–RA regulatory chains

Since correlation analysis alone does not determine the causative effect, we performed in-depth CIT analyses to explore whether DNA methylation causes RA through regulating gene expression, in other words, to assess the potential regulatory chain of causative factor (DNA methylation)–mediator (mRNA)–outcome (RA). Among the above 81 identified DMPs, we selected 36 significantly correlated methylation–mRNA pairs (p<0.05 and Pearson correlation coefficients <-0.4 or >0.4) for CIT filtering. Six DMPs corresponding to six unique genes, that is, B lymphoid tyrosine kinase (BLK) (cg16861076), leucocyte-associated immunoglobulin-like receptor 2 (LAIR2) (cg08905487), Lin-54 DREAM MuvB core component (LIN54) (cg01882498), MX dynamin-like GTPase 1 (MXI1) (cg26312951), PARP9 (cg00959259) and RAS-like family 10 member A (RASL10A) (cg23754382), were affirmed to be causative among the regulatory chains of DNA methylation–mRNA–RA (table 1, figure 1 and online supplementary table S4). Notably, PARP9 was also highlighted. Figure 1 shows the significant Pearson correlations between the six DMPs and genes identified by CIT.

Identified DMPs and genes formed an interferon-inducible gene interaction network

To illustrate and better understand the regulatory effects of DMGs, we first constructed the predicted protein–protein interactions among the above 445 DMGs, 91 DMGs and 67 DMGs, respectively, using STRING, a database of known and predicted protein interactions. The interaction network was very complicated among the 445 DMGs (online supplementary figure S5) but much distinguishable among the 91 DMGs (online supplementary figure S6). In the network of the 91 DMGs, we found an interesting interferon-inducible gene interaction subnetwork, including MX dynamin-like GTPase 1 (MXI1), ISG15 ubiquitin-like modifier (ISG15), IFN-induced protein 44-like (IFI44L), deltax E ubiquitin ligase 3L (DTX3L), PARP9

Rheumatoid arthritis

Table 1  Causal inference test (CIT) identified six DNA methylation–mRNA–RA regulatory chains

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Cpg site</th>
<th>MAPINFO</th>
<th>CHR</th>
<th>Gene region</th>
<th>Δβ</th>
<th>p values</th>
<th>Fold change (RA/HC)</th>
<th>p values</th>
<th>S1 p values</th>
<th>S2 p values</th>
<th>S3 p values</th>
<th>S4 p values</th>
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<tbody>
<tr>
<td>BLK</td>
<td>cg16861076</td>
<td>11421594</td>
<td>S</td>
<td>Body</td>
<td>−0.064</td>
<td>6.85E−04</td>
<td>0.58</td>
<td>5.19E−04</td>
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<td>2.23E−02</td>
<td>2.66E−02</td>
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<td>LAIR2</td>
<td>cg08905487</td>
<td>55013821</td>
<td>TSS</td>
<td>−0.057</td>
<td>3.37E−02</td>
<td>2.05</td>
<td>4.47E−04</td>
<td>4.93E−02</td>
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</tr>
<tr>
<td>LIN54</td>
<td>cg01882498</td>
<td>83934956</td>
<td>TSS</td>
<td>0.077</td>
<td>3.48E−02</td>
<td>1.24</td>
<td>6.56E−04</td>
<td>4.65E−02</td>
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<tr>
<td>MX1</td>
<td>cg26312951</td>
<td>42797847</td>
<td>TSS</td>
<td>−0.075</td>
<td>6.83E−03</td>
<td>2.12</td>
<td>2.19E−04</td>
<td>1.67E−02</td>
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<tr>
<td>PARP9</td>
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<td>TSS</td>
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<tr>
<td>RASL10A</td>
<td>cg23754382</td>
<td>29712472</td>
<td>TSS</td>
<td>0.056</td>
<td>1.02E−02</td>
<td>0.69</td>
<td>9.89E−04</td>
<td>2.10E−02</td>
<td>1.58E−02</td>
<td>1.66E−02</td>
<td>1.58E−02</td>
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</table>

A total of 36 selected trios were tested by CIT (online supplementary table S4). Steps 1–4 in CIT tested the association between DNA methylation and RA, between DNA methylation and mRNA, between mRNA and RA, and whether DNA methylation is independent of RA after adjusting for age and mRNA, respectively. S1–S3 p values indicated the significance of testing in steps 1–3, respectively. For all the six DMPs, the p values of testing in step 4 were larger than 0.05, that is, insignificant (data not shown). CIT p value is the most significant one from S1 to S3.

CHR, chromosome; DEG, differentially expressed gene; Δβ, difference of methylation between patients with RA and healthy controls; DMP, differential methylation position; HC, healthy control; MAPINFO, position in Build 37; RA, rheumatoid arthritis.

Figure 1  DNA methylation/mRNA correlation plots for six differential methylation positions/genes identified by causal inference test. Note: The log2 transformation was applied to the gene expression data using the Adjust Data function of Multi-experiment Viewer (MeV) software. r: Pearson correlation coefficient.

Key DMPs and corresponding genes were validated their differences in additional samples

To verify the major results of microarray analysis, we selected 10 DMGs including PARP9, IFI44L, MX1, ISG15, family with sequence similarity 8 member A1 (FAM8A1), serine/threonine kinase 17a (STK17A), BLK, canopy FGF signalling regulator 1 (CNPY1), Chromobox 7 (CBX7) and solute carrier family 7 member 14 (SLC7A14) to validate differential DNA methylation/expression in two additional sample sets. A total of 15 DNA methylation positions and 15 mRNA expression genes were validated.

and signal transducer and activator of transcription 3 (STAT3). Such a network was present in the network of 67 DMGs as well (figure 2A).

We further constructed coexpression integrative networks of methylation and mRNA by using DNA methylation values (β value) of 81 DMPs and the mRNA expression data of the 67 corresponding genes into Cytoscape V.3.2.1.11 As shown in figure 2B, among the five identified subnetworks, two (figure 2B-b1, b2) contain only mRNA expression values, and PARP9 is a node gene in the network (figure 2B–b1). Two subnetworks (figure 2B-b4, b5) that contain both DNA methylation and mRNA showed the coexpression network containing MX1, PARP9, DTX3L, ISG15 and IFI44L. The results obtained through STRING and Cytoscape both pointed to an interaction network including four hub genes, MX1, IFI44L, DTX3L and PARP9.

To further validate the major results of microarray analysis, we selected 10 DMGs including PARP9, IFI44L, MX1, ISG15, family with sequence similarity 8 member A1 (FAM8A1), serine/threonine kinase 17a (STK17A), BLK, canopy FGF signalling regulator 1 (CNPY1), Chromobox 7 (CBX7) and solute carrier family 7 member 14 (SLC7A14) to validate differential DNA methylation/expression in two additional sample sets. A total of 15 DNA methylation positions and 15 mRNA expression genes were validated.

Figure 1  DNA methylation/mRNA correlation plots for six differential methylation positions/genes identified by causal inference test. Note: The log2 transformation was applied to the gene expression data using the Adjust Data function of Multi-experiment Viewer (MeV) software. r: Pearson correlation coefficient.
The expression level of PARP9 and cg22930808 were negatively correlated with mRNA showed that the methylation levels of two DMPs (cg08122652 and cg22930808) were located in PARP9. In addition, the quantitative RT-PCR (qRT-PCR) showed that 6 of the 10 DEGs (BLK, CBX7, IFI44L, MX1, PARP9 and STK17A) presented significant differential expressions in PBMCs between 35 patients with RA and 35 healthy controls. Four of the six genes (BLK, IFI44L, MX1 and PARP9) showed consistent regulation direction with the discovery sample (online supplementary table S5). Interestingly, most of the verified genes were involved in the interferon-inducible gene interaction network (figure 2A).

Methylation of PARP9 was correlated with mRNA level in Jurkat cells and T lymphocytes isolated from patients with RA. Since the above combined evidence highlighted the significance of DMPs in PARP9, we analysed the methylation status of the three verified DMPs and the mRNA level of PARP9 at different timepoints after 5-Aza (DNA methyltransferase inhibitor) treatment in Jurkat cells, an immortalised cell line of human T lymphocytes that are frequently used as cell model in studies of immune-related diseases. Pearson correlation analysis showed that the methylation levels of two DMPs (cg08122652 and cg22930808) were negatively correlated with mRNA expression level of PARP9 (p<0.05, r=0.356 and 0.350, respectively). The ΔCT was used in the correlation analysis in vitro. We also performed similar experiment in T cells isolated from seven patients with active RA and found a significant correlation between the methylation level of DMP (cg00959259) and PARP9 gene expression (r=0.752, p=0.019).

PARP9 gene has functional effects on Jurkat cells. We further assessed the effects of PARP9 on behaviours of Jurkat cells by constructing PARP9 over-expression (PARP9-OE) Jurkat cell line and PARP9 down-expression (shRNA) (PARP9-SH) Jurkat cell line. Comparing with its negative control, the mRNA and protein level of PARP9 was 7.9-fold and 2.15-fold upregulated, respectively, in PARP9-OE cells, and 3.5-fold and 6.3-fold downregulated, respectively, in PARP9-SH cells (figure 3A).

As expected, the PARP9-SH cells showed significantly lower proliferation rate than the negative control cells (p=0.035). Conversely, PARP9-OE cells exhibited significantly higher proliferation rate compared with the negative controls (p=0.024) (figure 3B). We also carried out cell-cycle analysis to explain the results of proliferation tests (see online supplementary figure S7) and found that reduced expression of PARP9 was associated with G1 cell-cycle arrest, as evidenced by the increased percentage of G1 and the reduced percentage of S and G2. Meanwhile, over-expression of PARP9 promoted cell proliferation, as evidenced by the reduced percentage of G1 and S and increased percentage of G2 (p<0.05) (figure 3C).

Next, we assessed the expression level of PARP9 in PARP9-OE and PARP9-SH with phytohaemagglutinin (PHA) stimulation. As shown in online supplementary figure S8, the expression level of PARP9 was significantly elevated in all the four PHA-stimulated cell lines (PARP9-OE/NC and PARP9-SH/NC), as compared with the unstimulated controls, respectively. Further, we determined the effect of PARP9 on inflammatory cytokine expression, such as IL-1, IL-2, IL-4, IL-6, IL-8, TNFα and IFNγ using the qRT-PCR tests. Among the seven tested cytokines, only IL-2 presented upregulated effect in PARP9-OE and downregulated effect in PARP9-SH Jurkat cells, as compared with the negative controls, respectively. To confirm the findings, we repeated experiments by treating the cells with 5-Aza and found a similar trend (figure 3B).
the qRT-PCR tests for IL-2 and validated the significant positive regulatory effect of PARP9 on IL-2 (p<0.05) (figure 3D).

Besides, we assessed the effect of PARP9 on IL-2 expression under PHA-stimulated condition. As expected, we found that PHA could stimulate IL-2 expression in both PARP9-OE and PARP9-SH cell line. Consistent with the prior observations on unstimulated cells, the positive regulatory effects of PARP9 on IL-2 expression were also observed in PHA-stimulated PARP9-OE and PARP9-SH cell lines (figure 3E).

To further ascertain the role of PARP9 in the process of activation in Jurkat T cell, flow cytometry was employed to test the expression of antigen CD69, a commonly used early activation biomarker of T cells. We found that the CD69 expression was promoted by PHA stimulation in negative control cells. Under PHA stimulation, PARP9 over-expression slightly increased the amount of CD69 (figure 3F–f1), and PARP9 silencing evidently decreased CD69 expression (figure 3F–f2). These results indicated that PARP9 expression level could affect the degree of Jurkat T-cell activation.

**DISCUSSION**

In this study, we identified an IFN regulatory gene interaction network associated with RA and ascertained the PARP9-regulated cellular mechanisms underlying RA pathogenesis. IFN has been widely used in clinic settings because of its positive effects of antiviral, inhibiting tumour cell proliferation and immune regulation. At present, the pathogenesis of autoimmune diseases including RA is poorly understood. Environmental triggers, such as virus infections, are involved in these diseases. It was hypothesised that human chronic autoimmune diseases are due to infection of autoreactive B lymphocytes by Epstein-Barr virus (EBV) and a subsequent series of T-lymphocyte reaction. Indeed, EBV-induced lymphoproliferative disorders were reported in about 40% of rheumatic patients. IFN was previously reported to be a potential therapy for RA that might help to diminish both joint inflammation and destruction by cytokine modulation. Meanwhile, a systemic upregulation of IFN type I inducible genes, the so-called IFN signature, has been demonstrated in several autoimmune diseases such as systemic lupus erythematosus (SLE), Primary Sjögren’s syndrome (pSS) and RA. Several studies have demonstrated that the IFN signatures in RA do have potential clinical relevance, and it could be served as a biomarker of preclinical RA. Among hundreds of IFN-inducible genes, the elevated expression of MX1 and IFI44L were most often observed in autoimmune diseases.

For example, MX1 was suggested as a biomarker for IFN bioactivity in pSS, and the mRNA expression levels of IFI44L were increased in CD4+ T cells from patients with SLE. Here, we
identified several IFN-regulated genes related to RA, including MX1, IFI44L, PARP9 and DTX3L. Hypomethylation of these IFN-regulated genes was correlated with enhanced gene expression. Our pathway analysis of DMGs also revealed IFN signaling among the top associated pathways. These findings suggest a role of type I IFN activity in the development of RA.

Epigenetic mechanisms may serve as a dynamic link between genotype, environment and phenotype. Increasing evidence suggests an epigenetic contribution to the pathogenesis of autoimmune disease.32 33 Considering that epigenetic alterations are potentially reversible, characterising epigenic dysfunction in disease has the potential to identify new biomarkers and novel therapeutic targets.34 DNA methylation is the best characterised epigenetic modification in humans, and it has emerged as an important contributor to human complex diseases. Although the exact mechanisms remain to be elucidated, global hypomethylation and demethylation of IFN-regulated genes in multi-rheumatoid diseases are the main features to date.34 35 36 Loss of DNA methylation in these genes was associated with an increase in mRNA expression. Therefore, the interferon signature pathway could be featured by hypomethylation of IFN-regulatory genes.37

Moreover, a recent study revealed that significant hypomethylation of two CpG sites within IFI44L promoter was identified in patients with SLE, RA and pSS, and promising to be the first epigenetic diagnostic marker for SLE.34 Previous studies identified the significant hypomethylation of CpG site (cg06872964) within IFI44L in SLE.34 CpG site (cg00959259) within PARP9 in pSS24 and CpG site (cg26312951) within MX1 in pSS.34 The findings expand current knowledge of epigenetic changes of IFN type I inducible genes in autoimmune diseases. Since these epigenomic markers were also identified for RA in our study, they may serve as common sites shared by the autoimmune diseases. On the other hand, some identified DMPs may be unique to RA. For example, for the six DMPs within the CIT regulation chains (table 1), four DMPs (cg16861076, cg08905487, cg01882498, cg23754382) may be unique to RA, as they were not reported in other autoimmune diseases according to our current search (online supplementary table S6). Further research would be needed to explore whether the RA-related DMPs/DEGs identified in the present study are unique to RA or common to other autoimmune diseases.

In our study, the gene PARP9 was the most distinctive DMG in RA. Little was known about its function. In 2000, Aguiar et al35 reported that PARP9 was a novel risk-related gene in diffuse large B-cell lymphomas that enhances cellular migration. Then, it was found that PARP9 and DTX3L are located in a head-to-head orientation, regulated by a gamma IFN-responsive bidirectional promoter, and are over-expressed in diffuse large B-cell lymphomas with a prominent inflammatory infiltrate.36

Recently, PARP9–DTX3L complex was observed with a double benefit for IFN-dependent host defense.37 As described above, our current knowledge of PARP9 was mainly focused on its antiviral or IFN response. As a member of PARPs superfamily that has anti-inflammatory properties, DNA repair and gene transcription regulation, the functional mechanisms of PARP9 in RA remain unexplored previously. The present study demonstrated that PARP9 could influence cell cycle and cell proliferation in vitro. Reduced expression of PARP9 was associated with G1-phase arrest, and over-expression of PARP9 promoted cell proliferation in Jurkat cells. We also found that PARP9 positively regulated IL-2, a cytokine glycoprotein that stimulates a wide range of leucocytes, including T cells and natural killer cells and involved in the Th1 cytokine pathway of the immune response.38 39 Subsequent functional tests indicated that the degree of Jurkat T-cell activation was also positively regulated by PARP9 expression level. These results suggested that PARP9 could be implicated in inflammatory reactions and enhance immunoreactions of RA.

The main strength of this study is the multistage research strategy, which enables us to systematically identify both methyl-ation sites and their target genes, and to thoroughly investigate molecular and cellular mechanisms underlying RA pathogenesis. The results enhanced our understanding of the roles of methylation in pathology of RA and provided helpful clues for developing diagnostics, classification and therapy for RA.

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Contributors HZ participated in study design, recruiting patients with RA, data analysis, molecular testing, drafted and revised the manuscript. LFW participated in molecular testing, data analysis and revised the manuscript. XBM participated in data interpretation and revised the manuscript. XL coordinated the sample collection. HT participated in sample collection, data analysis. WXZ participated in sample collection, cell culture, molecular testing and revised the manuscript. WX participated in cell culture, molecular testing, data interpretation and revised the manuscript. YFG, MJW, KQZ and JW participated in recruiting patients with RA, data analysis and revised the manuscript. YHQ and XBM participated in sample collection, revised and finalised the manuscript. All authors read and approved the final manuscript.

Funding The study was supported by Natural Science Foundation of China (81401343, 81473046, 81872681, 81373010, 81502868, 31041079, 81541068), the Natural Science Foundation of Jiangsu Province (BK20150346), the Natural Science Research Project of Jiangsu Provincial Higher Education (16KJA330001), the Startup Fund from Soochow University (Q413900112, Q413900712) and a Project of the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Competing interests None declared.

Patient consent Obtained.

Ethics approval The ethical committee of Soochow University.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement The microarray data for methylation have been submitted to the GEO database with accession number GSE119942.

REFERENCES
Rheumatoid arthritis


Identification of a distinct imaging phenotype may improve the management of palindromic rheumatism

Kulveer Mankia, Maria-Antonietta D’Agostino, Richard J Wakefield, Jackie L Nam, Waqar Mahmood, Andrew J Grainger, Paul Emery

ABSTRACT

Objectives To use high-resolution imaging to characterise palindromic rheumatism (PR) and to compare the imaging pattern observed to that seen in new-onset rheumatoid arthritis (NORA).

Methods Ultrasound (US) assessment of synovitis, tenosynovitis and non-synovial extracapsular inflammation (ECI) was performed during and between flares in a prospective treatment-naive PR cohort. MRI of the flaring region was performed where possible. For comparison, the same US assessment was also performed in anticyclic citrullinated peptide (CCP) positive individuals with musculoskeletal symptoms (CCP+ at risk) and patients with NORA.

Results Thirty-one of 79 patients with PR recruited were assessed during a flare. A high frequency of ECI was identified on US; 19/31 (61%) of patients had ECI including 12/19 (63%) in whom ECI was identified in the absence of synovitis. Only 7/31 (23%) patients with PR had synovitis (greyscale ≥1 and power Doppler ≥1) during flare. In the hands/wrists, ECI was more prevalent in PR compared with NORA and CCP+ at risk (65% vs 29% vs 6%, p<0.05). Furthermore, ECI without synovitis was specific for PR (42% PR vs 4% NORA (p=0.003) and 6% CCP+ at risk (p=0.0012)). Eleven PR flares were captured by MRI, which was more sensitive than US for synovitis and ECI. 8/31 (26%) patients with PR developed RA and had a similar US phenotype to NORA at progression.

Conclusion PR has a distinct US pattern characterised by reversible ECI, often without synovitis. In patients presenting with new joint swelling, US may refine management by distinguishing relapsing from persistent arthritis.

Early diagnosis and treatment of inflammatory arthritis (IA) is associated with less joint damage and a higher chance of achieving remission. However, identifying and treating IA at the earliest opportunity can be challenging as many patients with disease-specific autoantibodies and/or inflammatory joint symptoms do not necessarily develop persistent arthritis. An important example is patients with palindromic rheumatism (PR). PR is characterised by intermittent flares of articular and periarticular inflammation. Up to 50% of patients with PR will eventually develop rheumatoid arthritis (RA), with those that are anticyclic citrullinated peptide (CCP) antibody positive at highest risk of progression. However, the time to progression is variable and many anti-CCP positive patients with PR do not develop persistent arthritis, even after several years of follow-up. Identifying patients with this favourable prognosis from those with early persistent IA is important; the latter require early disease-modifying therapy, whereas the former can often be monitored with a more conservative approach.

In clinical practice, distinguishing true PR from a new presentation of IA can be challenging; many patients require multiple assessments before a diagnosis is made. High-resolution imaging, particularly ultrasound (US), is recommended as part of the diagnostic workup for suspected RA with many rheumatologists now using US in their routine practice. Imaging studies in PR have, however, been limited. This is likely due to the difficulty in capturing this group of patients and the sporadic nature of flares. We therefore aimed to comprehensively describe the imaging phenotype of PR in a prospective treatment-naive cohort, both during and between flares. We then sought to compare this to the imaging findings in (1) anti-CCP positive individuals with musculoskeletal (MSK) symptoms (CCP+ at risk) and (2) patients with new-onset RA (NORA). We hypothesised that both synovial and non-synovial extracapsular (EC) structures are important disease targets in PR and that imaging would reveal a distinct pattern of inflammation which may be used to distinguish PR from patients presenting with early persistent IA.
Inflammatory arthritis

Figure 1  Flow chart showing patient visits for the palindromic rheumatism cohort. The cohort was followed according to patient-reported flares. Patients in Group A were not in flare at visit 1 and were in flare at visit 2. Patients in Group B were in flare at visit 1 but not at visit 2. Ultrasound assessments were performed at both visit 1 and visit 2. Ten out of 15 (67%) patients with PR who were flaring at the initial visit had US abnormalities. Eleven of these patients subsequently attended a non-flare visit where only one (9%) patient had US abnormalities. Of the patients who were not in flare at the initial visit and who subsequently attended for a flare visit, 9/16 (56%) had US abnormalities. Patients were monitored for the development of persistent arthritis. PR, palindromic rheumatism; RA, rheumatoid arthritis; US, ultrasound.

METHODS

Design
A prospective analysis of a regional PR cohort was performed. For comparison, both a prospective and retrospective analysis of a cohort of CCP+ at-risk individuals and patients with NORA was also undertaken.

Patients with PR
Patients with PR were recruited from rheumatology clinics in Leeds and the Yorkshire region. Some patients with PR were also recruited through a national primary care programme adopted by the National Institute for Health Research Clinical Research Network. All patients were assessed at Chapel Allerton Hospital, Leeds, UK, and were recruited if the study rheumatologist diagnosed PR. In the absence of accepted classification criteria, PR was defined as ‘a confirmed history or physical examination consistent with episodes of joint pain and swelling that returned to normal between episodes in the absence of an alternative diagnosis’.

Patients underwent clinical and US assessment at baseline and were followed according to patient-reported flares: those patients who were flaring at the initial visit were invited to reattend when they were not flaring; likewise, the patients who were not flaring at the initial visit were asked to telephone when they were having a flare and were seen within 48 hours. A flare episode was defined as two or more features of pain, swelling and erythema in or around at least one joint region that later normalised. Patients were divided into two subgroups according to the disease phase at the first assessment: patients in Group A were not in flare at their first assessment (ie, ‘non-flare’), whereas patients in Group B were ‘in flare’ at their first assessment. For both groups, patients were re-evaluated at a second visit when the disease phase changed. US was performed at all flare and non-flare visits. MRI of the most affected region was performed during flare visits where possible. Patients were monitored for the development of persistent arthritis.

Anti-CCP+ at-risk individuals
CCP+ at-risk individuals were recruited through a national primary care programme. All patients with NORA met American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 classification criteria for RA. All patients with NORA were anti-CCP positive and disease-modifying antirheumatic drug (DMARD) naïve at the time of assessment. Clinical and US assessments performed at RA diagnosis were included in the current analysis.

Patients with NORA
All patients with NORA met American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 classification criteria for RA. All patients with NORA were anti-CCP positive and disease-modifying antirheumatic drug (DMARD) naïve at the time of assessment. Clinical and US assessments performed at RA diagnosis were included in the current analysis.


**Table 1** Patient characteristics

<table>
<thead>
<tr>
<th>Age (years), mean (SD)</th>
<th>CCP+ at risk (n=33)</th>
<th>PR (n=31)</th>
<th>During flare (n=31)</th>
<th>During non-flare (n=27)</th>
<th>NORA (n=24)</th>
<th>P values</th>
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<td></td>
<td>47 (15)</td>
<td>49 (14)</td>
<td>55 (15)</td>
<td>58 (14/24)</td>
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<td>Sex (% F)</td>
<td>88% (29/33)</td>
<td>55% (17/31)</td>
<td>100% (24/24)</td>
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<td></td>
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<td>Anti-CCP positive (%)</td>
<td>100% (33/33)</td>
<td>68% (21/31)</td>
<td>100% (24/24)</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RF positive (%)</td>
<td>18% (6/33)</td>
<td>48% (15/31)</td>
<td>75% (18/24)</td>
<td>&lt;0.01†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMARD naive (%)</td>
<td>100% (33/33)</td>
<td>90% (28/31)</td>
<td>100% (24/24)</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker (% yes)</td>
<td>21% (7/33)</td>
<td>39% (12/31)</td>
<td>29% (7/24)</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker (% yes)</td>
<td>58% (19/33)</td>
<td>32% (10/31)</td>
<td>25% (6/24)</td>
<td>0.03†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol consumer (% yes)</td>
<td>42% (14/33)</td>
<td>58% (18/31)</td>
<td>67% (16/24)</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDR with RA (% yes)</td>
<td>24% (8/33)</td>
<td>13% (4/31)</td>
<td>25% (6/24)</td>
<td>0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of symptoms (months)</td>
<td>13 (6,60)</td>
<td>30 (9, 57)</td>
<td>19 (8.5, 46)</td>
<td>14 (10, 40)</td>
<td>0.542</td>
<td></td>
</tr>
<tr>
<td>EMS (mins)</td>
<td>0 (0.30)</td>
<td>90 (0,120)</td>
<td>0 (0,2.5)</td>
<td>60 (10, 120)</td>
<td>&lt;0.01†</td>
<td></td>
</tr>
<tr>
<td>Symptoms in hands§</td>
<td>61% (20/33)</td>
<td>84% (26/31)</td>
<td>100% (24/24)</td>
<td>0.01†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptoms in feet§</td>
<td>33% (11/33)</td>
<td>26% (8/31)</td>
<td>58% (14/24)</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptoms in large joints§</td>
<td>64% (21/33)</td>
<td>48% (15/31)</td>
<td>71% (17/24)</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain VAS (mm)</td>
<td>23 (4,50) n=31</td>
<td>58 (25,81)</td>
<td>39 (24, 59) n=19</td>
<td>0.03*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatigue VAS (mm)</td>
<td>38 (6.65) n=31</td>
<td>42 (22,64)</td>
<td>42 (23, 60) n=19</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Global health VAS (mm)</td>
<td>18(7, 40) n=31</td>
<td>41 (16, 55)</td>
<td>29(16, 50) n=21</td>
<td>0.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TJC 28</td>
<td>0 (0, 2)</td>
<td>2 (1, 3)</td>
<td>0 (0, 0)</td>
<td>5 (3, 9)</td>
<td>&lt;0.01††</td>
<td></td>
</tr>
<tr>
<td>TJC 53</td>
<td>1 (0, 2)</td>
<td>1 (1, 2)</td>
<td>0 (0, 1)</td>
<td>5 (3, 7)</td>
<td>&lt;0.01††</td>
<td></td>
</tr>
<tr>
<td>SJC 28</td>
<td>0 (0, 0)</td>
<td>1 (1, 2)</td>
<td>0 (0, 0)</td>
<td>2 (1, 6)</td>
<td>&lt;0.01††</td>
<td></td>
</tr>
<tr>
<td>SJC 44</td>
<td>0 (0, 0)</td>
<td>1 (1, 2)</td>
<td>0 (0, 0)</td>
<td>3 (2, 6)</td>
<td>&lt;0.01††</td>
<td></td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>1.31 (0.24, 5.24) n=28</td>
<td>9.9 (1, 26)</td>
<td>6.5 (0, 9.38) n=23</td>
<td>0.01*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAS28CRP</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>3.48 (3,18, 4.56) n=21</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>DAS28CRP</td>
<td>n/a</td>
<td>n/a</td>
<td>3.48 (3,18, 4.56) n=21</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Baseline characteristics of PR patients seen in flare, CCP+ at-risk individuals and patients with NORA. Median and IQR are presented for scale variables. P values are given for comparisons between CCP+ at risk, PR flare and patients with NORA (Kruskall-Wallis and Fisher’s exact tests). For significant results, pairwise tests were performed (Mann-Whitney U test for scale variables).

*CCP+ vs PR flare p<0.05.

†CCP+ vs NORA p<0.05.

‡PR flare vs NORA p<0.05.

§Symptoms over the past week.

US scans were mainly performed using a General Electric (GE) Logiq E9 machine employing a 15–6 MHz transducer. Copious gel was used as a standoff to avoid excessive transducer pressure. A small number of US scans were performed using a GE S7 machine. Power Doppler (PD) was assessed using a pulse repetition frequency set between 0.7 and 1.0 kHz, medium wall filter and gain adjusted until background noise was suppressed. Doppler frequency was 10 MHz.

Scoring of grey scale (GS) and PD synovitis was according to the OMERACT scoring system.15 16 Tenosynovitis was defined according to EULAR Outcome Measures in Rheumatology (OMERACT) scoring system.

US evaluation: US evaluation was performed by rheumatologists (RJW, MADA, KM, JLN) and sonographers (LH, KS) with extensive experience in MSK US who were blinded to patient group, symptoms and clinical assessment. All US examiners participated in a training session and agreed on the scanning protocol. A standardised 38-joint, 10-tendon US protocol was used at all visits (see online supplementary material 1). All available recorded images were scored by a single expert reader (MADA) who was blinded to all patient details, and this score was used in the analysis.
the OMERACT definition\(^\text{17}\) and scored as present or absent. To avoid overestimation, as scoring used for the analysis was based on central reading of images, synovitis was defined as GS ≥1 and PD ≥1.

Non-synovial EC abnormalities were frequently identified in our initial US assessments of PR flares and have previously been observed in patients with PR.\(^\text{18}\) Therefore, the following classification system for EC abnormalities was agreed by consensus (KM, MADA, RJW) after review of a randomised selection of flare images of different joint regions: periarticular inflammation: localised non-synovial soft tissue inflammation with or without PD signal outside the joint capsule and around the joint region; peritendinous oedema: oedema with or without PD signal occurring around a tendon without a tenosynovium; subcutaneous oedema: diffuse non-synovial soft tissue oedema with or without PD signal occurring outside the capsule and extending beyond the joint region. The figure 2 shows example images for each of these definitions. EC abnormalities were subsequently scored as present or absent.

**MRI evaluation**

MRI scanning was performed on the most symptomatic region during PR flare. Patients were scanned using a 3T Siemens Verio MRI scanner (Erlangen, Germany) (see online supplementary methods 1).

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**Figure 2** Ultrasound findings in flares of palindromic rheumatism. Representative images of the different types of ultrasound pathology detected at the flaring region are shown in the panels. (1) Peri-articular inflammation shown at a PIPJ in (a) LT and (b) TV. Joint effusion is also present. (2) Peri-tendinous oedema shown at (a) a PIPJ in LT and (b) a MCPJ in TV. (3) Subcutaneous oedema (indicated by the symbol “\(\)”) shown at a MCPJ and midfoot. (4) Flexor tenosynovitis shown in (a) LT and (b) TV. Subcutaneous oedema is also present. (5) Synovitis shown at (a) MCPJ and (b) wrist ICJ. ICJ, intercarpal joint; LT, longitudinal; MCPJ, metacarpophalangeal joint; PIPJ, proximal interphalangeal joint; TV, transverse.
All MRI scans were scored by an experienced reader (MADA) who was blinded to all patient and clinical details. The presence or absence of synovitis, bone marrow oedema (BME), tenosynovitis, erosions, peri-tendinous oedema and peri-articular inflammation was reported for the imaged region (ie, hand, knee, shoulder). Due to interference from coil artefacts, subcutaneous oedema was not included in this analysis. Synovitis and BME were reported according to the OMERACT RA MRI scoring system (RAMRIS). 

Tenosynovitis was defined according to the OMERACT MRI Tenosynovitis Scoring System and scored as present or absent. In the absence of an accepted definition for EC MRI abnormalities (ie, peri-articular inflammation and tendo-articular oedema), these lesions were identified and scored using T1 fat-sat gadolinium enhanced sequences and reported descriptively. Periarticular inflammation was defined as EC effusion and/or postcontrast enhancement of the EC tissues on axial and coronal sequences over ≥3 consecutive slices. Peritendinous oedema was defined as peritendinous effusion and/or postcontrast enhancement outside the tendon sheath, seen on axial and coronal sequences over ≥3 consecutive slices.

Statistical analysis

We tested the hypothesis that the frequency of synovial and EC US abnormalities during the flare episode would be different in patients with PR compared with anti-CCP+ at-risk individuals and patients with NORA. Therefore, the proportion of patients with PR with US abnormalities during a clinically defined flare in the hand(s)/wrist(s) was compared with the proportion of anti-CCP+ at-risk individuals and patients with NORA with US abnormalities in the hands/wrists using χ² or Fisher’s exact test (where expected counts were ≤5 cases). We also tested the hypothesis that the proportion of patients with PR with synovial and EC non-synovial US abnormalities would increase between non-flare and flare disease phases. Therefore, the proportion of patients with PR with US abnormalities in the clinically flaring region was compared in flare and non-flare disease phases using McNemar’s exact test. Kruskall-Wallis and Fisher’s exact tests were used to compare patient characteristics between groups. For significant results, pairwise tests were performed using Mann-Whitney U test for scale variables.

RESULTS

Patients

Seventy-nine patients with PR met the study inclusion criteria and were recruited between May 2015 and May 2017. The cohort was followed prospectively according to patient-reported flares (figure 1). Fifteen out of 79 patients were flaring at the initial visit and 11 of these patients re-attended when they were not flaring. Sixteen out of 64 patients who were not flaring at their initial visit subsequently attended during a flare. In total, the 31/79 patients who had an US assessment during a flare episode were included in the analysis. Seven out of 31 (23%) patients developed persistent IA during the subsequent follow-up period; all these patients met the ACR/
EULAR 2010 classification criteria for RA. Of the complete cohort, 13/79 (16%) patients developed persistent IA; 47/79 patients were anti-CCP positive and of these 35 (74%) did not develop persistent IA during the follow-up period.

Thirty-three CCP+ at-risk individuals and 24 patients with NORA were included as control groups and were matched for age with patients with PR. Demographic and clinical characteristics are shown in table 1.

**Ultrasound findings in patients with PR**

US characteristics of patients with PR during flare (31 scans recorded) compared with US findings of the same region when the patient was not flaring (27 scans recorded) are shown in table 2 and online supplementary figure 1. US abnormalities were infrequently identified during non-flare and none had GS ≥2 and PD ≥1. Similarly, EC inflammation (ECI) was identified in only 4/27 (15%) of non-flare US scans. GS synovitis, tenosynovitis, periaricular inflammation and subcutaneous oedema were all less prevalent in non-flare scans compared with flare scans (p<0.05). In contrast, there was no difference in the frequency of PD signal and peritendinous oedema between flare and non-flare US scans (p=0.289 and p=0.625, respectively). No erosions were identified on flare or non-flare scans.

Of the 27 patients who had non-flare scans, 11 were performed after the flare scan was captured. There was improvement in US abnormalities in all but one of these patients.

**Ultrasound findings during PR flare**

In the 31 patients in whom US features were captured, the flaring region was the hands/wrists in 26 patients, the foot/ankle in one patient, the knee in three patients and the shoulder in one patient.

A high frequency of ECI was seen (figures 2 and 3) during flare: in 19/31 (61%) patients, one or more of periarticular inflammation, peritendinous oedema and/or subcutaneous oedema was identified. Interestingly in 12 patients, ECI was seen in the absence of GS (GS ≥2) or PD synovitis. GS alone (GS ≥2) was present in 12/31 (39%) patients. Tenosynovitis and peritendinous oedema were detected in 7/31 (23%) and 3/31 (10%) of patients, respectively. PD signal was present in only 7/31 (23%) of patients. No differences in either synovial inflammation or ECI were found between in patients with PR according to anti-CCP status.

Five patients attended with more than one flare (online supplementary data). Overall, US inflammation did not appear to increase with sequential flares (online supplementary figure 4).

No patients had tophi, double contour sign, hyperechoic aggregates or any other US features suggestive of crystal arthritis.

**Comparison of PR with anti-CCP+ at-risk individuals and patients with NORA**

US abnormalities identified in patients with PR during flares involving the hands/wrists were compared with US abnormalities in the hands/wrists of anti-CCP+ at-risk individuals and patients with NORA (table 3 and online supplementary figures 2 and 3). PD signal was observed less frequently in patients with PR compared with patients with NORA (p<0.05). In contrast, ECI was identified in the majority (65%) of patients with PR but only 7/24 (29%) of patients with NORA (p=0.023). No patients with PR had synovitis on US of the flaring region without ECI also being present. Of note, the identification of ECI without synovitis at the flare site appeared to be specific for PR; 42% of patients with PR had this US phenotype but this occurred in only one patient with NORA (p=0.003) and two CCP+ at-risk individuals (p=0.0012).

**Comparison of MRI and ultrasound**

Eleven flares were captured by both MRI and US (in one patient two flares were captured by both imaging modalities). MRI appeared more sensitive than US for synovitis, tenosynovitis, peritendinous oedema and periaricular inflammation (online supplementary figure 5). Synovitis (taken as cut-off of RAMRIS >1) was identified in 7/11 (64%) flares, whereas BME was reported in only one flare. Tenosynovitis and peritendinous oedema were identified by MRI in 5/11 (45%) and 6/11 (55%) flares, respectively. Periaricular inflammation was identified by MRI in 6/11 (55%) flares. No MRI erosions were identified.

**Ultrasound features at progression to RA**

The US phenotype of seven patients with PR who developed RA during the follow-up period was similar to the patients with NORA who did not have a history of PR. US synovitis and/or tenosynovitis of the hands/wrists was present in 5/7 (71%) of patients at progression to RA. In contrast, ECI was only present in 2/7 (29%) of patients (online supplementary figure 3).

**DISCUSSION**

In the early stages of IA, identifying patients with persistent disease from those with a better prognosis can be difficult. While the presence of anti-CCP antibodies in patients with early synovitis is generally associated with poor prognosis, many...
patients with anti-CCP positive PR do not develop persistent IA.6 7 Indeed 74% of anti-CCP positive patients with PR in our cohort did not develop IA during follow-up. In clinical practice, these patients may be inappropriately treated (eg, with methotrexate) as they often meet ACR/EULAR criteria for RA.14

To our knowledge, this is the first study to demonstrate high-resolution imaging, especially US, may be used to distinguish PR from NORA at a single assessment. Isolated ECI appears to be specific for PR whereas synovitis and tenosynovitis is more frequently identified in NORA. This is important as PR carries a more favourable prognosis but often takes several assessments to diagnose clinically; we have identified a specific imaging phenotype which may facilitate earlier identification and therefore more appropriate management of these patients.

This is also the first study to use imaging to characterise ECI, synovitis and tenosynovitis in both flare and non-flare phases of PR. The high prevalence of peri-articular soft tissue inflammation and subcutaneous oedema on US during flare may explain clinical peri-arthritis in these patients. Tenosynovitis and peri-tendinous oedema, both identified on US and MRI, could also cause this. The specific US phenotype of ECI without synovitis suggests first that intra-articular inflammation may often not be responsible for the clinical features of PR flare and second that ECI may be mechanistically important rather than a secondary effect of an adjacent synovitis. This highlights the value of US in identifying the site of inflammation, particularly as most studies (including ours) have identified patients with PR clinically as having recurrent ‘joint’ swelling. Extra-articular abnormalities have been previously described in patients with PR who do not have US synovitis.18 However, contrary to our findings, a relatively high frequency of GS and PD synovitis has previously been reported.9 10 18 23 Differences in patient characteristics may be one explanation. Our patients were comparatively early in their disease course (median 2.5 years) and all but three were DMARD-naive at the time of imaging. In contrast, patients in the other studies had experienced several years of disease.9 10 23 and 45%–61% were on DMARD therapy at the time of assessment.9 21 It is possible that the phenotype we have described reflects de novo PR and this may change towards an RA phenotype with more prolonged disease duration and/or under the influence of immunomodulation.

The mechanism of ECI in PR is unclear and requires investigation; clinically, there are similarities with autoinflammatory disease,6 24 and the role of autoinflammation in PR is an important area for future research.

The low frequency of US abnormalities when patients were not flaring supports the notion that flares of PR are truly relapsing and are important to distinguish from early IA. This is consistent with previous published data.23 The use of MRI is a strength of this study. In the majority of cases, MRI findings concurred with US findings as well as identifying additional abnormalities. Also, 2/11 patients had ECI on MRI in the absence of synovitis. The absence of erosions on MRI and identification of BME in only one patient confirms a distinct imaging pattern to early RA. Previous reports of MRI findings in PR flare are limited to a case report23 and a study of four patients in whom BME was identified in all cases and synovitis in three.20 Both studies describe a phenotype more akin to RA than we have observed.

Due to the transient and unpredictable nature of flares, it was not possible for the same US examiner to perform all scans. However, all sonographers were trained in the same centre and followed the same US protocol. In addition, all US and MRI scans were scored by an expert reader who was blinded to all clinical details. We acknowledge that the reliability of the proposed classification system for ECI should be assessed in future work; our findings should also be validated in other PR cohorts.

In conclusion, we identified a specific imaging phenotype in PR, which may be used to distinguish true PR from persistent IA in patients presenting with early arthritis. These findings may refine diagnosis and improve the management of this important condition.

Acknowledgements The authors would like to acknowledge Laura Horton and Kate Smith for performing ultrasound scans, Rob Evans and Brian Chaka for radiography support, and Ian Weatherill and Philip Luxford for administrative support.

Contributors KM designed the study, collected and analysed the data and wrote the manuscript. MADA designed the study, scored the ultrasound and MRI images and helped write the manuscript. RIW helped design the ultrasound protocol and performed some of the ultrasound scans. JLN was one of the study clinicians and performed some of the ultrasound scans. WM helped with data analysis. AJG was responsible for MRI protocols. PE designed and led the study. All co-authors read and revised the manuscript.

Funding The study was supported by the National Institute for Health Research (NIHR) Leeds Clinical Research Facility. Additional support was provided by Arthritis Research UK (ARUK grant number 7174).

Competing interests None declared.

Patient consent Not required.

Ethics approval NHS Health Research Authority National Research Ethics Service Committee Yorkshire and the Humber, Leeds West.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available from this study.

REFERENCES
Inflammatory arthritis


CLINICAL SCIENCE

Treat to target (drug-free) inactive disease in DMARD-naive juvenile idiopathic arthritis: 24-month clinical outcomes of a three-armed randomised trial

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INTRODUCTION

Juvenile idiopathic arthritis (JIA) is the most common autoimmune disease in children.1 In recent years, earlier introduction of conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) and the development of biological (b) DMARDs have improved the outcome for patients with JIA.2–4 but ongoing inflammation in JIA may still cause functional disability and joint damage.5 Early inactive disease may be the optimal therapeutic target.5–10 Studies in JIA support the window of opportunity hypothesis when the disease is optimally responding to treatment and chronicity may be prevented.10–14

Once inactive disease is achieved, discontinuation of treatment might be possible.13–19 Comparative drug studies have shown that initial treatment with csDMARD results in less rapid response than initial treatment including glucocorticoids or a bDMARD,10 20 but the latter two have not been directly compared. If the initial treatment is not effective, subsequent treatment adjustments should still aim at achieving the treatment target. In adults with rheumatoid arthritis, such targeted therapy has been proven effective in long-term prevention of damage progression and maintaining functional ability, even irrespective of initial treatment success.21–23 In JIA, continuous treatment-to-target therapy in a tight control setting, with treatment adjustments based on frequent evaluations of disease activity, has not yet been studied. Recent recommendations agree that treatment-to-target should be implemented in daily practice.24

The aim of the BeSt (acronym for Dutch ‘treatment strategies’) for Kids study was to investigate which of three treatment-to-target strategies, using treatment-to-target aimed at inactive disease, is most effective and safe. Here, we report the results of one of the first treat-to-target study in patients with recent-onset JIA.

METHODS

Patients

Patients, 2–16 years, with new-onset (oligoarticular, juvenile psoriatic arthritis or rheumatoid factor (RF)-negative polyarticular) JIA, without previous DMARD therapy and symptom duration...
<18 months were eligible. RF-positive patients with JIA were excluded because monotherapy might be inappropriate for this severe category. Also the number was too low to stratify. Uveitis at enrolment was an exclusion criterion. Rest of the exclusion criteria are summarised in online supplementary file 1.

**Study design and medical intervention**

The BeSt for Kids study is an investigator-initiated multicentre randomised study with 2 years of follow-up. To minimise the risk of bias of the open design, all outcome measurements were assessed by trained research nurses, physiotherapists and physicians who remained unaware of the allocated treatment strategy during entire study period (single-blind design). The trial was registered in the Dutch Trial Register, number 1574.

Patients were enrolled starting October 2009 to April 2014 by diagnosing paediatric rheumatologists. Randomisation was by variable block, stratified per centre and per oligoarticular or polyarticular disease, into three strategy arms: (1) initial treatment with csDMARD monotherapy (methotrexate (MTX) or sulfasalazine (SSZ) if preferred by treating physician); (2) initial treatment with MTX and 6 weeks of tapered prednisolone (‘bridging therapy’); and (3) initial treatment with MTX and etanercept. For all arms, the treatment protocol described a number of subsequent treatment steps in case patients failed to fulfil treatment targets (figure 1 and online supplementary file 2).

In case of side effects, the responsible drug was reduced to the lowest tolerated dose, but if it was not tolerated at all or contraindicated, patients on monotherapy proceeded to the next step in the allocated treatment group, and patients on combination therapy continued with the other drug of the combination. Additional treatment with non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular injections with glucocorticoids were permitted without a maximum and registered per strategy. All patients on MTX received folic acid 5 mg/week. The use of DMARD or oral glucocorticoids was only permitted as dictated by the protocol. All protocol violations were recorded.

After 3 months of treatment, the initial target was an adjusted ACRPed50%, calculated as described previously, and scored by a research nurse or physiotherapist who remained blinded to the allocated treatment group.

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**Figure 1** Flow diagram of the three treatment strategies compared in the BeSt for Kids study. Revised diagnosis were localised scleroderma with arthritis (arm 1) and polyarteritis nodosa (arm 3). See ‘Patients and methods’ section for description of treatment groups. n=21 patients had ≥18 months of complaints’ duration at first consultation, n=7 had comorbidities considered (relative) contraindication for the DMARD therapy by either the paediatric rheumatologist or reason for (parents of) patients to refuse participation. These were morphea (one patient), morbid obesity (n=1), hashimoto thyreoiditis (n=1), type 1 diabetes (n=1) and previous uveitis (n=3). DMARD, disease-modifying anti-rheumatic drug; ETN, etanercept; MTX, methotrexate; po, orally; sc, subcutaneous; SSZ, sulfasalazine.
during the entire study period. Treatment was continued if this target was met, escalated according to protocol if not.

After 6 months of treatment, the treatment target was inactive disease, defined according to Wallace 2004 criteria\textsuperscript{26} (online supplementary file 3) modified by Physicians Global Assessment<10 mm indicating no disease activity.

In all three arms, in case of inactive disease for at least three (oligoarticular disease) or six (polyarticular disease) consecutive months, DMARD(s) were tapered and stopped. In case of combination therapy, first etanercept was tapered to once per 2 weeks, only once, directly followed by 50\% dose reduction, then stopped. On the same requirements, MTX or SSZ dose was reduced with 25\% per week to zero. Following tapering strategies (online supplementary file 4), in case of a disease flare, defined by recurrence of arthritis (online supplementary file 5), the last discontinued drug and/or the last effective dose was reintroduced. By protocol, prednisolone could not be restarted, and etanercept could be restarted but not discontinued for a second time.

Outcomes and analyses
Primary outcome measures are time-to-inactive disease and time-to-flare after tapering and stopping all DMARD therapy. Time-to-flare was defined as the time between first moment of drug-free inactive disease (DFID) and the first arthritis judged as flare by the treating physician (online supplementary file 5). Secondary outcome measures were adjusted ACRPedi30/50/70/90 scores, adverse events and functional ability. The Juvenile Disease Activity Score (JADAS)–10 score, JADAS-minimal disease activity (JADAS-MDA) and JADAS-inactive disease (JADAS-ID) were calculated as described previously (online supplementary file 6).\textsuperscript{27,28} Functional ability was determined by the Childhood Health Assessment Questionnaire (CHAQ).\textsuperscript{29} Side effects were registered through open-end interviewing at each study visit combined with incidental reports in the intervals, and routine safety laboratory tests at each study visit (complete blood count, serum liver transaminases and creatinine). Severe adverse events (SAEs) were defined as any adverse reaction resulting in any of the following outcomes: a life-threatening condition or death, significant or permanent disability, malignancy and (prolonged) hospitalisation.

Statistical methods
Multiple imputation using package mice in software package R (V.3.4.0, http://r-project.org) was used to deal with missing values with n=10 imputed data sets. Imputation variables were gender, age at inclusion, duration of symptoms, ANA positivity, diagnosis, number of affected joints and all outcome variables. In case of drug-free clinically inactive disease, often intentionally no blood was drawn causing non-random missing erythrocyte sedimentation rate (ESR), and here ‘0’ was imputed for analysis of inactive disease.

Where measured repeatedly, measurements were treated as separate variables (wide format). Student’s t-test was used to compare continuous normally distributed variables between groups. Non-parametric Kruskal-Wallis tests were used otherwise. For dichotomous variables, Pearson’s X\(^2\) test was used. A two-tailed probability value of <0.05 was considered statistically significant. P values were not adjusted for multiple statistical tests. Time-to-inactive disease and time-to-flare was evaluated using log-rank test. The comparison of the groups over time in reaching aACRPedi 30/50/70/90, JADAS-10 and CHAQ-score was analysed by generalised estimation equation models for continuous outcomes with time-by-strategy interaction as variable of interest. The third arm was treated as reference arm since we hypothesised that arm 3 would be superior compared with arm 1 or arm 2, based on previous results.\textsuperscript{12,21}

RESULTS
Patient characteristics
Baseline demographics and disease characteristics are summarised in table 1. Figure 1 summarises the study in a flow diagram. Ninety-four patients were randomised to one of three treatment groups: 32 patients were assigned to initial monotherapy (arm 1), 32 patients to initial combination of MTX with 6 weeks prednisolone-bridging therapy (arm 2) and 30 patients to arm 3, initial combination of MTX/etanercept. Median symptom duration was 7.5 (IQR 5–12.5) months and median duration between diagnosis and inclusion was 6 (IQR 3–14) weeks. During follow-up two patients left the study because of revised diagnosis, one patient with localised scleroderma (in arm 1) and one (arm 3) with polyarteritis nodosa.\textsuperscript{32} They were left out of further analyses. Two patients who were lost-to-follow-up, one in arm one after inclusion and one in arm 2 after 15 months, were included in the intention-to-treat (ITT) analysis.

Median time-to-inactive disease was 9.0 (5.3–15.0) months in arm 1, 9.0 (6.0–12.8) months in arm 2 and 9.0 (6.0–12.0) months in arm 3 (overall 9.0 (6.0–12.0) months (log-rank test p=0.3)). After 1 year 54\% of patients in arm 1, 47\% in arm 2 and 62\% in arm 3 were in inactive disease (figure 2).

During 24 months 59\% (19 (3 oligo)/31 (61\%)) of patients in arm 1, 16 (1 oligo)/32 (50\%) in arm 2 and 19 (1 oligo)/29 (63\%) in arm 3) had tapered and stopped all DMARDs (DFID), after median 15.0 (IQR 12.0–18.0) months (arm 1), 19.5 (12.0–24.0) months (arm 2) and 18.0 (12.0–21.0) months (arm 3) of therapy. However, 26\% (6 (one oligo) patients in arm 1, 3 in arm 2 and 5 in arm 3) subsequently had to restart treatment before the end of the study, in arm 1 median after 4.5 (3.0–9.0) months, in arm 2 after 3.0 (3.0–3.0) months and in arm 3 after 3.0 (3.0–7.5) months (overall 3.0 (3.0–6.8) months (p=0.7)). Three months later, inactive disease was regained by 10/14 (71\%) (six in arm 1, one in arm 2 and three in arm 3). After 24 months, 71\% (arm 1), 70\% (arm 2) and 72\% (arm 3) of patients had inactive disease and 45\% (arm 1), 31\% (arm 2) and 41\% (arm 3) had DFID.
### Table 1 Baseline demographic and disease characteristics

<table>
<thead>
<tr>
<th>JIA category</th>
<th>Arm 1 (Sequential monotherapy)</th>
<th>Arm 2 (MTX + 6 weeks prednisolone)</th>
<th>Arm 3 (MTX+ etanercept)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), median (IQR)</td>
<td>9.0 (4.7–12.9)</td>
<td>10.2 (6.6–13.9)</td>
<td>8.6 (4.2–12.4)</td>
</tr>
<tr>
<td>Symptom duration (months), median (IQR)</td>
<td>8.1 (5.5–11.9)</td>
<td>5.9 (4.6–13.3)</td>
<td>8.6 (5.2–13.4)</td>
</tr>
<tr>
<td>ANA pos, n (%)</td>
<td>14 (45.2)</td>
<td>11 (34.4)</td>
<td>9 (31.0)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>23 (74.2)</td>
<td>19 (59.4)</td>
<td>19 (65.5)</td>
</tr>
<tr>
<td>JIA category</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligo, n (%)</td>
<td>5 (16.1)</td>
<td>3 (9.4)</td>
<td>3 (10.3)</td>
</tr>
<tr>
<td>Oligoarticular &lt;6 months</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Oligoarticular ≥6 months</td>
<td>4 (12.9)</td>
<td>2 (6.3)</td>
<td>0</td>
</tr>
<tr>
<td>Poly*, n (%)</td>
<td>24 (77.4)</td>
<td>25 (78.1)</td>
<td>24 (82.8)</td>
</tr>
<tr>
<td>Psoriatic, n (%)</td>
<td>2 (6.4)</td>
<td>4 (12.5)</td>
<td>2 (6.9)</td>
</tr>
<tr>
<td>No. active joints, median (IQR)</td>
<td>7.0 (5.0–13.0)</td>
<td>7.5 (6.0–11.8)</td>
<td>8.0 (5.5–13.0)</td>
</tr>
<tr>
<td>No. limited joints, median (IQR)</td>
<td>2.0 (0.0–3.0)</td>
<td>2.0 (1.0–3.8)</td>
<td>3.0 (1.5–5.0)</td>
</tr>
<tr>
<td>ESR, median (IQR)</td>
<td>6.0 (2.0–11.0)</td>
<td>6.0 (2.0–23.5)</td>
<td>9.0 (3.5–26.0)</td>
</tr>
<tr>
<td>JADAS-10, mean (SD)</td>
<td>16.5±4.2</td>
<td>18.8±4.4</td>
<td>18.8±5.4</td>
</tr>
</tbody>
</table>

*Missing follow-up data occurred in 4% for active joint count, in 4% for limited joint count and physician VAS, 7% for parent/patient VAS, 7% for CHAQ score and 16% for ESR.

**ANA**, antinuclear antibodies; CHAQ, Child Health Assessment Questionnaire; ESR, erythrocyte sedimentation rate; JIA, juvenile idiopathic arthritis; JADAS-10, juvenile arthritis disease activity score in up to maximum 10 joints; MTX, methotrexate; VAS, visual analogue scale; oligo, oligoarticular JIA; poly, polyarticular RF-negative JIA; pos, positive; psoriatic, JIA with psoriasis.

**Adjusted ACRPedi30/50/70/90, JADAS-10 and CHAQ score**

Adjusted ACRPedi30/50/70/90 scores were reached in similar high percentages over time in all three arms (figure 2 and online supplementary table S1) JADAS-10 scores after 24 months improved comparably (figure 2), JADAS MDA and ID-criteria are given in online supplementary table S3. Overall, flares were characterised by a JADAS-10 of 9.7 (8.1–11.3), which improved 3 months after restart of treatment to JADAS-10 of 3.9 (1.8–6.0). In all three arms, CHAQ values improved from mean 1.0 (SD 0.6) to 0.5 (0.6).

**Medication changes and protocol violations**

Figure 3 shows all medication actually used in the study per arm (ie, including protocol violations). In arm 1, treating physicians prescribed SSZ (n=15) almost as often as MTX (n=17). By t=3 months 10/15 patients had switched from SSZ to MTX, two due to side effects, 8 because of insufficient response. After 3 months, patients who remained on SSZ had similar ACRPedi50% scores as patients who started on MTX (data not shown). During 24 months in arm 1, nine patients in arm 1 reached inactive disease while still on monotherapy, four on initial SSZ (one flared later) and five on initial MTX (three flared later). In arm 2 (17/32), 53% of patients who started on MTX plus 6 weeks of prednisolone switched to MTX with ETN before end of year 1. Overall 17 patients (55%) in arm 1 and 23 patients (72%) in arm 2 progressed to a biological, at various time points, according to protocol. Treatment was left to treating physician due to end of protocol in four patients in arm 1, versus 15 and 18 in arms 2 and 3. In arm 3 significantly less treatment adjustments were needed to achieve first inactive disease: 0.6 (0.3–1.0) treatment steps compared with 1.4 (0.9–1.8) steps in arm 1 and 1.5 (1.0–1.9) steps in arm 2 (p=0.011). Across all arms, 10 (two in arm 1, two in arm 2, six in arm 3) patients failed to achieve inactive disease on ETN and switched to adalimumab (9) or infliximab (1). After 24 months, 5 of these 10 patients gained inactive disease on the second anti-tumour necrosis factor.

Online supplementary table 2 summarises protocol violations including outside of protocol glucocorticoid use across the three arms. Incorrect glucocorticoid treatments were given in the first months in arm 1 (three times) and in arm 2 (four times) compared with none in arm 3. Overall, treatment was not escalated according to protocol in all three arms for refusal to start or increase the dose of MTX or etanercept (Online supplementary table 2).

**ADVERSE EVENTS**

Adverse events (AEs) were similar across the arms. AEs are summarised in table 2. AEs were mild in general and involved mostly gastrointestinal complaints, upper respiratory tract and other infections and general malaise. One patient in arm 1 while on MTX developed de novo uveitis anterior after 6 months of treatment. No patients had permanent sequelae.

**Discussion**

This is one of the first treatment-to-target studies, tightly controlled and single-blinded, in newly diagnosed DMARD-naïve patients with JIA, aiming at inactive disease. Efficacy and safety of three treatment strategies were compared that are frequently used and comparable with the Childhood Arthritis and Rheumatology Research Alliance American Consensus Treatment Plans. Abrogation of inflammation by treating JIA to target has recently been recommended.24 Our results show that after 24 months inactive disease was achieved by >70% of patients, irrespective of initial treatment, including tapering and stop strategies. Fifty-nine per cent achieved DFID, although early flares occurred that were successfully retreated.

After 3 months of treatment, more patients who started with MTX and etanercept (arm 3) had achieved rapid improvement as...
Figure 2  Clinical outcomes after 24 months: adjusted ACRPedi30/50/70/90, inactive disease, CHAQ and JADAS-10 score, based on generalised estimating equations (GEE) analyses on imputed data. Error bars indicate 95% CIs. Adjusted ACRPedi30/50/70/90=30/50/70/90% improvement according to adjusted American College of Rheumatology Pediatric response criteria. CHAQ, Dutch version of the Child Health Assessment Questionnaire; JADAS-10, juvenile arthritis disease activity score up to maximum of 10 joints; MTX, methotrexate.
Figure 3  Treatment of patients during 2 years of follow-up. Treatment was started and when necessary adapted to reach inactive disease. Within the first year of therapy more treatment changes occurred in arms 1 and 2 compared with arm 3. When inactive disease was reached for a consecutive period of 3 months in case of oligoarticular disease, and 6 months for polyarticular disease, all disease-modifying anti-rheumatic drugs where tapered and stopped according to protocol within approximately 2 months.
determined by aACRPedi70 scores, but time-to-inactive disease was similar across the arms. Due to treatment adjustments in case of active disease, which were needed more often in arms 1 and 2 than in arm 3, aACRPedi improvement scores were met in similar percentages of patients over time across the arms. After 24 months of treatment-to-target JADAS-10-scores were considerably reduced and functional ability as assessed by CHAQ was lowered substantially across the arms.

Our results show higher percentages of patients achieving inactive disease than in the prospective randomised double-blinded TREAT-study, which included only polyarticular patients with JIA (n=85) including 30%–40% RF positives. In the ACUTE-JIA study (n=59), 68% achieved inactive disease after 1 year in the infliximab arm. This unblinded study allowed one treatment intensification step but did not include tapering or stop strategies. In the daily practice-based ReACCh-out-cohort, polyarticular and oligoarticular JIA achieved inactive disease after 24 months in 71% and 86% mainly by additional glucocorticoid use.

The current study also aimed at systematically tapering and discontinuing treatment when inactive disease was achieved. DFID was achieved by 54/92 (59%) of all patients, although in 14 patients (six (one oligo) in arm 1, three in arm 2 and five in arm 3), flares occurred, requiring restart of treatment, resulting in overall 39% of patients still in DFID at the 2 years end point. Time-to-flare was similar across the arms. Overall flare rates (26%) were lower than 37%–60% mentioned in previous cohorts, which may also depend on our limited total follow-up period of 24 months.

Contrary to previous studies we included oligoarticular patients (n=11) because they can have substantial disease burden and adverse outcomes, but used a rapid drug-tapering scheme (tapering and stopping medication after 3 months of inactive disease compared with after 6 months in polyarticular disease) as we hypothesised that DFID could be achieved earlier in patients with less inflamed joints. We could not establish this difference significantly, possibly due to low numbers. Only one oligoarticular patient out of five who achieved DFID flared. These limited results suggest that oligoarticular JIA patients could benefit from a treatment-to-target strategy.

There are several limitations to our study. First, the sample size, which may obscure differences between groups that in a larger population might have become clear. This can be explained by rarity of the disease, delays in referral (21 patients had ≥18 months symptom duration at the first consultation), comorbidities preventing DMARD use (seven patients) and reluctance of parents to enrol their children in a clinical trial. Data on the clinical course of non-participating patients receiving ‘routine care’ are currently not available. Recent retrospective studies in polyarticular JIA showed that despite achieving inactive disease for some time most patients had active disease during follow-up. Second, this study was performed in a single-blinded setting, with the clinical assessors remaining unaware of the treatment received. Third, there was a relatively high frequency of protocol violations or intra-articular injections. (Not allowed) glucocorticoid treatments were given in the first months in arms 1 (three times) and 2 (four times) compared with none in arm 3. These findings may indicate that the clinical efficacy of treatment in arm 3 was better, and that with less effective csDMARDs, additional glucocorticoid courses are required to achieve similar results. These protocol violations suggest that physicians at least tried to follow the treatment-to-target approach. However, in a larger number of patients across the three arms the physicians did not follow protocol for various reasons, mainly reluctance to intensify therapy based on shared decision-making.
Based on the results from our study, we conclude that DFID is a feasible goal in treatment of children with JIA, as was recently recommended,24 resulting in >70% achieving inactive disease and 39% stopping all DMARDs after 24 months. In addition, we showed that tapering and discontinuation of treatment is a realistic goal. On the other hand, treatment-to-target resulted in a relatively high use of bDMARDs, >50% of patients in all arms. The AEs were nonetheless mostly mild, as previously reported.31 Long-term follow-up of the BeSt for Kids cohort, including radiology results, is initiated to investigate possible lasting positive results of treatment-to-target in JIA.

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Acknowledgements The authors thank all participating patients and parents as well as the following physiotherapists and research nurses: Ingrid Honkoop AMC, Annette Hummelman-van Dijk SKZ, Susan Moors physiotherapist SKZ, Jacqueline Bouts, LUMC, Piroска de Boer, LUMC, Veronique van de Lugt, ARC-Reade.

Contributors All authors were involved in drafting the article or revising it critically for important intellectual content. All authors approved the final version to be submitted. HM had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: DMCB, CFA, RTC, LWAS-V, MVR. Acquisition of data: PHM, DMCB, DS-M, Y-KK, JMVdB, TKW, ICJ, PBW, MVR, LWASV-S, CFA, RTC. Analysis and interpretation of data: PHM, JMVdB, SB, DMCB, LWASV-S, CFA, RTC. Critically revising the manuscript: DMCB, DS-M, Y-KK, IB, WVBdB, WPB, TKW, MAJvb, LWASV-S, JMVdB, CFA, RTC.

Funding This is an investigator-initiated study which received financial support from Pfizer, who had no role in study design, data collection, data analysis, data interpretation, writing of an abstract, or decision to submit a manuscript for submission.

Competing interests None declared.

Ethics approval Approval of the Medical Ethical Committee of the Leiden University Medical Center and local Ethical Committees was obtained prior to start at each study site.

Provenance and peer review Not commissioned; externally peer reviewed.

Data statement The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

REFERENCES


CLINICAL SCIENCE

Low incidence of vertebral fractures in early spondyloarthritis: 5-year prospective data of the DESIR cohort

Julie Sahuguet,1 Jacques Fechtenbaum,1 Anna Molto,1,2 Adrien Etcheto,1 Clementina López-Medina,2 Pascal Richette,3 Maxime Dougados,1,2,4 Christian Roux,1,2,4 Karine Briot1,2

ABSTRACT

Objectives An increased risk of vertebral fractures (VF) has been reported in spondyloarthritis (SpA). Our hypothesis is that the prevalence of VFs is lower than reported in previous studies, especially in early SpA. This study aimed at assessing the incidence of radiographical VFs over 5 years in early axial SpA.

Methods The DESIR (DEvenir des Spondylarthropathies Indifférenciées Récentes) cohort, which included patients with inflammatory back pain highly suggestive of axial SpA, is the basis of this study. All radiographs of the DESIR cohort had been assessed at a central facility, by one investigator specialised in the field of the diagnosis of VFs according to Genant’s method. We assessed the prevalence and incidence of VFs and vertebral deformities at baseline and over 5 years.

Results Five-year X-rays were available for 432 patients (mean age 34.3±8.7 years, 53% women). Diagnosis of VF was doubtful and needed adjudication for 19 patients (4.4%). 13 patients had prevalent VFs (3.0%) which were located at the thoracic spine (12 were grade 1). At 5 years, five patients had an incident VF (1.15%); seven vertebral fractures were located at the thoracic spine (n=6/7), and of grade 1 (n=6/7).

Conclusion In the DESIR cohort, a population of early SpA, we found a low prevalence and incidence of VFs in a cohort of early inflammatory back pain suggestive of early axial SpA over 5 years.

BACKGROUND

Osteoporosis is a frequent complication of inflammatory rheumatic disorders and a well-recognised feature of axial spondyloarthopathy (axial SpA). The disease is characterised by osteoporosis, osteopenification and spine ankylosis. The ankylosed spine is at risk of fractures. A case-control study of 53 108 patients with fractures using the Swedish National Hospital Discharge Register showed that the risk of fractures was higher in SpA than in rheumatoid arthritis (RA), and that the largest increase was for vertebral fractures (VF) (OR 7.1 and 2.7 for SpA and RA, respectively). However, decreased mobility might not be the single mechanism of bone fragility because low bone mineral density has also been observed in patients with early SpA. Systemic inflammation itself has a deleterious effect on bone remodelling, and this is the rationale for studying the potential positive bone effects of potent anti-inflammatory drugs.

The prevalence of VFs in patients with axial SpA is highly variable in different studies, up to 30%. These data are unexpected in a disease affecting a young population, predominantly males and without treatment with glucocorticoids. Actually, the definition of a VF varies among studies. The objective of our study was to assess the incidence of VFs in a cohort of early inflammatory back pain suggestive of early axial SpA over 5 years.

METHODS

Population The DESIR (DEvenir des Spondylarthropathies Indifférenciées Récentes) cohort is a longitudinal prospective cohort studying subjects with inflammatory back pain (IBP) of recent onset and recruited from 25 regional centres in France. A detailed description of the centres, organisation of the cohort and full detailed protocol are available online (please visit the journal online published online only).

What is already known about this subject?

► An increased risk of vertebral fractures (VFs) has been reported in spondyloarthritis (SpA).

► However, the prevalence of VFs in patients with axial SpA is highly variable in different studies.

What does this study add?

► In this cohort of early SpA, the prevalence and incidence of VF in SpA was lower than that reported in previous studies.

► These discrepancies can be explained by the differences in the characteristics of the population and the methods of VF assessment.

How might this impact on clinical practice or future developments?

► Deformities of vertebral bodies are frequent in axial SpA, particularly at the thoracic spine, and some deformities may be confounded as a fracture, leading to an overestimation of fracture.

► This should be taken into account for the diagnosis of VF.

Key messages

What is already known about this subject?

► An increased risk of vertebral fractures (VFs) has been reported in spondyloarthritis (SpA).

► However, the prevalence of VFs in patients with axial SpA is highly variable in different studies.

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► This should be taken into account for the diagnosis of VF.

The cohort included patients aged over 18 years and under 50 years with IBP as defined by Calin and/or Berlin criteria for more than 3 months and less than 3 years and symptoms suggestive of SpA according to the local rheumatologist's assessment (eg, score ≥5 on a numerical rating scale of 0–10, where 0 is not suggestive and 10 is very suggestive of SpA).11

The exclusion criteria were other spinal disease clearly defined (eg, discarthrosis), history of any biotherapy, and history or current disorders that might interfere with the validity of the informed consent and/or prevent optimal compliance of the patient with the cohort. Corticosteroid intake was permitted only in doses of less than 10 mg prednisone per day and had to be stable for at least 4 weeks before baseline. A total of 708 patients with IBP were included between October 2007 and April 2010. Patients were evaluated every 6 months during the first 2 years and then on a yearly basis for an expected total follow-up duration of 10 years. For this study, we used the baseline data of the cohort. For the assessment of the VF, we used the X-rays of baseline and 5 years.

Parameters collected
Baseline parameters were activity and severity parameters of the disease using questionnaires self-assessed by the patient: BASDAI (Bath Ankylosing Spondylitis Disease Activity Index (0–100)), BASFI (Bath Ankylosing Spondylitis Functional Index (0–100)) and medication including use of non-steroidal anti-inflammatory drugs (NSAIDs) and TNF blocker use.12-15 Risk factors for osteoporosis were assessed at baseline: age, gender, current smoking, height, weight and Body Mass Index (BMI, kg/m²) were collected. Erythrocyte sedimentation rate and C reactive protein (CRP) were assessed at baseline. The structural damage at spine was evaluated on X-rays by the modified Stokes Ankylosing Spondylitis Spinal Score (mSASSS).16 The structural damage at the sacroiliac joint was evaluated by the modified New York criteria. The SpondyloArthritis Research Consortium of Canada method was used for scoring inflammation using MRI.17

Bone mineral density (BMD) measurements
BMD was measured by dual-energy X-ray absorptiometry at baseline for all included patients in 12 centres (ie, half of the participating centres) with investigators having expertise in BMD measurements. BMD was determined at the lumbar spine (second to fourth vertebrae) and the upper part of the left femur (total femur and femoral neck). The results were given as BMD (g/cm²), Z and T scores. The International Society of Clinical Densitometry recommends using the threshold of −2 SD in Z score for the definition of low BMD in young adults. Z scores were determined according to references provided by the manufacturers. All examinations were performed according to the manufacturer’s recommendations. Devices were controlled by measuring a spine phantom at least three times a week throughout the study; all examinations were performed according to the manufacturer’s recommendations.

VF diagnosis
For the assessment of VF, anteroposterior and lateral views of the entire thoracic and lumbar spine were considered. All radiographs of the DESIR cohort (baseline and 5 years) were assessed at a central facility (by a single investigator specialised in the field of the diagnosis of VFs) from the fourth thoracic vertebra to the fourth lumbar vertebra according to Genant’s method.19-22 The semiquantitative grading scale is as follows: normal, grade 0; mild fracture, a decrease in any vertebral body height of 20% to 25% (grade 1); moderate fracture, a decrease of 25% to 40% (grade 2); and severe fracture, a decrease of 40% or more (grade 3). We used the same method (and definition of height reduction) to define a prevalent VF (present at baseline) and an incident VF. Using a temporal sequence of reading (ie, unblinded for chronological order), an incident VF was defined as a change in the score of a vertebra from grade 0 to a subsequent grade 1 or more at 5 years. In doubtful cases, adjudication by two other senior experts was performed. Careful attention was paid to discriminate vertebral deformities (VFs) of non-osteooporotic origin and VFs as aspects of vertebrae are modified by acquired or constitutional deformations. The deformations due to an infection, a tumour and a metabolic disease are more easily moved away. Among the vertebral height decreases, it is necessary to eliminate those who are not of osteoporotic origin: bad quality of the acquisition with excessive obliquity of the incidental beam responsible for an overlap endplate, a disease of Scheuermann and Schmorl nodes, degenerative deformations of discarthrosis and the variants of normality as short vertebral height. To diagnose a VF from a VD, we use the classification of Genant taking care of decreases superior to 20% but not due to fracture and the fractured decreases not reaching the 20% threshold.21 We also use the presence of a cortical defect, a depression of a vertebral endplate, an angulation of the endplate and the comparison with vertebrae of the over and under levels. Deformities of similar appearances or contiguous vertebrae and presence of degenerative changes on adjacent intervertebral discs were considered for the deformity origin.

Statistical analysis
Data are expressed as mean (±SD). Analysis on VFs was based on patients having a baseline and a 5-year X-ray follow-up. We assessed the incidence of VFs and VDs and the prevalence of VFs (at least one grade 1) and VDs at baseline and over 5 years. The database used in our study was locked on 30 June 2015 (intended follow-up of the cohort: 10 years). All analyses were performed using SAS software, V9.1.

RESULTS
Characteristics of the population
Seven hundred and eight (n=708) patients with IBP highly suggestive of axial SpA were included in the DESIR cohort. Six hundred and ninety-four patients had spinal X-rays at baseline and 432 patients had also X-rays at the 5-year visit. This population of 432 patients (mean age was 34.3 (±8.7) years, 53% women) was the basis of our study to assess the incidence of VFs. Their baseline characteristics are described in table 1; they were not different from the DESIR population. For the assessment of the prevalence of VFs and deformities, we used the 262 patients who had spinal X-rays at baseline. Their characteristics are similar to the DESIR population (table 1). A total of 197 patients (45.60%) received a TNF blocker therapy during the 5-year follow-up. BMD measurements were available for 223 patients at baseline and 14.3% had a low BMD defined by a Z-score ≤−2 at least one site.

Prevalence of VFs and deformities
At baseline, 21 patients had prevalent VFs (3.0%); all of them were located at the thoracic spine; 14 were grade 1 and six grade 2 and one grade 3. VFs were not associated with larger occulto-wall distance. Sixty-six patients (9.5%) had a VD which was not a VF. In patients with a higher confidence for the diagnosis
of SpA (≥8) (n=328), the proportion of patients with at least one prevalent VF was 1.8% (n=6) and with at least one VD was 7.9% (n=26). Most frequent causes of VDs were a disease of Scheuermann and Schmorl nodes (n=24), short vertebral height (SVH) (N=21), degenerative deformations of discarthrosis (n=9) and kyphosis/scoliosis (n=4), structural changes related to SpA (squaring, discitis) (n=6), error of parallax (n=2) and presence of a meganucleus (n=1) (online supplementary figures 3–5).

Prevalence of VFs was not different in patients with and without structural damage in sacroiliac joints. Among the 21 prevalent VFs, 4 VFs (19.0%) were found in patients with r-axSpA and 17 in the patients nr-axSpA (p=0.431). Mean values of mSASSS were 0.357 (0.59) in patients with prevalent VF and 0.500 (1.86) in patients without (p=0.04). However, there is the question of clinical relevance of a difference of 0.15 points over a score of 0 to 72. Among 21 prevalent VFs, 20 were observed in patients with NSAIDs and 1 without NSAIDs.

Incidence of VFs and VDs
Diagnosis of VF was doubtful and needed adjudication for 19 patients (4.4%). At 5 years, five patients had an incident VF (1.15%), in a total of seven vertebrae. They were mostly located at the thoracic spine (n=6/7). Most of them were grade 1 (n=6/7) and one VF was a grade 2 (figure 1).

The incidence of vertebral deformities over 5 years was 2.30% and a total of 14 VDs (figure 2). All of them were located at the thoracic spine and were explained by either degenerative changes (discarthrosis) (N=5), increase in kyphosis (N=4) and structural changes related to SpA (squaring, discitis) (N=5).

In patients with a higher confidence for the diagnosis of SpA (≥8) (n=328), the proportion of patients with at least one incident VF was 1.4% (n=3) and at least one incident VD was 3.7% (n=8).

Patients with incident VFs were not different from patients with incident deformities for age (34.9±5.6 and 32.9±8.1 years) and BMI (21.6±2.3 and 22.3±3.7 kg/m², respectively). In contrast, a low BMD (Z score ≤−2) was observed in 50% and 16.7% of patients with incident VFs and VDs, respectively. Incidence of VFs was not different in patients with and without structural damage in sacroiliac joints. Among the five incident VFs, two were observed in patients with r-axSpA and three in patients with nr-axSpA (p=0.232). The mean values of mSASSS

### Table 1

<table>
<thead>
<tr>
<th>Characteristics of the DESIR (DEvenir des Spondylarthropathies Indifférenciées Récentes) population and of analysis population</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total population N=708</strong></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Age (years) (mean±SD)</td>
</tr>
<tr>
<td>Gender (female, %)</td>
</tr>
<tr>
<td>Menopausal status (n, %)</td>
</tr>
<tr>
<td>BMI (mean±SD)</td>
</tr>
<tr>
<td>CRP (mean±SD)</td>
</tr>
<tr>
<td>BASDAI (mean±SD)</td>
</tr>
<tr>
<td>BASFI (mean±SD)</td>
</tr>
<tr>
<td>Presence of IBD</td>
</tr>
<tr>
<td>Disease duration (years)</td>
</tr>
<tr>
<td>Past use of corticosteroids (n, %)</td>
</tr>
<tr>
<td>Score NSAIDs (mean±SD)</td>
</tr>
<tr>
<td>Use of at least one anti-TNF treatment during the 5 years (n, %)</td>
</tr>
<tr>
<td>Lumbar spine BMD l (mean±SD)</td>
</tr>
<tr>
<td>Femoral neck BMD (mean±SD)</td>
</tr>
<tr>
<td>Presence of Z score ≤−2 at least one site (n, %)</td>
</tr>
<tr>
<td>mSASSS baseline (mean±SD)</td>
</tr>
<tr>
<td>S1 SPARCC score baseline (mean±SD)</td>
</tr>
<tr>
<td>HLA-B27 antigen (n, %)</td>
</tr>
<tr>
<td>Current smoking (n, %)</td>
</tr>
</tbody>
</table>

BSADAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; BMD, bone mineral density; BMI, Body Mass Index; CRP, C-reactive protein; IBD, inflammatory bowel disease; mSASSS, modified Stokes Ankylosing Spondylitis Spinal Score; NSAID, non-steroidal anti-inflammatory drug; SIJ, sacroiliac joint; SPARCC, SpondyloArthritis Research Consortium of Canada; TNF, tumour necrosis factor.
were similar between patients with and without incident VFs (0.500 (1.0) vs 0.496 (1.48); p=0.806). None of the risk factors attributable to SpA (age, BASDAI, BASFI, C-reactive protein, mSASSS) was associated with the presence of incident VFs. The 5 incident VFs and the 14 incident VDs were observed in patients with NSAIDs. Among five incident VFs, three occurred in patients receiving TNF blocker and two without (no statistical difference).

DISCUSSION

This study conducted in a large cohort of young adults with early IBP suggestive of SpA followed in tertiary centres showed a very low incidence of VFs (1.15%). Most of these VFs were mild (grade 1), located at the thoracic spine and occurred in patients without any prevalent VFs.

Both the prevalence and the incidence of VFs reported in our study are lower than that reported in previous studies. Prevalence of VFs ranges from 4% to 42%.22–28 Data from large databases assessed the relationship between SpA and clinical VFs.29 30 A nested case–control study performed in the large General Practice Research Database showed that patients with SpA have a threefold increased risk of clinical VF (OR 3.26 (1.51–7.02)).29 In a large database in Catalonia, Spain, accounting for 80% of the population (6474 patients with axial SpA followed for a median time of 5 years), 0.86% and 3.4% sustained a clinical vertebral and a non-vertebral fracture, respectively. This represents a twofold increased risk of clinical VFs, as compared with controls.30 In this study, the association between SpA duration and clinical spine fractures was strongest in the first year (OR 8.03 (2.13–30.3)) (short-term SpA ≤1 year since diagnosis), significant for midterm duration (OR 7.52 (1.46–38.8)) and not significant for long-term duration (OR 3.01 (0.87–10.4)). For these two studies, the population was recruited from database with retrospective analysis of data, precluding accurate diagnosis of a flare of the disease or an incident VF as a determinant of pain.

The prevalence of VFs is higher in longstanding SpA disease. In 176 patients (79% men, aged 48.6 ± 13.1 years), with a mean disease duration of 22 years, the prevalence of VFs using a semiautomated software was 32.4%, 82% of the fractures were at the thoracic spine and 65% of them were mild. In a large prospective cohort of 292 patients (mean age of 42.8 years, 70% men) with ankylosing spondylitis, the mean time since the diagnosis of 6 years and a mean duration of symptoms of 16 years), 13 (6%) developed new VFs over 2 years.24 Most fractures were mild and located at the midthoracic and thoracolumbar regions of the spine.24 In this study, VFs were frequently observed in older patients, with advanced disease, low hip BMD and less healthy life. In patients with early SpA (7 months of disease duration but 5.7 years of symptom duration), 15% of the 113 patients (66% men, age 37.3 ± 9.0 years) had a vertebral fracture; most of them were located at the mid-thoracic spine, half of the fractures were moderate and none were severe.23 These VFs were associated with low BMD of the lumbar spine and with axial psoriatic arthritis.

These discrepancies might be explained by the characteristics of our population: 53% of them were women with a symptom duration of 12 months on average and 43% of them used biologics during the 5 years of follow-up. There is no evidence that these treatments have an antifracture efficacy, but it has been reported in different studies and in patients with different disease duration that these treatments increase BMD, a surrogate marker of bone strength. In our study, prevalent and incident VFs were observed in patients receiving NSAIDs and TNF blockers, even if it is impossible to definitively conclude because of the low number of incidental events. Unlike other studies,23 24 we did not identify any risk factors of VF related to SpA disease but the small number of events, making impossible any definitive interpretation. These discrepancies can also be explained by the different methods used for vertebral fracture diagnosis (semiautomated, semiquantitative, qualitative, etc). VFs are often defined in studies as a reduction in vertebral height relative to the other vertebras, but this definition does not consider the deformities of vertebral bodies. Hence, some deformities associated with the disease may be confounded as a fracture, leading to an overestimation of fracture.31

Deformities of vertebral bodies are frequent in axial SpA, particularly at the thoracic spine, for various reasons: erosions of the anterior corners, squaring, wedging secondary to discitis and so on. In our study, we showed that the main causes of vertebral deformities were SVH and Scheuermann disease. These deformities are captured by semiautomated methods of morphometry, which use automatically positioning of points on vertebral contours; with such methods, ‘fractures’ are defined as any reduction of the anterior or middle height of the vertebral body higher than 20% as compared with the posterior height, or adjacent vertebral body heights. These methods are very sensitive but need expert adjudication; otherwise, they increase the risk of false positives.32 Moreover, the thoracic and lumbar vertebrae can be normally wedged and biconcave, respectively. SVHs are frequent at the thoracic spine and are not related to osteoporosis or fracture risk.31 Knowing that, we paid attention to direct and indirect signs of endplate fracture mainly at the middle part of the vertebral body. The necessity of the vertebral endplate depression for the VF diagnosis at the thoracic level permits the elimination the non-osteoporotic VDs. However, this depression is much more difficult to appreciate at the lumbar spine where the vertebra is spontaneously biconcave. Moreover, in men, there are difficulties in the diagnosis of VFs because of the frequency of the non-osteoporotic VD due to traumatisms, disc and vertebral degeneration, and lesions secondary to Scheuermann disease.19

VFs are the hallmark of osteoporosis, and it has been shown that the presence of VFs is the main determinant of the risk of future osteoporotic vertebral and non-vertebral fractures. The strongest
associations were observed between prior and subsequent VFs, and this risk increases with the number of prior vertebral fractures.33–35

Low BMD is also a strong risk factor of future fracture, with a strong relationship between the decrease in BMD and the risk of further fracture.36 We observed that the proportion of patients with osteoporosis was higher in patients with incident VFs than in other patients, but the very low number of events preclude any analysis of other risk factors. In our study, most of the VFs were mild (grade 1). Such deformities are sometimes considered as an hypnosis in our study conducted in a young population. The relevance of mild VFs has been shown in studies conducted in osteoporotic postmenopausal women showing in this population that these mild fractures are a risk factor for sustaining other VFs.37 However, their relevance in young adults needs further studies.

The strengths of our study were that we had a large prospective cohort of axial SpA over 5 years, we made a careful interpretation of every X-ray using a semiquantitative method and if needed we made adjudication for doubtful cases in few cases (4.4%). We confirmed the prevalence and incidence of VFs and VDs in patients with a higher confidence of the axSpA diagnosis (eg, ≥8). The limits of this study were the small number of events, making impossible the interpretation of some secondary analysis, like the identification of risk factors for VFs. It will be interesting to use the MRI follow-up to distinguish VDs and VFs, but MRI is only available for 190 patients at 5 years.

We found in the DESIR cohort, a population of early SpA, a prevalence of VF of 3.0% and 1.15% of incidental VFs. This confirms our hypothesis that the actual prevalence and incidence of VF in SpA is lower than that reported in previous studies, probably depending on the characteristics of the population and the methods of vertebral fracture’s assessment avoiding any misclassification of VDs.

**Correction notice** This article has been corrected since it published Online First. The sixth author’s name has been corrected to Pascal Richette.

**Acknowledgements** The DESIR study is conducted as a Programme Hospitalier de Recherche Clinique with Assistance Publique Hopitaux de Paris as the sponsor. The DESIR study is also under the umbrella of the French Society of Rheumatology, which financially supports the cohort. An unrestricted grant from Pfizer has been allocated for the first 5 years. The DESIR cohort is conducted under the control of Assistance Publique Hopitaux de Paris via the Clinical Research Unit Paris Centre and under the umbrella of the French Society of Rheumatology and Institut national de la sante et de la recherche medicale (Inserm). Database management is performed within the Department of Epidemiology and Biostatistics (Professeur Jean-Pierre Daures, DLM, Nimes, France). We also wish to thank the different regional participating centres: MD (Paris-Cochin B), Andre Kahn (Paris-Cochin A), Philippe Dieude (Paris-Bichat), Bruno Fautrel (Paris-La Pitié-Salpetriere), Francis Borenbaum (Paris-Saint-Antoine), Pascal Claudapierre (Crestet), Maxime Breban (Boulogne-Billancourt), Bernadette Saint-Marcoux (Aulnay-sous-Bois), Philippe Goupille (Tours), Jean Francois Mallet (Dijon), Emmanuelle Demis (Le Mans), Daniel Wendling (Besancon), Bernard Combe (Montpellier), Liana Euler-Ziegler (Nice), Pascal Richette (ParisLariboisiere), Pierre Lafforgue (Marseille), Patrick Bouzier (Amiens), Martin Soubrier (Clermontferrand), Nele Vanhove (Bordeaux), Soline Loeuille (Nancy), Bene-Marc Flipo (Ulle), Alain Saraux (Brest), Xavier Mariette (Le Kremlin-Bicetre), Alain Cantegrel (Toulouse) and Olivier Vittecoq (Rouen). We wish to thank the research nurses, the staff members of the Clinical Research Unit of Paris Centre, the staff members of the Biological Resource Center of Bichat Hospital, the staff members of the Department of Statistics of Nimes and all the investigators, and in particular Jerome Allain, Emmanuelle Denis, Salah Ferkal, Clement Prat, Marie-Agnes Timsit and Eric Toussirot, for active patient recruitment and monitoring.

**Contributors** The author and the coauthors contributed equally to this paper: design of the work, analysis and/or interpretation of data, drafting the work or revising it critically, and final approval of the version to be published.

**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 consent** Obtained.

**Provenance and peer review** Not commissioned; externally peer reviewed.

### References


HLA class I and II alleles in susceptibility to ankylosing spondylitis

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ABSTRACT
Objective To examine associations of HLA class I and class II alleles with ankylosing spondylitis (AS) in three cohorts of patients of European, Asian and African ancestry.

Methods HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1 and HLA-DPB1 alleles were genotyped in 1948 unrelated white and 67 African-American patients with AS from the Prospective Study of Outcomes in Ankylosing Spondylitis cohort, the North American Spondylitis Consortium and Australo-Anglo-American Spondyloarthritis Consortium, 990 white and 245 African-American Controls and HLA alleles in 442 Han Chinese patients with AS and 346 controls from Shanghai and Gansu, China. In addition to the case:control analyses, HLA-B*27-negative patients with AS were analysed separately, and logistic regression and ‘relative predispositional effects’ (RPE) analyses were carried out to control for the major effect of HLA-B*27 on disease susceptibility.

Results Although numerous associations were seen between HLA alleles and AS in whites, among HLA-B*27-negative patients with AS, positive associations were seen with HLA-A*29, B*38, B*49, B*52, DRB1*11 and DPB1*03:01 and negative associations with HLA-B*07, HLA-B*57, HLA-DRB1*15:01, HLA-DQB1*02:01 and HLA-DQB1*06:02. Additional associations with HLA-B*14 and B*40 (B60) were observed via RPE analysis, which excludes the HLA-B*27 alleles. The increased frequency of HLA-B*40:01 and decreased frequency of HLA-B*07 was also seen in Han Chinese and African-Americans with AS. HLA-B*08 was decreased in whites with acute anterior uveitis.

Conclusions These data, analysing the largest number of patients with AS examined to date in three ethnic groups, confirm that other HLA class I and II alleles other than HLA-B*27 to be operative in AS predisposition.

INTRODUCTION
Studies of the contribution of major histocompatibility complex (MHC)-encoded variants to the heritability of ankylosing spondylitis (AS) suggest that it is responsible for 20.44% of the genetic risk for the disease, with over 114 non-MHC variants identified to date contributing another 7.38%.

What is already known about this subject?
► Although the association of HLA-B27 with ankylosing spondylitis (AS) is well established, as is the association with B60 (B*40), those of other HLA alleles that have been reported in smaller studies have been inconsistent or unconfirmed.
► The role of HLA alleles other than HLA-B27 have not been studied in African-Americans.
► In a large recent study imputing HLA alleles in whites from our group, a number of new HLA associations have been reported.

What does this study add?
► In this study, the largest to date in whites and Asians and the first to date in African-Americans (other than HLA-B27) to employ direct HLA typing, a number of the HLA-associations we described by imputing HLA alleles in whites are confirmed in whites and in Chinese and African-American patients with AS.
► A number of other HLA association not seen in the imputation analysis, including some seen in previous small studies, especially in the largest cohort of HLA-B27-negative patients with AS reported to date, are seen with other HLA-B, DRB1, DQB1 and DPB1 alleles, some reported in earlier smaller studies and other novel associations (though the latter because of possible power or stratification issues will need confirmation).
► This study shows a role for HLA-class I alleles other than HLA-B27 and class II HLA genes in predisposition to AS and subsets thereof, independent of linkage with HLA-B27.

How might this impact on clinical practice or future developments?
► This study gives the clinician a better understanding of the relative inputs of HLA alleles other than HLA-B27 in predisposition to AS and better informs how to interpret HLA-B27 typing results in blacks.
Table 1  Selected HLA Class I and II associations with AS in whites overall

<table>
<thead>
<tr>
<th>Allele</th>
<th>Cases frequency</th>
<th>Control frequency</th>
<th># Cases</th>
<th># Controls</th>
<th>OR</th>
<th>P values</th>
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<td>8.6</td>
<td>13.9</td>
<td>1326</td>
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<td>1326</td>
<td>1134</td>
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<td>1.5x10^-4</td>
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<td>1326</td>
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<td>1980</td>
<td>0.36</td>
<td>&lt;1x10^-4</td>
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**AS, ankylosing spondylitis.**

HLA class I genes (HLA-A, HLA-B and HLA-C) and HLA class-II genes (HLA-DRA1, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1 and HLA-DPB1) encode cell-surface molecules that play an essential role in the immune defence against intracellular infections and in initiating an immune response to invading pathogens, respectively. Linkage disequilibrium (LD) with HLA-B*27 makes it difficult to resolve whether they play an independent role themselves, which would provide additional clues to AS pathogenesis, and potentially serve as biomarkers of diagnosis. Studies of direct HLA typing to date have been small and underpowered, providing inconsistent results.

Recently, we genotyped 7264 MHC single-nucleotide polymorphisms (SNPs) in 9069 AS cases and 13 578 population controls of European descent using the Illumina Immunochip microarray and controlling for the effects of HLA-B*27:02 and B*27:05, identified several other HLA allele associations with AS, including significantly increased frequencies of HLA-B*13:02, B*40:01, B*40:02, B*47:01, B*51:01 and negative associations with HLA-B*07:02 and B*57:01 and association of HLA-A*02:01, HLA-DRB1*01:03 and HLA-DPB1. In a Korean study, imputation was used to additionally show that HLA-C*15:02 is associated with AS susceptibility.

The purpose of this study was, in the largest reported series of patients to date for directly genotyped HLA alleles, to examine the relative contributions of HLA-class I and class II alleles, analysing overall disease associations and conditioning on the presence of HLA-B*27 in three ethnic groups: 1948 whites of European ancestry, 442 Han Chinese and 67 African-Americans, the latter never having been examined for MHC associations (other than HLA-B*27 per se) previously.

**METHODS**

**Patient enrolment**

White patients with AS in this study came mostly from the Prospective Study of Outcomes in Ankylosing Spondylitis (PSOAS) cohort, a multiethnic study conducted at four US academic institutions (The University of Texas Health-McGovern Medical School (UTH-H), Cedars-Sinai Medical Center, the University of California at San Francisco and the National Institutes of Health Clinical Center) and from The North American Spondylitis Consortium (NASC) (n=408). The black patients included 53 from the PSOAS study, 7 from NASC and 7 other either from patients followed from the outpatient clinic at UTH or referred by Dr Joel Taurog from the University of Texas Southwestern Medical School. All cases met modified New York criteria for AS. Controls were self-identified whites from the USA with no history of rheumatic disease obtained from unaffected non-consanguineous spouses or household or friend controls the Scleroderma Family Registry and DNA repository or the NASC (unrelated spouses or friends-75%) or from University of Texas-Houston Division Controls from Texas (25%). It should be noted that both the NASC and Scleroderma Family Registry controls and families came from all over the USA, although were enriched from Texas and California (NASC) and from Texas, Maryland, Pennsylvania and Michigan (Scleroderma Family Registry). Approximately one-third of the US patients with AS were probands in the NASC study and 2/3 came from the PSOAS study (from which approximately half came from California, 30% from Texas and 20% from the Mid-Atlantic region). All controls were screened by questionnaire for the presence of autoimmune disease or spondylarthropathy and were excluded if having such. For the HLA-B locus analyses, an additional 555 white British and Australian patients with AS from the Australo-Anglo-American Spondylitis Consortium were also analysed. HLA-B locus typing from 442 Han Chinese patients with AS and 346 unrelated Chinese controls were analysed by the same statistical approaches. Though some of the HLA-typing was reported previously by us, additional statistical analyses were carried out for the present study on a significantly larger AS cohort heretofore unreported. The patients with AS came from the clinics and hospitals in Shanghai and Jianguo Province of China and the Chinese controls (who were free of any history of rheumatic disease) were obtained from a study project of Chinese population genetics at Fudan University in Shanghai. All subjects provided written informed consent. The study was approved by the Institutional Review Boards of all participating medical centres.

**HLA genotyping**

Single Stranded Conformational Polymorphism (SSCP) typing, of HLA-A, HLA-B and HLA-C alleles, was performed using commercially available kits (Dynal) on genomic DNA extracted from peripheral blood. HLA-DRB1, HLA-DQA1, HLA-DQB1...
The HLA SBT uTYPE 6.0 program (Life Technologies) was used in sequencing analysis and assigning HLA-B alleles. The HLA SBT uTYPE 6.0 program (Life Technologies) was used in sequencing analysis and assigning HLA-B alleles.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Case frequency</th>
<th>Control frequency</th>
<th>No. cases</th>
<th>No. controls</th>
<th>OR</th>
<th>P values</th>
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<td>0.45</td>
<td>0.04</td>
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</table>

Table 2 Associations in white HLA-B*27-negative patients with AS

The white patients in the PSOAS and NARC cohorts were strikingly similar in their clinical features, with psoriasis occurring in 10.4%, Crohn’s disease in 5.9%, ulcerative colitis in 3.5% and reactive arthritis in 5.0%. Minor differences between the two cohorts included a slightly lower frequency of HLA-B*27 (85.4%) and uveitis (37%) in the PSOAS cohort compared with 98.5% and 44%, respectively, in the probands from the NARC cohort (the latter enriched for multicase families). The prevalence of HLA-B*27 and uveitis is higher in familial AS. Among the 61 blacks with AS studied who had clinical information available, uveitis occurred in 37.7%, psoriasis in 10.2% and inflammatory bowel disease in 11.5%.

EXAMINATION OF HLA-A, HLA-B AND HLA-C Allele Frequencies in all white patients with AS and controls showed a number of highly significant associations at each locus (table 1). HLA-B*27 occurred in 87.8% of the white patients with AS compared with 7.6% of controls (p<1×10^-9, OR=87.7, 95% CI 66.8 to 115.0), while HLA-B*27 homozygosity occurred in 3.5% of patients and 0.1% of controls (p<1×10^-9, OR=35.9, 95% CI 4.96 to 258.0) (data not shown—table 1 shows allele frequencies). In addition to HLA-B*27, several other MHC class I alleles, including HLA-A*02, C*01 and C*02 were increased in frequency, whereas HLA-A*01, A*03, B*07, B*08, B*13, B*35, B*40, B*44, B*51, B*57, C*03, C*06 and C*07 were decreased in frequency (table 1). Examination of HLA-DRB1 and HLA-DQB1 allele frequencies in 790 patients and 704 controls demonstrated positive associations with HLA-DRB1*01:01, DRB1*01:03 and DRB1*04:04 as well as with DQB1*02:01 (in LD with DRB1*01:03, DQB1*03:02 (in LD with DRB1*04:04) and DQB1*05:01 (in LD with DRB1*01:01). Examination of HLA-DPB1 alleles in 635 patients and 385 controls revealed an association with HLA-DPB1*03:01. HLA-DRB1*15:01 and DRB1*03:01 and their respective linked alleles DQB1*06:02 and DQB1*02:01 were deceased in frequency in the white patients with AS.

Statistical analysis

We constructed 2×2 tables and tested the proportion of alleles in cases vs controls with adjusted X^2 test using EPI-INFO (cdc.gov/epiinfo/index.html). Another method of ‘adjusting’ for the effect of HLA-B*27 at the HLA-B locus is to mask the HLA-B*27 alleles and analyse the remaining alleles for association with phenotype by comparing their frequencies (relative predispositional effects analysis (RPE)). Typically, RPEs are assessed by sequentially removing the alleles with the largest effect among those remaining. Such a procedure has no impact on loci other than HLA-B, as we are not removing individuals, but rather alleles. In addition to the univariable associations of each allele with AS, interaction effects between each allele and HLA-B*27 in relation to AS were examined using logistic regression models where the effects of each allele on AS were estimated for HLA-B*27-negative and positive patients separately. For the HLA-B analyses, this study has 94% power to detect an association with an additive OR of 1.5 for an allele with minor allele frequency of 0.1 at nominal significance.

RESULTS

and HLA-DPB1 typing was performed by standard oligotyping techniques with high resolution HLA-DRB1 typing further achieved by sequence analysis of PCR-amplified HLA-DRB1 exon 2, except for 18 of the African-American patients with AS, where the MHC class II alleles were determined by SSCP typing using commercially available kits (One Lambda). Genomic DNA from the Chinese patients with AS and controls underwent the allele-specific PCR using primers supplied in the SeCore kits and then were followed by sequencing exons 2 and 3 of the HLA-B gene. The HLA SBT uTYPE 6.0 program (Life Technologies) was used in sequencing analysis and assigning HLA-B alleles. The HLA-B genotyping in HLA-B*40 carriers was confirmed with sequence-based typing using SeCore Kits (Life Technologies, USA). The HLA SBT uTYPE 6.0 program (Life Technologies) was used in sequencing analysis.

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The HLA-B genotyping in HLA-B*40 carriers was confirmed with sequence-based typing using SeCore Kits (Life Technologies, USA). The HLA SBT uTYPE 6.0 program (Life Technologies) was used in sequencing analysis.
Removing the effect of the presence of HLA-B*27 using RPE analysis, new associations emerged with HLA-B*14 and B*40:01 (table 3) and the positive associations with HLA-B*38 and B*52 persisted, as did the negative associations with HLA-B*07 and B*57. Based on findings from HLA-B*27 stratified models for each allele, evidence for a significant interaction effect was observed for B*44 and B*49 (table 3).

Among the Han Chinese patients with AS, HLA-B*27 carriage was observed in 93.0% of the patients with AS and 7.5% of the controls (p<1×10^-4, OR=163) (data not shown). Ten (2.3%) of the 442 Chinese patients were homozygous for HLA-B*27, which occurred in 40 of 67 patients with AS compared with 2 of 245 controls (p=1.4×10^-4, OR=2.3). Neither of the two most common HLA-B*27 subtypes seen in whites (B*27:02 and B*27:05) were selectively associated with AAU, independent of their association with AS. Among blacks with AAU, 78.3% were HLA-B*27-positive compared with 42.1% without AAU (p=0.013, OR=4.95). The frequencies of IBD, psoriasis and reactive arthritis were too low for meaningful statistical analyses.

**DISCUSSION**

HLA-B*27 is the major genetic association with AS in all major ethnic groups. Association of other MHC Class I and II loci with either AS specifically or spondyloarthritis in general have been suggested, but have been confounded due to LD between HLA-B and the MHC class II loci. In this study, by conducting stratified analyses of HLA-B*27, we have shown associations with other MHC alleles that cannot be attributed to LD with HLA-B*27 in a moderately large number of patients with AS from three ethnic groups. This study also analyses these alleles in the largest collection of HLA-B*27-negative patients with AS reported to date. We confirm by direct LA typing some of the associations we previously reported by imputation.  

HLA-B60 is a serologically defined specificity that correlates at the DNA level with HLA-B*40:01.  

The frequencies of IBD, psoriasis and reactive arthritis were too low for meaningful statistical analyses.

AAU occurred in 380 of 1389 with clinical data available (27.4%) (table 6). HLA-B*27 occurred in 92.9% compared with 85.0% of those without AAU (p=1.4×10^-4, OR=2.3). Neither of the two most common HLA-B*27 subtypes seen in whites (B*27:02 and B*27:05) were selectively associated with AAU, independent of their association with AS. Among blacks with AAU, 78.3% were HLA-B*27-positive compared with 42.1% without AAU (p=0.013, OR=4.95). The frequencies of IBD, psoriasis and reactive arthritis were too low for meaningful statistical analyses.

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association with HLA-B*40:01 with AS in three ethnic groups. In this study, the largest to date employing direct HLA-typing, we were able to confirm the positive association with HLA-B60 (B*40), especially B*40:01, seen in other studies of whites by direct typing27 and by imputed alleles19 and of Taiwanese Chinese. We were also able to confirm the negative association with HLA-B*07 and B*57 that we reported previously in whites by imputation28 and in the case of B*07 in Han Chinese by DNA sequencing.24 The association seen with HLA-B*14, although only by RPE analysis, is compatible with what has been observed in French SpA families2 and in African blacks.3 The association with HLA-B*38 seen most strikingly in HLA-B*27-negative patients is interesting, given its known association with psoriatic arthritis,29 although this was not seen in the Chinese cohort, where a weak association was described with HLA-B*39 (HLA-B*38 and B*39 being ‘splits’ of the parent specificity HLA-B*16 (http://alaalleles.org/antigens/broads_splits.html).

We observed an association with HLA-A*02 in the overall cohort that was not seen in the smaller HLA-B*27-negative cohort. This is compatible with the independent association with HLA-A*02:01 that we observed in a much larger patient cohort by imputation,28 although in the current study we were not able to resolve the association to the 4-digit level. Associations with HLA-DRBI*01 and HLA-DRBI*04:04 seen here have previously been described in UK patients with AS and from French spondyloarthritis families,28 29 although the lack of confirmation in HLA-B*27-negatives does not allow us to rule out that this may reflect LD with HLA-B*27. Similarly, the association with HLA-DRB1*01:03 that we observed by imputation19 was again seen here, but was not seen in the HLA-B*27 negatives. HLA-DRB1*01:03 is also strongly associated with inflammatory bowel disease,30 in particular where associated with peripheral spondyloarthritis.31 On the other hand, the decreased frequency of HLA-DRB1*15:01 and its linked allele DQB1*06:02 overall and in the HLA-B*27 negatives confirms what we have observed by imputation analysis in a larger cohort,19 now seen by direct HLA typing. An increased frequency of HLA-B*14 has been observed in French SpA families2 and in African patients with AS3 in other studies.

We were not able to confirm all the associations by direct HLA typing we previously described by imputation.19 In some cases, even with the rather large number of patients studied (1948 whites), given the low ORs (<1.5), there may have not have been adequate power. Alternatively, although the patients and controls were from all over the USA, ancestry informative markers were not examined in the controls and we cannot rule out potential stratification issues. We previously described an association with HLA-B*51 by imputation, seen only in a conditional regression analysis, which we could not confirm here, even by RPE analysis. However, we did find an association with HLA-B*52 in whites, both in the HLA-B*27 negatives and in the overall cohort. HLA-B*51 and B*52 are well-recognised ‘splits’ of the parent specificity HLA-B5 (http://alaalleles.org/antigens/broads_splits.html) and although HLA-B*51 was decreased overall, it was increased in frequency in the HLA-B*27 negatives and by RPE analysis, although not significantly. HLA-B*51 was significantly reduced

### Table 4 HLA-B allele frequency analysis in 442 Han Chinese patients with AS and 346 controls

<table>
<thead>
<tr>
<th>Allele</th>
<th>Controls %</th>
<th>OR</th>
<th>P values</th>
<th>OR</th>
<th>P values</th>
<th>OR</th>
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<th>P values</th>
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<td>1.24</td>
<td>0.32</td>
<td>0.03</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

n/a: OR is not estimable due to frequencies of zero in contingency tables of AS (case/control) and each allele (+/-).
Significant associations are highlighted in bold numbers.

1Significant interaction effect found with B*27 (+/−).
2Significant interaction effect found with B*27 (+/−).
3AS, ankylosing spondylitis.
Table 5  Selected HLA allele frequencies in African-American patients with AS and controls

<table>
<thead>
<tr>
<th>Allele</th>
<th>Patients with AS (%)</th>
<th>2xN†</th>
<th>Controls (%)</th>
<th>2xN</th>
<th>OR</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>B*07:00</td>
<td>3.7</td>
<td>134</td>
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<td>490</td>
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<td>0.15</td>
</tr>
<tr>
<td>B*08:00</td>
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<td>134</td>
<td>6.1</td>
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<td>0.35</td>
<td>0.12</td>
</tr>
<tr>
<td>B*14:00</td>
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<td>134</td>
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<td>490</td>
<td>0.52</td>
<td>0.56</td>
</tr>
<tr>
<td>B*15:00</td>
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<td>134</td>
<td>11.0</td>
<td>490</td>
<td>0.94</td>
<td>0.97</td>
</tr>
<tr>
<td>B*27:00</td>
<td>29.9</td>
<td>134</td>
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<td>490</td>
<td>41.3</td>
<td>&lt;1×10⁻⁵</td>
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<tr>
<td>B*35:00</td>
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</tr>
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<tr>
<td>B*40:01</td>
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<td>0.03§</td>
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<td>0.48</td>
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<tr>
<td>B*51:00</td>
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<td>B*57:00</td>
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<td>0.05</td>
</tr>
<tr>
<td>B*58:00</td>
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<td>7.8</td>
<td>490</td>
<td>0.56</td>
<td>0.26</td>
</tr>
<tr>
<td>DRB1*01:01</td>
<td>7.7</td>
<td>130</td>
<td>4.6</td>
<td>482</td>
<td>1.74</td>
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</tr>
<tr>
<td>DRB1*15:01</td>
<td>13.1</td>
<td>130</td>
<td>11.6</td>
<td>482</td>
<td>1.14</td>
<td>0.76</td>
</tr>
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<td>DRB1*03:01</td>
<td>3.1</td>
<td>130</td>
<td>6.2</td>
<td>482</td>
<td>0.48</td>
<td>0.24</td>
</tr>
<tr>
<td>DRB1*03:02</td>
<td>2.3</td>
<td>130</td>
<td>6.2</td>
<td>482</td>
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<td>0.13</td>
</tr>
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<td>DRB1*04</td>
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<td>130</td>
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<td>482</td>
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</tr>
<tr>
<td>DRB1*07:00</td>
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<td>130</td>
<td>7.3</td>
<td>482</td>
<td>0.51</td>
<td>0.23</td>
</tr>
<tr>
<td>DRB1*08:00</td>
<td>10.0</td>
<td>130</td>
<td>5.0</td>
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<td>482</td>
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<tr>
<td>DRB1*13</td>
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<tr>
<td>DQB1*03:02</td>
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<td>130</td>
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<td>482</td>
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<td>4.6</td>
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<td>7.9</td>
<td>482</td>
<td>0.56</td>
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<td>DQB1*06:02</td>
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<td>13.9</td>
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</tr>
<tr>
<td>DPB1*03:01</td>
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<td>130</td>
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</tbody>
</table>

Significant associations are highlighted in bold numbers.

*Two patients previously typed for HLA-B did not have DNA available for MHC class II typing.
†Unless stated, the p-values shown were multiplied by the number of alleles examined at each locus. Alleles with frequencies of <5% were not examined unless potential biological relevance was perceived.
‡After removing the effect of B*27, HLA-B*40:01 occurred in 4 of 94 genotypes in patients compared with 7 of 450 genotypes in controls, p=0.157, OR=3.03.
§These represent uncorrected p values. These were no longer significant after applying Bonferroni’s correction.
AS, ankyllosing spondylitis.

in the Chinese cohort overall, but having controlled for the presence of HLA-B*27 by RPE, no association was observed. We were unable to establish an independent association with HLA-B*13, an allele long associated with psoriasis, described previously by imputation in whites.19 However, as with HLA-B*51, HLA-B*13 was reduced in the overall Han Chinese dataset, but having controlled for HLA-B*27, no association was observed. HLA-B*47, observed by imputation as AS-associated,19 only occurred in five patients with AS and hence was too uncommon to establish as an AS association (data not shown).

The association of HLA-DPB1*03:01 with AS seen in whites further extends what was demonstrated previously in smaller cohorts.11 12 This is compatible with the association of SNPs around the HLA-DPB1 locus recently established by imputation.19 We did not examine HLA-DPA1 alleles and so could not confirm the associations with HLA-DPA1*01:02 and DPA1*01:03 reported by Díaz-Peña et al.12 However, we did not observe any association with HLA-DPB1*13:01 that was seen in their cohort.

We could not confirm some other associations described in smaller AS or SpA cohorts elsewhere13–24 perhaps due to clinical heterogeneity. One small recent study of HLA-A, HLA-B, HLA-C and DRB1 alleles in 75 Moroccan patients with AS reported an allele frequency of HLA-B*27 of 32%, with associations also
seen with B*57, C*02 and DRB1*15 and negative associations with HLA-B*35 and B*49.32 The reasons for the discrepancies of these data and ours are hard to interpret given the small size of the cohort, which precluded examination of HLA-B*27 negatives. Another recent study of 189 Colombian patients with SpA (including 87 with AS, but also reactive arthritis and undifferentiated SpA) found associations with HLA-B*15 as well as with HLA-DRB1*01 and HLA-DRB1*04 as well as with HLA-B*27, which occurred at an allele frequency of 26.2%.33 Again, the small size of the cohort of patients with AS did not allow examination of HLA-B*27 negatives.

The size of the black AS cohort was small (n=67 patients), which would have restricted the statistical power of our observations. Nevertheless, we were able to confirm the positive association with HLA-B*40:01 and the negative association with B*07, for the first time in this ethnic group. The finding of HLA-B*27 in 60% of the black patients with AS is compatible with what was reported by Khan et al in a smaller cohort several years ago.13

We were able to confirm the association with HLA-DRB1*01:03 as observed by Cortes et al by imputation,39 although not an association of HLA-DRB1*08 with either AS or the uveitis phenotype, as has been reported elsewhere,10,11 in fact HLA-DRB1*08 was actually decreased in those with AAU. We did observe a significant association with the presence of HLA-B*27 and the occurrence of AAU in our patients, as also seen by imputation16 but not the higher frequency of HLA-B*27 homozygosity seen there. Otherwise the most robust association, although negative, was the decreased frequency of HLA-B*08 in those with AAU, which contrasts what was observed by imputation, where an increased frequency of a SNP associated with HLA-B*08, namely rs115937001, was observed.36 The reasons for this was unclear, as the OR of 1.8 in that larger group of 1711 patients with uveitis would suggest that there should have been sufficient power in this cohort of 380 patients with AAU to confirm this.

Thus, in the largest study of direct HLA typing of patients with AS and controls from three ethnic groups (and the only one examining African-American blacks other than HLA-B*27), these data show that the impact of the MHC on AS susceptibility extends beyond HLA-B*27. Many (though not all) of the observations made in a prior larger study of imputed HLA alleles39 are seen here. That positive and negative associations of some of these alleles cross ethnic boundaries suggests an independent role of both MHC class I and II alleles in influencing susceptibility to AS and subsets thereof.

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Contributors JDR contributed many of the patients, carried out the HLA typing, entered the data, interpreted the results, did many of the statistical analyses, wrote the manuscript and provided the funding for the study. XZ established the Chinese collaboration and oversaw its completion, reviewed the manuscript and provided some of the funding for this study. ML carried out the multivariable and regression statistical analyses and participated in the writing and drafting of the manuscript. MHW contributed a large amount of the patients for this study as well as some of the controls. He also reviewed the manuscript. LY contributed a large amount of the Chinese patients for this study as well as some of the controls and also q’d the Chinese HLA genotyping and reviewed the manuscript. TJS contributed the blood of the US patients for this study. She also reviewed the manuscript. Hejian Zou contributed a large amount of the Chinese patients for this study as well as some of the controls. He also reviewed the manuscript. MMW contributed a number of US patients for this study. He also reviewed the manuscript and obtained funding from the NIH Clinical Center to carry on the project there. MJL contributed a number of US patients for this study as well as some of the controls. She also reviewed the manuscript. TJL read all the X-rays for the study to determine who qualified for inclusion. He also reviewed the manuscript. DH contributed a large amount of the Chinese patients for this study as well as some of the controls. He also reviewed the manuscript. MHR oversaw the qc process of the datasets and worked with ML in the statistical analyses. JW oversaw the Chinese segment of this study, carrying out the HLA typing and coordinating the participating centers as well as providing funding for the Chinese portion of this project. MAB provided the Australian patients in this study. He also q’d the HLA typing with the imputed data from our GWAS, confirmed the statistical analyses and assisted JDR in manuscript preparation.

Funding This study was supported by the National Institutes of Health-National Institute of Allergy and Infectious Diseases (NIH-NIAID) grant U01 AI09090 (Drs Zhou, Reveille), National Institute of Arthritis, Musculoskeletal and Skin Diseases (NIH-NIAIMS) grants R01 19 AR-46208 and 2P01AR052915-06A1 (Dr Reveille) and by University Clinical Research Grants M01-RR-02555 (The University of Texas Health Science Center at Houston) and M01-RR-000425 (Cedars-Sinai Medical Center) and by a grant from the Spondylitis Association of America. MAB was funded by a National Health and Medical Research Council (Australia) Senior Principal Research Fellowship. LSG is supported by the Rosalind Engelman Research Center at the University of California, San Francisco. MMW is funded by the Intramural Research Program, NIAMS/NIH.

Competing interests None declared.

Patient consent Not required.

Ethics approval The Internal Review Boards at The University of Texas-Houston McGovern Medical School, The University of California-San Francisco, Cedars-Sinai Medical Center, The NIH-NIAIMS Clinical Center, The Princess Alexandra Hospital (Brisbane), Fudan University reviewed and approved this study.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement We would be happy to share the data published in this manuscript. There are no unpublished data referable to the work included here.

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33 Londono J, Santos AM, Peña P, et al. Analysis of HLA-B15 and HLA-B*27 in spondyloarthropathy with peripheral and axial clinical patterns BMI Open. 2015;Sw009902.


**Salmonella** exploits HLA-B27 and host unfolded protein responses to promote intracellular replication

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**ABSTRACT**

**Objective** *Salmonella enterica* infections can lead to Reactive Arthritis (ReA), which can exhibit an association with human leucocyte antigen (HLA)-B*27:05, a molecule prone to misfolding and initiation of the unfolded protein response (UPR). This study examined how HLA-B*27:05 expression and the UPR affect the *Salmonella* life-cycle within epithelial cells.

**Methods** Isogenic epithelial cell lines expressing two copies of either HLA-B*27:05 or a control HLA-B*35:01 heavy chain (HC) were generated to determine the effect on the *Salmonella* infection life-cycle. A cell line expressing HLA-B*27:05:HC physically linked to the light chain beta-2-microglobulin and a specific peptide (referred to as a single chain trimer, SCT) was also generated to determine the effects of HLA-B27 folding status on *S. enterica* life-cycle. XBP-1 venus and AMP dependent Transcription Factor (ATF6)-FLAG reporters were used to monitor UPR activation in infected cells. Triacin C was used to inhibit *de novo* lipid synthesis during UPR, and confocal imaging of ER tracker stained membrane allowed quantification of glibenclamide-associated membrane.

**Results** *S. enterica* demonstrated enhanced replication with an altered cellular localisation in the presence of HLA-B*27:05:HC but not in the presence of HLA-B*27:05:SCT or HLA-B*35:01. HLA-B*27:05:HC altered the threshold for UPR induction. *Salmonella* activated the UPR and required XBP-1 for replication, which was associated with endo-terumic membrane expansion and lipid metabolism.

**Conclusions** HLA-B27 misfolding and a UPR cellular environment are associated with enhanced *Salmonella* replication, while *Salmonella* itself can activate XBP-1 and ATF6. These data provide a potential mechanism linking the life-cycle of *Salmonella* with the physicochemical properties of HLA-B27 and cellular events that may contribute to ReA pathogenesis. Our observations suggest that the UPR pathway maybe targeted for future therapeutic intervention.
diluted into 1% bovine serum albumin/0.1% Tween-80% and plated on Luria Broth (LB) agar at room temperature for 16 hours. Each experimental condition was performed in triplicate and each plating in duplicate. For microscopic analysis, coverslips containing infected cells were washed with 1× PBS, fixed for 10 min with 3.8% PFA (pH 7.4), washed twice with 1× PBS and stored at 4°C.

**UPR-mediated membrane expansion during infection**
Glibenclamide BODIPY FL (green; Invitrogen) was used to quantitate endo-teric membrane size and localisation. Henrietta Lacks (HeLa) cells were treated with UPR-inducing drugs and labelled with glibenclamide according to the manufacturer's protocol. Labelled cells were analysed by fluorescence activated cell sorting (FACS). Cell nuclei were counterstained with DAPI, visualised by fluorescence microscopy. For control and drug-treated cells, equivalent exposures were collected.

To determine endo-teric-derived membrane expansion during infection, HeLa cells were grown either on sterile glass, infected with *S. enterica* Typhimurium expressing mCherry (see online supplementary materials and methods) and stained with glibenclamide green. Cells were fixed, washed and counterstained with DAPI, followed by fluorescence microscopy or automated confocal analysis. Images were acquired by an Opera LX (PerkinElmer) plate reader with a confocal microscope (NA=0.6, 40× air objective). Exposure times were 100 ms for the DAPI channel (365 nm), 2000 ms for the ER channel (488 nm) and 2000 ms for the *Salmonella* channel (561 nm). Camera pixels were binned by two resulting in a pixel size of 0.323×0.323 µm, and 4800 images were acquired per 96-well plate (50 images per well), which were processed in one batch using the same image analysis pipeline, algorithms and parameters (see online supplementary materials and methods for analysis of glibenclamide mean fluorescence intensity (MFI)).

**RESULTS**

**XBP-1 and ATF6 activation following *Salmonella* infection**
We used our previously described epithelial cells with identical sites of transgene integration (and therefore isogenic) expressing physiological levels of HLA-B2721 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E).

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Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred to as Empty (E)84) were generated (integration at the same sites of transgene integration and therefore isogenic) expressing physiological levels of HLA-B27 (online supplementary figure S1A–E).

**XBP-1 pathway is required for efficient *S. enterica* Typhimurium replication**
Our observations suggested that activation of the XBP-1 pathway plays a significant role in *S. enterica* growth and replication. To support our hypothesis, we analysed *S. enterica* replication in XBP-1-deficient mouse embryonic fibroblasts (MEF). XBP-1+/+ and XBP-1−/− MEF cell lines were infected with ST.mCherry, harvested and analysed by flow cytometry. Importantly the MFI, which is a measure of the average number of bacteria per cell, for both cell types were similar at 1 and 4 hours pi, indicating no significant differences in either bacterial invasion or early infection stages. However, when the bacteria began replication, the XBP-1−/− MEF cell line exhibited significantly lower MFI values than the XBP-1+/+ cell line, indicating reduced bacterial growth (figure 2A). XBP-1+/+ cells had approximately threefold more bacteria than in XBP-1−/− cells during the replication phase (8–24 hours pi) (figure 2B–C).

**Enhanced bacterial replication is linked to UPR-induced lipid metabolism**
Next we wished to test the hypothesis that UPR promotes *Salmonella* replication. To mimic UPR conditions, HeLa cells were pretreated with the following UPR-inducing pharmacological agents: A23187 (0.5 mM), MG132 (0.5 µg/mL), TUN (0.5 µg/mL) and TPG (200 nM) (see figure 3A for mode of action). HeLa cells were treated with DMSO (vehicle control) or the respective UPR inducer 16 hours prior to infection with ST.mCherry, and the fold increase in infected cells over the control was determined 24 hours pi. Overall the data indicate
that a UPR environment leads to an increase in intracellular *Salmonella* (figure 3B).

TPG appeared to have a more pronounced effect on *Salmonella* replication and also has the most direct effect on ER homeostasis (figure 3A). We employed TPG further to determine the effect of a pre-existing endoplasmic reticulum (ER) stress environment. HeLa cells were treated with TPG at 25, 100 and 400 nM 16 hours prior to infection. TPG-treated cells showed significant increases in the MFI of infected cells when compared with the controls (figure 3C). A fourfold to sevenfold increase in TPG-treated cells was recorded 24 hours pi (figure 3D). Interestingly, flow cytometry revealed no significant difference in the percentage of HeLa cells infected with increasing TPG concentrations (online supplementary figure 2). Direct quantification of bacterial replication by cfu recovery also demonstrated significant increase in bacterial numbers in TPG-treated cells at 8 and 24 hours pi (figure 3E). Depending on the signals and duration, UPR can lead to production of proapoptotic or antiapoptotic factors. The observed increase in bacterial counts 8 hours pi may be due to the production of antiapoptotic factors. To assess the level of cell death, activation of caspases was followed by staining with the fluorescein isothiocyanate (FITC) conjugated pan caspase detection reagent FLICA and the live dead dye 647. Treatment of cells with UPR-inducing drugs did not alter activation of caspases or induction of cell death during infection, indicating that the observed enhanced bacterial counts post-UPR are not linked to increased cellular death (online supplementary figure 3).

*Salmonella* replication and intracellular niche development require a source of membrane, which would depend in part on lipid biosynthetic pathways, which can be activated and/or enhanced by the UPR. We therefore inhibited de novo synthesis of long-chain fatty acid synthesis during the induction of the UPR with Triacin C (TRC), a potent inhibitor of long fatty acyl CoA synthetase isoforms 1, 3 and 4. HeLa cells were treated with DMSO, TRC (200 nM) and TPG (200 nM) or cotreated with TRC (200 nM) and TPG (200 nM) 16 hours prior to infection. TRC treatment prior to infection did not significantly alter the percentage of cells infected either in the presence or absence of TPG (online supplementary figure 2). Cells treated with TPG alone showed significant increases in...
in MFI values of the infected cells when compared with the DMSO or TRC controls (figure 4A). However, cotreatment of cells with TRC and TPG significantly reduced the effect of the UPR induction on the levels of intracellular bacteria 24 hours pi (figure 4A, B). These observations were confirmed by recovery of viable bacteria from similarly infected HeLa cells (figure 4C), indicating inhibition of lipid metabolism during UPR induction reduces the increase in intracellular bacteria observed at late time points.

Salmonella infection leads to endoreticular membrane expansion. To determine whether the increase in intracellular bacteria from cells undergoing the UPR was dependent on replication within the SCV, we used the isogenic S. enterica Typhimurium ΔsifA mutant, which can infect but is impaired in intracellular growth and can escape the SCV.22–24 HeLa cells were treated with DMSO or 200 nM TPG 16 hours prior to infection with ST.mCherry or the isogenic ΔsifA mutant. ST.mCherry exhibited significant increases after TPG treatment as already described. In marked contrast, UPR-induced cells infected with the ΔsifA mutant demonstrated no increase in bacterial numbers (figure 5A). Thus, intracellular localisation within the SCV is required for UPR-mediated effects on bacterial replication.

As UPR activation is associated with endoreticular membrane expansion and lipid metabolism,25 26 we determined whether Salmonella infection could lead to expansion of endoreticular membrane during the replication/growth phase of its life-cycle using glibenclamide green ER tracker dye as a marker for endoreticular membranes. HeLa cells were infected with either wild-type or ΔsifA ST.mCherry as a control strain. At 4 and 24 hours pi, cells were stained with glibenclamide green ER tracker dye and analysed by automated confocal microscopy. The levels of glibenclamide-labelled membrane in infected cells were quantified and compared with uninfected cells. An increase in glibenclamide labelling in cells infected with wild-type but not sifA-deficient bacteria was observed (figure 5B), which supports our observation that XBP-1 is not activated by ΔsifA bacteria (data not shown).

**The folding status and expression of HLA-B27 after Salmonella replication and cellular localisation**

To address the role of the folding status of HLA-B27, we generated an HLA-B27 molecule fused to the light chain β2m and an HLA-B27-specific peptide derived from the influenza nucleoprotein (referred to as HLA-B27.SCT). As a control we also generated a similar fusion protein for HLA-B35 with EBNA1 peptide (figure 6A). The HLA-B27 and HLA-B35 SCTs were transfected into the original FLIPIN HeLa founder line. Functional activity of the HLA-B27.SCT was determined by incubation±NP383-391 peptide. The HLA-B27.SCT line was an effective CTL target in the absence (figure 6B, top left panel) and presence (figure 6B, top right panel) of exogenously added peptide, whereas the HLA-B35.SCT line did not activate HLA-B27-NP-restricted CTL lines (figure 6B, bottom left and right panels). We next determined whether HLA-B27.SCT could form dimeric conformations. Both cell lines were treated with N-ethylmaleimide (NEM) and lysates were separated by charge and Mw as described previously. Immunoblotting with the anti-V5 tag antibody pK revealed that neither HLA-B27.SCT s were transfected into the original FLIPIN HeLa founder line. Functional activity of the HLA-B27.SCT was determined by incubation±NP383-391 peptide. The HLA-B27.SCT line was an effective CTL target in the absence (figure 6B, top left panel) and presence (figure 6B, top right panel) of exogenously added peptide, whereas the HLA-B35.SCT line did not activate HLA-B27-NP-restricted CTL lines (figure 6B, bottom left and right panels). We next determined whether HLA-B27.SCT could form dimeric conformations. Both cell lines were treated with N-ethylmaleimide (NEM) and lysates were separated by charge and Mw as described previously. Immunoblotting with the anti-V5 tag antibody pK revealed that neither HLA-B27.SCT nor HLA-B35.SCT lines form high Mw conformers (figure 6C, top and bottom panels, respectively).

Next, we wished to know whether HLA-B27.SCT cells could support enhanced Salmonella replication. HLA-B27.SCT, along with HLA-B35 and B27.HC lines, were infected with ST. Green fluorescent protein (GFP), CFU recovery, determined 24 hours pi, demonstrated enhanced numbers of bacteria in the B27.HC but not in the B27.SCT line (figure 6D).

As Salmonella survival can correlate with their intracellular localisation,27 we tracked ST.GFP within the different HLA-class I expressing cell lines using confocal microscopy. Following infection, cells were stained for the trans-Golgi specific marker giantin (red) and the nucleus with DAPI (blue) (figure 6E). In the E84 and HL-A.B35.HC cell lines, we detected ST.GFP concentrated in juxtaposition to the Golgi apparatus, which reflects Salmonella within the SCV. Surprisingly, in the presence of HLA-B27.HC, we noted that Salmonella markedly do not reside in close proximity to the Golgi, but instead was located more within the periphery (figure 6F, panel ii). In contrast, in...
Figure 3  

Salmonella exhibits enhanced recovery from cells undergoing UPR. (A) Pharmacological UPR-inducing agents and their mode of action. A23187 is a Ca^{2+} ionophore that disrupts intracellular Ca^{2+} levels. Thapsigargin is a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump leading to reduced Ca^{2+} endoplasmic reticulum (ER) concentrations. Tunicamycin inhibits the addition of carbohydrate (CHO) moieties to newly synthesised proteins. MG132 inhibits proteasome-mediated degradation leading to accumulation of misfolded proteins within the ER. Triacin C is also shown and inhibits de novo lipid synthesis. (B) Treatment of cells with UPR-inducing drugs increases the number of intracellular bacteria 24 hours pi. Cells were treated with A23187 (0.5 mM), MG132 (0.5 µg/mL), TPG (200 nM) and TUN (0.5 µg/mL). The mean fold increases in mCherry MFI values±SEM are shown (n=3). ANOVA was performed on mCherry MFI values (p<0.0001) with Tukey’s multiple comparison post-test to determine significant differences between individual groups (*p<0.05, ***p<0.001. (C–D) TPG-treated cells exhibit significant increases in MFI values of the infected cells when compared with dimethyl sulfoxide (DMSO) controls (C) and in the fold difference in MFI values between the DMSO-treated and TPG-treated cells (D) in a concentration-dependent manner. The mean MFI values±SEM (n=3) are shown and ANOVA was performed (p<0.0001) with Tukey’s multiple comparison post-test used to determine if there were significant differences between DMSO and all drug-treated groups at 24 hours pi (***p<0.001). (E) Recovery of viable bacteria from infected cells exhibits similar fold differences between the DMSO-treated and TPG-treated cells infected at 24 hours pi. The mean fold differences in cfu values (n=6)±SEM are shown. The Mann-Whitney test was used to compare cfu recoveries between DMSO-treated and TPG-treated and the exact p values were calculated (*p=0.004, **p=0.015). ANOVA, analysis of variance; cfu, colony-forming unit; ER, endoplasmic reticulum; MFI, mean fluorescence intensity; pi, postinfection; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; TPG, thapsigargin; TUN, tunicamycin; UPR, unfolded protein response.
infected B27.SCT cells (figure 6F, panel iv), the bacteria resided in similar locations to the B35 and E84 cells (figure 6F, panel iii), suggesting that bacterial location is associated with enhanced replication, in a process influenced by the folding efficiency of HLA-B27.

DISCUSSION
Why *Salmonella* exhibits an association with ReA and HLA-B27 remains undetermined. Here we have analysed *Salmonella* growth where HLA-B27 misfolding and the UPR are limiting parameters. Our study demonstrates that it is not the expression of HLA-B27 alone that results in enhanced bacterial replication, but HLA-B27 misfolding, which influences the ER stress environment. Our demonstration that HLA-B27 expression can reduce the threshold of ER stress induction and that *Salmonella* can induce the UPR provides key additional insight as to why such bacteria manipulate and exploit ER stress pathways to their benefit.

In the presence of misfolding HLA-B27, *Salmonella* predominantly resides in an altered peripheral cellular localisation.

Figure 4  *Salmonella* requires de novo lipid synthesis during enhanced recovery from cells undergoing UPR. (A) HeLa cells were treated with DMSO, TRC (200 nM) and TPG (200 nM) or cotreated with 200 nM TRC and 200 nM TPG at 16 hours prior to infection with ST.mCherry. TPG-treated cells exhibit significant increases in the MFI values of the infected cells when compared with DMSO or TRC controls. Cotreatment of cells with TRC and TPG significantly reduces the effect of the TPG on the levels of intracellular bacteria at 24 hours pi. The mean mCherry MFI values±SEM are shown (n=3) and analysis of variance was performed (p<0.0001) with Tukey’s multiple comparison post-test to determine significant differences between individual drug treatment groups (**p<0.001). (B) The fold difference in the MFI values of the DMSO, TRC, TPG and TRC/TPG treated cells. (C) Observation by FACS was confirmed by recovery of viable bacteria from similarly treated and infected cells. Colonies were counted and the number of bacteria present per cell in each sample was calculated (n=12). The Mann-Whitney test was used to compare cfu recoveries between DMSO and TPG or TRC and TPG cotreated cells and the exact p values were calculated (**p=0.0001). cfu, colony-forming unit; DMSO, dimethyl sulfoxide; HeLa, Henrietta Lacks; MFI, mean fluorescence intensity; NT, not treated; pi, postinfection; TPG, thapsigargin; TRC, Triacin C; UPR, unfolded protein response.

Figure 5  *Salmonella* infection can increase endoreticular membranes and exhibit altered cellular localisation in the presence of HLA-B27. (A) Increase in the number of intracellular bacteria in cells treated with UPR-inducing drugs is dependent on the intracellular replication of bacteria within the SCV. HeLa cells were treated with DMSO or 200 nM TPG at 16 hours prior to infection with *Salmonella enterica* Typhimurium 12023 or ΔsifA strains expressing mCherry. Wild-type (WT) bacteria, which replicate within the SCV, show increases in intracellular bacteria in the TPG-treated samples, while those infected with the ΔsifA mutant show no increase in the TPG-treated cells. (B) HeLa cells were infected with WT or ΔsifA ST.mCherry, stained with ER tracker (green) at 4 and 24 hours pi and analysed by confocal microscopy using an Opera LX plate reader. Quantification of endoreticular membrane content in infected cells. MFI of glibenclamide staining in infected (INF) and non-infected (NI) cells were compared at 4 and 24 hours pi with either WT or ΔsifA 12023. MFI values±SEM are shown (n=135–1476). For (B) statistical analysis was performed using the Kruskal-Wallis test (p<0.0001) and was performed with Dunn’s multiple comparison post-test to determine significant differences between individual groups (**p<0.001). DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; HeLa, Henrietta Lacks; HLA, human leucocyte antigen; MFI, mean fluorescence intensity; pi, postinfection; SCV, *Salmonella*-containing vacuole; TPG, thapsigargin; UPR, unfolded protein response.
Figure 6 *Salmonella* exhibits altered cellular location in the presence of folding and misfolding HLA-B27. (A) Schematic of the MHC class I SCT used in this analysis. (B) HLA-B27.SCT (top left and right panels) can activate NP-specific T cell clones and act as efficient targets in the absence of exogenous NP peptide (top left panel). HLA-B35.SCT does not activate NP-B27-specific CTLs (bottom left and right panels). (C) Two-dimensional isoelectric focusing of lysates from B27.SCT and B35.SCT cells and immunoblotted with anti-V5 pk demonstrate no dimer formation. (D) Expression of HLA-B27 in the context of the SCT molecule reverses the enhanced bacterial recovery phenotype observed in HLA-B27 HC-expressing cells. ANOVA was performed (p=0.0033) with Tukey’s multiple comparison post-test to determine significance between individual groups (*, **p<0.05). (E) Differences in bacteria recovered at later time points is not due to increased adhesion or invasion of HeLa.B27 HC by ST.GFP. Flow cytometric analysis of cells infected with ST.GFP over time shows no observed difference in the number of cells infected or relative number of bacteria per cell (as determined by MFI) until later infection time points, that is, >8 hours pi. Data are presented relative to the results from control E84 cells. Two-way ANOVA was performed (p<0.0001) with multiple t-tests to determine significance between individual groups (****p<0.0001). (F) *Salmonella* (green) resides in SCVs associated with the Golgi apparatus, stained with giantin (red) in HeLa.FLP (E84, control i) and HeLa.B35.HC (ii). HeLa.B27.SCT-expressing cells (iv) exhibit no altered localisation of *Salmonella* when compared with HLA-B27.HC (iii). Arrow heads highlight *Salmonella* localisation. Nuclei are stained with DAPI (blue). ACTL, cytotoxic T lymphocytes; ANOVA, analysis of variance; cfu, colony-forming unit; APC, allophycocyanin; DAPI, 4’,6-diamidino-2-phenylindole; E84, Empty 84; FLP, flip; GFP, green fluorescent protein; HC, heavy chain; HLA, human leucocyte antigen; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; NP, nucleoprotein peptide; PE, phycoerythrin; pi, postinfection; SCT, single chain trimer; SCV, *Salmonella*-containing vacuole.
Thus HLA-B27 may alter SCV biogenesis and intracellular movement. Maturation of the SCV and bacterial cellular localisation can determine survival and replicative capability of *Salmonella*.14 22 27 28 During the early stages of infection, the SCV migrates by following an endosomal maturation route, to a juxtanuclear location associated with the microtubule organising centre and the Golgi apparatus in epithelial cells.13 14 It is possible that the bacteria fail to form an SCV or exit the SCV more rapidly in the presence of misfolding HLA-B27, or escape from the SCV and randomly redistribute throughout the cytoplasm. Alternatively, *Salmonella* could initially occupy SCVs in juxtaposition to the Golgi and the movement to the periphery is enhanced in the presence of HLA-B27.

HLA-B27-HC expression and endocellular *S. enterica* growth both independently cause activation of the XBP-1 ER-stress pathway. XBP-1 activation peaks at 8–16 hours pi (figure 1D,E), which correlates with enhanced bacterial numbers in cells undergoing ER stress and/or expressing HLA-B27-HC with a propensity to misfold. Activation of both XBP-1 and ATF6 coincides with the replication/growth phase of *S. enterica* Typhimurium. During this phase *Salmonella* membrane modifications have been reported to be at their peak,29 which might therefore lead to UPR activation. We do not currently know the complete cellular stress response to *Salmonella* in our model cell system, but plan to address this by RNA-Seq to map other significant differences in how HLA-B*27:05*-expressing and HLA-B*35:01*-expressing cells respond after infection. However, taken together, data reported here suggest the ER stress consequences of HLA-B27 misfolding provide a favourable environment for replication of *S. enterica* within the HeLa cells. Interestingly, *Salmonella* infections can affect interleukin-23 production,30 a cytokine that has been implicated to be important in the disease phenotype of spondyloarthropathies.31 It is possible that the co-occurrence of *Salmonella* and HLA-B27 could have a cumulative or multiplicative effect on the UPR, which could explain the enhanced risk of ReA and/or an increased risk of symptomatic *Salmonella* infection in HLA-B27-positive individuals in a population exposed to infection.5 6 8 However, the effects of HLA-B27 on *Salmonella* may depend on the temporal activation of the UPR.32 Our observations with pre-existing UPR activation do suggest that these aforementioned factors could indeed influence *Salmonella* replication.

The origin of the membrane that makes up the growing SCV remains poorly defined, but ER membrane markers have been reported in SCVs. The ER membrane-bound markers calnexin and protein disulfide isomerase (PDI) were demonstrated to codistribute with SCVs and up to 20% of intracellular bacteria.33 34 Proteome analysis of host cell membranes modified by *Salmonella* indicated that ER membranes can be redirected to their intracellular niche.35 UPR activation can regulate ER membrane by increasing phospholipids and ER protein levels, as well as modulating fatty acid, sphingolipid, phospholipid and sterol metabolism, which ultimately lead to expansion of ER membranes.25 26 28 36 37 Our experiments with TRC suggest that the long-chain fatty acid CoA synthase (ACSL) family of proteins, which are involved in fatty acid degradation, phospholipid remodelling and production of long-chain acyl-CoA esters, could well be involved in this pathway.35 38

Several intracellular bacteria such as *Brucella* and *Legionella* interact directly with ER membrane.39 Recently it has been reported that *Chlamydia*, which is also associated with ReA,40 can also induce ER stress responses for the purposes of exploiting host lipid metabolism.41 Interestingly both *Chlamydia* and *Salmonella* have been reported to associate with and/or recruit ER-derived membranes.29 42 Bacterial species such as *Salmonella* that depend on expansion of membrane compartments to accommodate their growth would benefit from the enhanced lipid production that results from UPR activation. Together, along with observations that HLA-B27 can induce or alter the ER stress environment, UPR induction may be a common feature of intracellular bacteria that reside in vacuoles and may link with the pathology associated with ReA.

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Acknowledgements

The authors thank Professor David Holden (Imperial College London), Dr Michael Hersel (Universität Osnabrück) and Dr J Baumler (UC Davis) for the *Salmonella* strains.

Contributors All authors contributed to experimental design and to performing the experiments and generating data. All authors contributed to the construction of the manuscript. ANA: contributed to the planning, designing of experiments, interpretation of data and writing of the manuscript. IL: contributed and performed the biochemical analysis of the respective cell lines employed throughout the study, and contributed to the writing of the manuscript. JK-V: contributed to planning, designing and interpretation of the data collected by the microscopic screening, and contributed to the writing of the manuscript. TI: contributed to the design and the use of the UPR reporters. MT: contributed to the microscopic analysis. KM: contributed to the cellular and biochemical analysis of the cell lines employed throughout the study. SA: contributed to the design and generation of the constructs used in the study. NB: contributed to the cellular analysis of the cell lines employed throughout the study. PB: contributed to the cellular and biochemical analyses of the cell lines employed throughout the study and contributed to the writing of the manuscript. MB-E: contributed to the experimental design, data interpretation and writing of the manuscript. KG: contributed to the generation of the constructs and cell lines used throughout the study, data interpretation, experimental design and writing of the manuscript. DIP: contributed to the data interpretation, experimental design and writing of the manuscript, and contributed to the biochemical analysis of the cell lines employed throughout the study.

Funding ANA was funded by ARUK Fellowships Non-Clinical Career Development Fellowship (ref no: 18440). IL was funded by an ARUK PhD studentship (ref no: 17868). ANA and SIP were also in part funded by ARUK (grant 21261).

Competing interests None declared.

Patient consent Not required.

Ethics approval All experiments and procedures were performed as approved by the local ethics.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement All data are available on request to antony.antoniou@northumbria.ac.uk.

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CLINICAL SCIENCE

Effectiveness of low-dose radiation therapy on symptoms in patients with knee osteoarthritis: a randomised, double-blinded, sham-controlled trial

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ABSTRACT

Objectives Low-dose radiation therapy (LDRT) for benign disorders such as knee osteoarthritis (OA) is widely used in some parts of the world, despite absence of controlled studies. We evaluated the effect of LDRT on symptoms and inflammation in patients with knee OA. Methods In this randomised, double-blinded, sham-controlled clinical trial (RCT), we recruited patients with knee OA (clinical ACR criteria) in the Netherlands, aged ≥50 years, pain score ≥5/10 and non-responding to analgesics and exercise therapy. Patients were randomised 1:1 to receive LDRT (1 Gray per fraction) or sham intervention six times in 2 weeks, stratified by pain (<8 versus ≥8/10). Primary outcome was the proportion of建筑业ACR-OARSI responders, 3 months postintervention. Secondary outcomes included pain, function and inflammatory signs assessed by ultrasound, MRI and serum inflammatory markers. Results We randomly assigned 55 patients: 27 (49%) to LDRT and 28 (51%) to sham. At 3 months postintervention, 12/27 patients (44%; 95% CI 26% to 63%) in the LDRT vs 12/28 patients (43%; 95% CI 25% to 61%) in the sham group responded; difference 2% (95% CI 25% to 28%), OR adjusted for the stratifying variable was 1.1 (95% CI 0.4 to 3.2). Also, for clinical and any of the inflammatory signs, no differences were observed. Conclusions We found no substantial beneficial effect on symptoms and inflammatory signs of LDRT in patients knee OA, compared with sham treatment. Therefore, based on this RCT and the absence of other high-quality evidence, we advise against the use of LDRT as treatment for knee OA. Trial registration number NTR4574.

Key messages

What is already known about this subject?

► Low-dose radiation therapy (LDRT) is a common treatment for benign disorders such as knee osteoarthritis (OA) in some parts of the world.

What does this study add?

► This first randomised controlled study showed that LDRT does not lead to a substantial reduction of symptoms in patients with knee OA compared to sham.
► We observed no significant effects on inflammatory signs assessed by ultrasound and serum inflammatory markers.

How might this impact on clinical practice or future developments?

► We advise against LDRT as treatment for knee OA considering the absence of other high-quality evidence.

INTRODUCTION

Osteoarthritis (OA) is considered to be the most prevalent chronic joint disease and is one of the leading causes of pain and disability worldwide, with the knee being the most frequently affected joint.1,2 Since there is no disease-modifying treatment available, current knee OA management is symptomatic. However, in general, limited effect sizes for the non-surgical treatments and therapies of knee OA have been shown.3 When non-surgical treatments do not result in satisfactory reduction in symptoms, surgical options are often considered, but for many patients with knee OA, total knee replacement (TKR) is not (yet) an option, considering the balance between the potential benefits and drawbacks. In general, TKR has good clinical outcomes.4 However, given the potential drawbacks with regard to the proportion of patients being dissatisfied after a TKR, the risks of complications, the limited lifespan of a prosthesis and poorer patient outcomes after revision arthroplasty, it is generally acknowledged that TKR should not be performed too early in the disease course.4-6 Therefore, there is a clear need for more effective non-surgical knee OA treatment options.

Subclinical synovial inflammation is prevalent in OA, and it has been suggested to play an important role in the pathophysiology of the disease.7-9 Furthermore, recent studies have suggested that synovitis in knee OA might play a more significant role than previously thought, since it is associated with pain and structural damage.10-12 Thus, synovial inflammation may be a potential target for therapeutic approaches.

Previous in vitro and animal studies have shown that low-dose radiation therapy (LDR) exerts anti-inflammatory effects.13 LDRT is indeed widely used for knee OA in some parts of the world, but this procedure is relatively unknown in other parts.14,15
However, our recent systematic literature review showed that there is currently insufficient evidence available to demonstrate indisputably the effectiveness of LDRT in clinical practice, due to the absence of high-quality studies with a randomised design. For that reason, we conducted a controlled trial primarily to evaluate the effectiveness of LDRT on symptoms in patients with knee OA and, second, to examine the effects of LDRT on inflammatory signs.

METHODS

Study design

This randomised, double-blinded, sham-controlled superiority trial (RCT) was performed in two centres in Nijmegen, the Netherlands. Patient screening and data collection took place at the rheumatology outpatient clinic of the Sint Maartenskliniek. The intervention was performed at the Department of Radiation Oncology of the Radboud University Medical Center. The local Medical Ethics committee approved the study (2014-275). All patients gave written informed consent. The study was registered in the Dutch Trial Register (trial number NTR4574).

Patients

We enrolled patients from the rheumatology outpatient clinic of the Sint Maartenskliniek and through advertisements in local newspapers. Patients were eligible if they fulfilled the clinical ACR knee OA criteria. Other inclusion criteria were: (1) age ≥50 years; (2) a numeric rating scale (NRS) pain score ≥5/10 in the index knee and (3) insufficient response to both analgesics and exercise therapy. Key exclusion criteria were: treatment by a physical therapist in the previous 6 months; NRS pain score >2/10 in the contralateral knee or hips; corticosteroids in the previous 4 weeks; fibromyalgia; Kellgren & Lawrence (K&L) score >3 (see online supplementary text S1).

Patients were encouraged not to change analgesics and were discouraged using corticosteroid injections or receiving active treatment by a physical therapist during the study. However, when needed, their use was allowed and monitored.

Randomisation and masking

Included patients were randomly allocated 1:1 to either the LDRT or sham intervention using a web-response system. Allocation was stratified for pain intensity (NRS pain <8 versus ≥8/10) using stratified block randomisation (random block size of 2, 4 or 6). The total process was performed blinded for patients and study personnel. After randomisation, the unblinded radiotherapy technologist and radiotherapist were not involved in direct patient contact anymore. In the sham arm, the radiation therapy device was not activated, and these patients were in direct patient contact anymore. In the sham arm, the radiation therapy device was not activated, and these patients were in direct patient contact anymore.

Procedures

For the LDRT group, radiation therapy consisted a total dose of 6 Gray, applied in six fractions of 1 Gray, delivered every other weekday over 2 weeks, according to the German guidelines for radiation therapy of benign diseases. The sham intervention consisted of six fractions of 0 Gray.

Assessments

Assessments were planned at baseline (T0, maximally 2 weeks before start intervention), and 1, 2 and 3 months (T1, T2 and T3) postintervention. At T0 and T3, assessments visits were scheduled, while at T1 and T2, a set of questionnaires was sent. To reduce random measurement error, questionnaires were administered twice at T0 and T3. Mean scores were used for analyses. At baseline, a weight-bearing posterior-anterior fixed flexion radiograph of the index knee was taken and scored using the K&L grading system.

Clinical parameters assessed by patient-reported outcome measures

At T0, demographic, OA-related characteristics and number of comorbidities were collected. According to the long version of the Dutch Arthritis Impact Measurement Scales. Furthermore, at T0 and during all follow-up assessments, a set of PROMs was completed, including the Dutch Knee injury and Osteoarthritis Outcome Score questionnaire from which the Western Ontario and McMaster University Osteoarthritis Index scale (WOMAC) scores for pain, function and stiffness subscales were derived and standardised ranging from 0 to 100, where higher scores reflect better health status. In addition, pain intensity and the patient global assessment (PGA) of knee OA impact during the previous week were measured on a 0–10 point NRS, where 0 equals no symptoms. Quality of life was measured by the physical and mental component scores (PCS, MCS) using corresponding subscales of the 36-item Short Form Health Survey (SF-36), standardised for general population (mean 50). In addition, the patient acceptable symptom state (PASS) was taken into account. Last, patients were asked about analgesic use and intra-articular corticosteroid injections during the previous month at T1–T3. Finally, patients filled out their presumption about the assigned treatment at T3, to estimate the quality of study blinding.

Inflammatory signs

Inflammatory signs were assessed at baseline and T3 by ultrasonography, MRI and serum inflammatory markers, that is, the erythrocyte sedimentation rate (ESR, upper level women: 20 mm/hour, men: 15 mm/hour) and C reactive protein (upper level 5 mg/L).

Ultrasound (Philips IU22 with a 50 mm linear transducer (frequency 5–12 MHz)) of the knee was performed by a trained researcher. The items measured were absolute synovial effusion (mm) and synovial thickness (mm) measured at suprapatellar and both medial and lateral parapatellar recesses (mean scores, based on protocol as described elsewhere). Non-contrast-enhanced MRI of the index knee was performed at baseline and T3, using a 3.0 Tesla whole-body scanner using a knee coil (see online supplementary text S2 and S3 for details). Three inflammatory signs were assessed semiquantitatively (all graded 0–3, with 0 representing normal situation), using validated scoring systems: (1) synovitis assessed as synovial membrane thickness at four regions: medial and lateral recesses and medial and lateral suprapatellar bursa. Scores from four locations were summed to a maximum of 12, (2) effusion-synovitis, for a maximum of 3 and (3) Hoffa’s fat pad synovitis, for a maximum of 3 in line with the MRI Osteoarthritis Knee Score scoring system. The reading was performed in a paired, chronological order, blinded for patient characteristics, clinical outcomes, ultrasound assessments and treatment allocation, by one musculoskeletal radiologist with ample experience in standardised semiquantitative assessment of knee OA. No reliability score analysis for ultrasound (US) and MRI assessments was performed.

Outcomes

The primary outcome was the proportion of the OMERACT-OARSI responders at T3 (either relative improvement
in pain or function ≥50% and an absolute improvement of ≥20/100 points or two of the following: pain, function or patient’s global assessment (relative improvement ≥20% and ≥20/100 points absolute for pain and function or ≥1/10 point absolute for PGA)).

Secondary outcomes were clinical parameters (WOMAC pain, function, stiffness, PGA, NRS pain, quality of life) and inflammatory signs assessed by imaging and inflammatory markers. Also, the proportion of responders at T1 and T2 was assessed. Adverse events (AEs) and serious adverse events (SAEs) were reported by guidance of a self-composed list of potential adverse effects that might be expected after radiation therapy. Special attention was paid to skin and subcutaneous tissue disorders.

**Sample size**
This study was powered to detect a large effect of LDRT. The following assumptions were made: (1) a difference of 40% in the proportion of responders between the LDRT and sham group, (2) 40% responders in the sham group and (3) 80% power and alpha 5% level. Allowing for dropouts, we aimed to include 27 persons per group.

**Statistical analysis**
The analyses were performed blinded for assignment. Intention-to-treat primary analysis was performed to estimate the difference in proportion of responders between baseline and T3 between the two treatment groups, and 95% CIs were calculated. To adjust for the stratifying variable (NRS pain <8 versus ≥8/10), we performed logistic regression for the primary analysis and linear or logistic regression when applicable for secondary analyses, corrected for stratum. In addition, we performed sensitivity analysis adjusting for potential imbalanced confounding variables. All analyses were performed using STATA V.13.1.

**RESULTS**
From October 2015 through February 2017, 55 patients were enrolled: 27 in the LDRT group and 28 in the sham group (see figure 1). Twenty-eight patients (51%) were female, mean age 65 years (SD 9), median body mass index (BMI) 27 (IQR 24–31) kg/m². The patients were moderately to severely disabled by their disease considering their scores for pain, function and PGA (table 1). Baseline characteristics were similar between the two groups, except for a slightly lower mean age, higher median BMI, worse PGA and higher proportion of patients with ESR above upper limit in the LDRT group (table 1). During the month prior to baseline, 19 (70%) and 16 (57%) patients, respectively, used analgesics in the LDRT and sham groups. Three patients (14%) in the LDRT group and four patients (11%) in the sham group were included in the stratum with an NRS pain ≥8/10. The majority (n=39; 71%) was recruited by advertisement, and these patients showed a slightly better WOMAC pain, but comparable NRS pain, a slightly worse PCS, comparable K&L scores and

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**Figure 1** Trial profile. LDRT, low-dose radiation therapy.

were more often male (54% vs 38%) than the patients recruited from the outpatient clinic (difference mean WOMAC pain 11; 95% CI 3 to 20 and normalised PCS 6; 95% CI 2 to 10).

All 55 randomised patients completed the study with very good adherence to (sham) treatment; one patient in the LDRT group discontinued prematurely because of severe back pain after a fall at home hampering further LDRT after two fractions. Missing data were very limited: mean 1.1% per item (range 0.5%–1.8%).

**Primary outcome**

At 3 months postintervention, 12/27 (44%, 95% CI 26% to 63%) patients in the LDRT group and 12/28 (43%, 95% CI 25% to 61%) in the sham group met the primary outcome, that is, OMERACT-OARSI responder criteria; this resulted in a difference of 2% (95% CI −25 to 28%, p=0.9, table 2, figure 2). Logistic regression for response adjusting for the stratified variable (NRS ≥8/10) yielded similar results with an OR of 1.1 (95% CI 0.4 to 3.2, p=0.9) for the LDRT group compared with the sham group. Subsequently, we cannot reject our null hypothesis of no effect of the treatment, and the CIs around the between group difference show that a difference in effectiveness of LDRT versus sham treatment of over 28% is highly unlikely. Sensitivity analyses adjusting for potential confounders (age, BMI, PGA) yielded similar results (OR 1.3; 95% CI 0.4 to 4.2 for the LDRT group compared with the sham group).

**Secondary outcomes**

Both groups showed small mean improvements in the clinical outcomes pain, function and PGA between baseline and T3. No significant differences were found between the two groups regarding WOMAC pain, function and PGA at T1–T3 (T3 data are shown in table 3, also figure 3). No differences in any other secondary outcome including the inflammatory signs measured by imaging or inflammatory markers were observed (figure 4).

The number of responders at T1 and T2 are shown in table 2 and figure 2. Analyses during the third follow-up month were used in 15 (56%) and 13 (43%) patients in the LDRT group and the sham group, respectively. One patient received an intra-articular injection during follow-up (sham group second month postintervention). This patient was a non-responder at 3 months postintervention. Seventeen patients (63%, 95% CI 44% to 78%) in the LDRT group vs 23 patients (82%, 95% CI 64%...
to 92%) in the sham group reported being in PASS at T3. The number of patients who thought they had received and actually had received LDRT was comparable: 10/25 (40%, two missings) and 12/26 (46%, two missings) for the LDRT group and sham group, respectively, demonstrating adequate blinding.

**Adverse events**

The occurrence of both AEs and SAEs was comparable for both groups. Two SAEs in the sham group were observed: colon carcinoma was diagnosed postintervention in two patients, which we expect not to be related to the intervention. Three AEs occurred: one LDRT patient suffered from a collapse as mentioned above, one patient in the sham group experienced severe knee pain during and after the intervention and one patient in the sham group experienced cold sensations in the lower index leg. Local reactions were comparable in both groups. Furthermore, fatigue was recorded in six (22%) patients versus three patients (11%) in the LDRT group and sham group, respectively.

**DISCUSSION**

To our knowledge, we performed the first RCT to evaluate the effectiveness of LDRT in patients with knee OA with the radiation dose as recommended in current guidelines.37 We showed that treatment with LDRT, compared with sham, does not lead to a substantial reduction of symptoms. Considering the limits of the 95% CIs, a difference exceeding 28% of responders between groups seems unlikely. We also found no differences in changes of pain, function, PGA between the LDRT and sham groups. In addition, we found no substantial impact on imaging or laboratory inflammatory signs.

Our study has a number of strengths. These include the randomised, sham-controlled design, blinding of both patients and study assessors, use of validated outcome measures and use of a well-defined patient population. Furthermore, the number of patients needed according to our sample size calculation was met, and both lost to follow-up and missing data were low.

Some methodological choices can be challenged. First, the prespecified 40% difference margin can be considered relatively large. However, this seems clinically justifiable, because we felt that LDRT could have a place in clinical practice only when its effect would outweigh the time investment, patients’ burden, radiation exposure and costs. As a result, we cannot rule out the existence of a small effect of LDRT in knee OA. However, considering the limits of the 95% CI of our results, a difference exceeding 28% of responders between groups seems unlikely. In addition, our data do not show a significant difference in response at 1 and 2 months, although short term, almost clinically relevant between group differences up to 35%–39% cannot be excluded with 95% certainty. However, the differences in the number in responders were not confirmed by differences in continuous measures of pain and function. This limited sample size could, despite randomisation, also have resulted in imbalance of potential confounders between the two groups. However, adjusted analyses for confounding yielded similar results.

It can be argued that the absence of effect of LDRT in our study reflects a poor choice of patient population, treatment or outcome measures. However, we did include the relevant patient population, being patients with established knee OA, considering the quite severe baseline symptoms, the K&L scores and
Osteoarthritis

Figure 3  Mean scores of WOMAC pain, function and stiffness at baseline, 1, 2 and 3 months postintervention with its SD. LDRT, low-dose radiation therapy; WOMAC, Western Ontario and McMaster University osteoarthritis index scale, score 0–100 with higher scores indicating better scores.

Figure 4  Median (IQR) sum scores of synovial effusion and thickness (both US in mm) and effusion-synovitis (MRI, score 0–12) at baseline and 3 months postintervention. LDRT, low-dose radiation therapy; •, outlier.

physically impaired but not mentally impaired quality of life. In addition, the dose of the LDRT is comparable with that used in previous studies and that currently recommended in clinical practice. Also, a follow-up of 3 months seems adequate, as short-to-medium term effects were to be expected. A valid point of criticism could be the low-to-moderate sensitivity and specificity of the OMERACT-OARSI responder criteria as this could have led to misclassification and, subsequently, underestimation of the effectiveness of LDRT. However, these are the best and most often used clinical outcome measures in knee OA intervention studies. Considering also the lack of effect of LDRT in all other outcome measures, we consider our results very robust.

How should our results be interpreted in the view of existing evidence on LDRT in knee OA treatment? There have been several studies on LDRT in knee OA showing improvement of pain and/or function; however, all of them suffered from methodological shortcomings, that is, uncontrolled and/or retrospective design without blinding and non-validated single-item outcome measures. Therefore, we concluded recently in a systematic literature review that there is insufficient high-level evidence for a positive effect of LDRT on pain and functioning in patients with OA. In addition, two low-quality RCTs published in the 1970s, relating to patients suffering from a range of painful locomotor ailments including patients with knee OA, showed no effect of radiation therapy as used at that time with a relatively high dose. Of note, we found a substantial 3 months response of 40% in both groups, illustrating the substantial effect of mainly a placebo effect and regression to the mean.
In view of the absence of other high-level quality evidence in favour of LDRT, we hypothesise that these two effects are also responsible for the previously reported improvements of LDRT on symptoms in studies suffering from several methodological shortcomings. This is in accordance with previous research, and in particular for rather invasive interventions such as LDRT, which are associated with higher placebo effects.21 22 In conclusion, we consider our results as valid, in contrast to previous clinical studies.

The external generalisability of our findings is strengthened by the similarity of baseline characteristics between current patients and patients previously included in previous OA research. However, selectivity of the sample could have influenced this external generalisability, given the relatively high proportion of men as well as better baseline pain and physical quality of life of patients recruited by newspaper advertisement compared with outpatients. Nevertheless, this mixture of recruitment strategies attracts a more heterogeneous group of patients with OA and has previously shown not to influence the efficacy of the intervention and even increases the external generalisability.23 24

In addition to the absence of clinical response, we also found no substantial impact on inflammatory signs assessed by ultrasound, MRI and serum inflammatory markers. We used validated MRI and US scores, which strengthens the internal validity of these findings.18 20 However, several weaknesses regarding these secondary outcomes should be mentioned. First, because our study was primarily not powered to detect substantial differences in inflammatory signs, we can only state that LDRT did not result in large differences between the two groups. Second, it can be debated whether we should have selected patients with a minimal threshold of inflammation at baseline. We decided not to, because an inflammatory phenotype is not well defined or validated and because synovitis is known to fluctuate during the disease course,11 and the majority of patients with knee OA have been shown to have inflammatory signs anyway.25 26 The last was also seen in our patients. In addition, our baseline values of inflammatory signs are similar to those of previous studies that selected patients with signs of synovial inflammation,19 20 Also, additional analyses comparing patients with knee OA with and without inflammatory signs at baseline yielded similar results (data not shown). Third, in general, the gold standard method for detecting synovitis is histological analysis of samples obtained by biopsy. However, non-invasive imaging techniques, including US and MRI, are reported to perform well when correlating them with histological observations of inflammation in OA.7 19 20 29

In conclusion, we were not able to show a substantial effect of LDRT on symptoms and inflammatory signs in knee OA, compared with sham treatment. Considering the limits of the 95% CIs, a difference exceeding 28% of responders between groups seems unlikely. In view of the absence of other high-level quality evidence in favour of LDRT, we advise against its use as treatment for knee OA. Because this treatment is still widely used in some countries, future efforts should focus on deimplementation of LDRT, by changing the beliefs of involved clinicians and health professionals about the efficacy of LDRT that are not based on scientific grounds. Additionally, it is important that future research should also focus on the quality of the scientific evidence of LDRT treatment for other benign (musculoskeletal) disorders, for which high-quality studies are also lacking.30

Acknowledgements We are indebted to all patients who participated in the study and Vincent HHP Strat, physician assistant, for helping to screen patients.

Contributors FHvdH and JWHL had the idea for the study. Study conception and design (EAMM, MJMM, MML-H, JWHL, AAaB, CHMVDe); inclusion and data collection (EAMM, MJMM, MML-H, SSB); literature search, data analysis, tables and figures (EAMM, MJMM) and interpretation of data (all authors); drafting of the manuscript (EAMM); critical revision of the manuscript for important intellectual content (all authors); final approval of the manuscript (all authors). All authors take responsibility for the integrity of the work and agreed to submit the article for publication.

Funding There was no external funding source for this study. The study costs were jointly covered by Sint Maartenskliniek and Radboud University Medical Center.

Disclaimer The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Competing interests None declared.

Patient consent Not required.

Ethics approval The local Medical Ethics committee Arnhem-Nijmegen, the Netherlands approved the study (2014/275).

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES


Osteoarthritis


CLINICAL SCIENCE

Development and validation of prediction models to estimate risk of primary total hip and knee replacements using data from the UK: two prospective open cohorts using the UK Clinical Practice Research Datalink

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Handling editor Prof Josef S Smolen
► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2018-213894)

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Received 7 June 2018
Revised 14 September 2018
Accepted 15 September 2018
Published Online First 18 October 2018

ABSTRACT

Objectives The ability to efficiently and accurately predict future risk of primary total hip and knee replacement (THR/TKR) in earlier stages of osteoarthritis (OA) has potentially important applications. We aimed to develop and validate two models to estimate an individual’s risk of primary THR and TKR in patients newly presenting to primary care.

Methods We identified two cohorts of patients aged ≥50 years newly consulting hip pain/OA and knee pain/OA in the Clinical Practice Research Datalink. Candidate predictors were identified by systematic review, novel hypothesis-free ‘Record-Wide Association Study’ with replication, and panel consensus. Cox proportional hazards models accounting for competing risk of death were applied to derive risk algorithms for THR and TKR. Internal–external cross-validation (IECV) was then applied over geographical regions to validate two models.

Results 45 predictors for THR and 53 for TKR were identified, reviewed and selected by the panel. 301 052 and 416 030 patients newly consulting between 1992 and 2015 were identified in the hip and knee cohorts, respectively (median follow-up 6 years). The resultant model C-statistics was 0.73 (0.72, 0.73) and 0.79 (0.78, 0.79) for THR (with 20 predictors) and TKR model (with 24 predictors), respectively. The IECV C-statistics ranged between 0.70–0.74 (THR model) and 0.76–0.82 (TKR model); the IECV calibration slope ranged between 0.93–1.07 (THR model) and 0.92–1.12 (TKR model).

Conclusions Two prediction models with good discrimination and calibration that estimate individuals’ risk of THR and TKR have been developed and validated in large-scale, nationally representative data, and are readily automated in electronic patient records.

INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis and a leading cause of disability in populations worldwide.1 Although characterised as a slowly progressive condition, recent studies have highlighted substantial heterogeneity between groups of patients in the course of symptoms,2–4 function5 and structural disease.6 Healthcare costs attributed to OA, driven largely by primary and revision arthroplasty, appear concentrated in a minority of patients.8 The development and application of prognostic models capable of identifying patients with OA at high risk of future progression is now recognised as a priority internationally and by patients, carers and health and social care professionals.9 Such models could have important clinical and research applications: better targeting of intensive non-surgical care; selection of patients for active monitoring;
timely assessment and discussion of appropriateness for referral and recruitment of ‘high-risk’ patients as part of efficient clinical trial design evaluating new secondary prevention treatments.

Models that rely on pooling data from existing clinical trials and bespoke cohorts may offer the prospect of carefully measured, highly relevant predictors and outcomes, but are limited by the availability of large, long-term studies with sufficient harmonised data. Furthermore, due to the high prevalence of OA and to time and cost constraints, models that require the collection of biomarkers, imaging or lengthy patient-reported instruments are unlikely to be implemented at scale in routine primary care, irrespective of their informativeness in research settings. An alternative approach, and the one chosen in our study, is to investigate whether data already routinely available in large, representative primary electronic healthcare databases could provide accurate predictions which are feasible for implementing in routine primary care. This approach has been used to derive and validate risk algorithms for condition-specific outcomes in other chronic non-communicable diseases and for complex events such as hospital admissions.10–18

We sought to develop and validate multivariable prediction models, based exclusively on information routinely recorded within the primary care electronic health record, to estimate the risk of primary total hip replacement (THR) and total knee replacement (TKR) in patients newly presenting with hip pain/OA and knee pain/OA in UK primary care. To achieve this, we included a novel approach to identify candidate prognostic factors recorded in the primary care patient record.

METHODS

Data source and study population
We used data from the Clinical Practice Research Datalink (CPRD) covering a representative sample of 7% of the UK general population.19 The definition20 and the selection of population were presented in online supplementary technical appendix, figures S1 and S2.

Defining THR/TKR
Primary THR and TKR were identified within CPRD using the Read code list developed and applied in CPRD by Culliford and colleagues21 and validated by Hawley et al.22 23 Details of outcome definition was presented in online supplementary technical appendix.

Candidate predictors
Candidate predictors were identified from three sources: (i) a systematic review of previously published studies (further details available in online supplementary technical appendix); (ii) potentially relevant general predictors used within 12 QRsearch risk algorithms and shown to be feasibly obtained from UK primary care (eg, sociodemographic, lifestyle related, comorbidities)10–18 24–26 (iii) a hypothesis-free record-wide association study (ReWAS) of all third-level Read morbidity and process of care codes and for prescribed medicine, third-level sections within the British National Formulary which had been recorded in ≥1% of cases in the 3 years prior to date of arthroplasty. There were 6109 third-level Read morbidity and process of care codes and 325 prescribed medications assessed. The ReWAS case-control analysis was conducted in CPRD, with replication of ‘hits’ in a separate UK regional primary care Electronic Health Record (EHR) dataset—Consultations in Primary Care Archive (further details available in online supplementary technical appendix). Morbiditp, processes of care and prescribed medications that were statistically significantly associated with THR or TKR in the screen were taken forward (online supplementary figures S3 and S5 for TKR; online supplementary figures S4 and S6 for THR). The candidate predictors were assessed for clinical relevance by a review panel including seven members (six clinicians and one lay member), and the predictors agreed as relevant by ≥4 members were included in the modelling stage (online supplementary table S1). This process identified 29 candidate predictors for THR and 34 for TKR. These were extracted from records in the 3 years prior to index consultation.

Statistical analysis for model derivation
Primary THR and TKR occurring since the patients’ index consultation for hip pain/OA and knee pain/OA in primary care were treated as time-to-event outcomes in the THR and TKR models, respectively. Statistical method of predictor selection was presented in online supplementary technical appendix.

We formed the risk (cumulative incidence) equations for predicting an individual’s 10-year probability of primary THR and TKR since the index consultation for hip pain/OA and knee pain/OA, by using the developed model’s baseline cumulative incidence function (CIF) at 10 years, along with the estimated regression coefficients ($\beta$) and the individual’s predictor values (X) using the following equation27:

$$\text{CIF}\left(t = 10\right) = 1 - \left(1 - \text{CIF}_0\left(t = 10\right)\right) \exp(X\hat{\beta})$$

Validation of prediction models
We assessed the model discrimination using Harrell’s C-statistic and the model calibration using calibration slope (details in online supplementary technical appendix)28–30 over the 10 years of follow-up.

We assessed the apparent performance of the models; that is, the observed performance in exactly the same data used to develop the model. However, we also used an internal–external cross-validation (IECV) approach (online supplementary technical appendix) to evaluate the two derived prediction models over 13 geographical regions in the UK (presented in table 1).31–33

Multiple imputation using chained equations was applied to handle missing values, and the imputation model included all candidate predictors and outcome (online supplementary technical appendix).34

Based on the 15 509 THRs and a total of 73 predictor parameters and 18 289 TKRs and 79 predictor parameters, we had an effective sample size of 212 events per predictor parameter for the THR derivation cohort and 232 events per predictor parameter for the TKR derivation cohort, above the minimum requirement suggested by Peduzzi.35

In a sensitivity analysis, we assessed the models’ performances when including patients with a THR and TKR within the first 2 years after the index consultation (an exclusion criteria for the main analysis). In the other sensitivity analysis, we derived model coefficients by applying final predictors into patients with a THR and TKR within the first 2 years after the index consultation (an exclusion criteria for the main analysis).

We used Stata MP V.15.1 version for all statistical analyses. This study was conducted and reported in line with the transparent reporting of a multivariable prediction model for
Table 1 Characteristics of study populations for the primary total knee replacement (TKR) model and primary total hip replacement (THR) models

<table>
<thead>
<tr>
<th>Predictor</th>
<th>THR model</th>
<th>TKR model</th>
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<tbody>
<tr>
<td>N=301 052</td>
<td>N=416 030</td>
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</tr>
</tbody>
</table>

Outcome, n (%)  
Median follow-up duration (range), years  
Gender (female)  
Ethnicity  
Region  
Smoking status  
Drinking status  
Physical activity

Table 1 Continued

<table>
<thead>
<tr>
<th>Predictor</th>
<th>THR model</th>
<th>TKR model</th>
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</thead>
<tbody>
<tr>
<td>N=301 052</td>
<td>N=416 030</td>
<td></td>
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</tbody>
</table>

Osteoporosis  
Knee effusion  
Diabetic foot  
Bleed  
Scoliosis/kyphosis  
Development dysplasia of the hip  
Chondrocalcinosis  
Hip OA for THR model/knee OA for TKR model  
Hand OA  
Generalised OA  
Other joint OA  
Recorded diagnosis of non-specific OA  
Low back pain  
Hypertension  
Atrial fibrillation  
Congestive cardiac failure  
Venous thromboembolism  
Valvular heart disease  
Joint injection  
Knee arthroscopy  
ACL reconstruction  
Phenytoin  
Physiotherapy  
Corticosteroids  
Glucocorticoids  
Antidepressant  
Analgescics  
Weak combination opioids  
Moderate combination opioids  
Strong very strong combination opioids  
Hormone treatment  
Bisphosphonates  
Topical NSAIDS  
NSAIDS  
Other  
Drsugs for rheumatoid disease and gout  
NSAIDS  
COX2  
Prostaglandins and oxtocics  
Rheumatoid factor test  
Age, mean±SD, years  
Body mass index, mean±SD, kg/m²  
Charlson comorbidity index, median (interquartile)  
Number of consultations, median (interquartile)  
Number of referrals, median (interquartile)  
Polypharmacy, median (interquartile)  
Missing information: body mass index  
Continued
Table 2: Adjusted subdistribution hazard ratios and final model coefficients

<table>
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<th>Beta coefficient</th>
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<td>Final model for primary total hip replacement</td>
<td>Gender: women vs men 1.00 (0.96 to 1.04)</td>
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<td>Light smoker</td>
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<td>−0.283425</td>
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<td>Drinking status</td>
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<tr>
<td>Ex-drinker</td>
<td>1.01 (0.91 to 1.11)</td>
<td>0.008542</td>
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<td>Light drinker</td>
<td>1.18 (1.13 to 1.24)</td>
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<td>1.36 (1.18 to 1.56)</td>
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<td>−0.154314</td>
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<td>Alcohol consumption</td>
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<tr>
<td>No</td>
<td>0.85 (0.80 to 0.90)</td>
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<tr>
<td>Depression</td>
<td>0.85 (0.80 to 0.90)</td>
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<td>NSAIDS/COX2</td>
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<td>COX2</td>
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<tr>
<td>Hormone treatment: yes vs no</td>
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<td>(Age/10)^3</td>
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<td>(Body mass index (BMI/10)^2</td>
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<td>(BMI/10)^3</td>
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<td>−0.024987</td>
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<td>(Charlson comorbidity index+1)/10</td>
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<td>(Charlson comorbidity index+1)/10</td>
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<td>(Number of referrals+1)/10</td>
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<td>(Number of consultations+1)/1000</td>
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</tr>
<tr>
<td>(Number of consultations+1)/1000</td>
<td>–</td>
<td>−0.017409</td>
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</table>

Individual prognosis or diagnosis (TRIPOD) guidelines (online supplementary file 2).10

RESULTS

Study population

Table 1 summarises the baseline characteristics of the study populations, showing broadly similar characteristics between THR and TKR cohorts.

Model development

Of 45 candidate categorical predictors of primary THR, 26 were excluded due to multivariable −1%≤PAR≤1% (online supplementary table S2). Of the remaining 19 categorical predictors and 6 continuous predictors considered for inclusion in the multivariable prediction model, 14 categorical predictors and 6 continuous predictors were retained after backward elimination (table 2). Previous hip injury recorded within 3 years prior to index consultation was a strong predictor of increased risk of future primary THR (adjusted subdistribution HR 1.54, 95% CI 1.40 to 1.69). Age at index consultation and body mass index (BMI) showed non-linear adjusted associations with THR, peaking at 75 years and 47 kg/m² respectively (online supplementary figure S7).

Of 53 candidate categorical predictors of primary TKR, 20 were excluded due to multivariable −1%≤PAR≤1% (online supplementary table S3). Of the remaining 33 categorical predictors and 6 continuous predictors entered into the multivariable prediction model, 14 categorical predictors and 5 continuous predictors were retained after backward elimination (table 2). Oral NSAID and opioid analgesic prescriptions, intra-articular injections and previous arthroscopic knee surgery in the 3 years prior to index consultation were strong predictors of increased risk of future primary TKR. Age and BMI showed non-linear adjusted associations, peaking at 70 years and 40 kg/m² respectively (online supplementary figure S8).

Apparent predictive performance of the models

Our final THR prediction model was able to discriminate between patients with and without a primary THR with a C-statistic of 0.73 (95% CI 0.72 to 0.73); our final TKR prediction model was also able to discriminate between patients with and without TKR with a C-statistic of 0.79 (0.78 to 0.79) over the 10-year follow-up period. The calibration slope was 1.00 (0.98 to 1.02) and 1.00 (0.99 to 1.01) for THR and THR, respectively, as we would expect.

Internal–external cross-validation

The internal-external cross-validation revealed that the C-statistic was similar in each of the 13 geographical regions, ranging from 0.70 (0.68 to 0.72) to 0.74 (0.73 to 0.75) for the THR model (figure 1 left panel) and between 0.76 (0.73 to 0.79) and 0.82 (0.80 to 0.84) for the TKR model (figure 1 right panel). After meta-analysis, the summary C-statistic was 0.72 (0.72 to 0.73) for the THR model and 0.78 (0.77 to 0.80) for the TKR model. Based on the 95% prediction intervals, if the models


Table 2 Continued

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Subdistribution hazard ratio (95 CI)</th>
<th>Beta coefficient</th>
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<td>(Number of BNF chapters+1)/10)^−2</td>
<td>–</td>
<td>0.110533</td>
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<tr>
<td>(Number of BNF chapters+1)/10)^−2*ln((number of BNF chapters+1)/10)</td>
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<td>0.046402</td>
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<td>Ethnicity</td>
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<td>White</td>
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<tr>
<td>Other ethnicity group</td>
<td>0.90 (0.75 to 1.08)</td>
<td>−0.105427</td>
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<tr>
<td>Not recorded</td>
<td>1.04 (1.01 to 1.08)</td>
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<td>Non-smoker/not recorded/ex-smoker</td>
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<td>Light smoker</td>
<td>0.75 (0.64 to 0.89)</td>
<td>−0.281159</td>
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<tr>
<td>Moderate/heavy smoker</td>
<td>0.75 (0.71 to 0.80)</td>
<td>−0.281584</td>
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<td>Drinking status</td>
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<td>Ex drinker</td>
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<td>Diabetes mellitus: yes vs no</td>
<td>0.88 (0.84 to 0.93)</td>
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<td>Mental disorders: yes vs no</td>
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<tr>
<td>Anxiety</td>
<td>0.76 (0.73 to 0.80)</td>
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<td>Depression</td>
<td>0.85 (0.81, 0.89)</td>
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<td>Previous knee injury: yes vs no</td>
<td>1.29 (1.24 to 1.35)</td>
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<tr>
<td>Recorded diagnosis of joint-specific OA</td>
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<tr>
<td>No/not recorded</td>
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<td>Hip OA</td>
<td>0.59 (0.55 to 0.63)</td>
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<td>Hand OA</td>
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<td>No prescription</td>
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<td></td>
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<td>1.27 (1.19 to 1.36)</td>
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<td>(BMI/10)^2</td>
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<td>(BMI/10)^3</td>
<td>–</td>
<td>−0.047226</td>
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Continued
Osteoarthritis

Figure 1  C-statistics in 13 validation cohorts and the overall estimation across validation cohorts. The left panel is for THR model and the right is for TKR model.

discrimination with C-statistics of greater than 0.70 for both models. To our knowledge, these are the first such risk prediction tools, for primary THR and TKR in osteoarthritis developed in large-scale cohort data.

Strengths and limitations of study

Our study was based on a large, representative and contemporary UK population with data obtained from a validated research database.19 Risk prediction tools relying on routinely collected primary care data are more readily implementable in primary care practice10, and this was an important motivation for our study design. Potential limitations include missing predictor data and known predictors that are not measured or recorded in the primary care EHR. Around 5% of cohort participants did not have a recorded value for BMI in the 3 years prior to index pain/osteoarthritis consultation, but we found little difference in findings between complete dataset and multiple imputed datasets. We assumed no consultation record of a morbidity or prescription meant, there had been no such event within primary care. Although it is a fairly standard approach to use the most recent record for time-varying exposures that are likely to be generally stable, this approach might still be conservative (ie, underestimate the exposure–outcome association) to the extent that it misclassifies the exposure level relevant to the outcome (eg, lifetime cumulative exposure to smoking).

Primary THR and TKR are complex, multiply determined outcomes and can be considered as a composite measure of osteoarthritis progression, since these procedures are indicated for a combination of pain, functional disability, impact on quality of life, radiological changes and failed conservative treatment.87 Joint replacement is an important outcome of osteoarthritis, and this is reflected in its role when judging the validity of imaging-related primary endpoints for clinical trials of structure-modifying drugs.37 However, it is important to recognise that a proportion of individuals with progressive OA may not be offered, or accept, TKR/THR. The receipt of TKR/THR can also reflect extraneous factors such as patient age, sex, ethnicity, willingness to undergo surgery, comorbidity, patients’ needs, patients’ coping skills, physician effect and prevailing supply-side factors.38 We found adjusted rates of THR and TKR were lower given the following patient characteristics at or before index hip/knee consultation: age over 80–85 years, non-white ethnicity,39 higher levels of comorbidity (including diagnosed mental health disorder, generalised OA and low back pain) and very high levels of obesity. Osteoarthritis progression in the context of these factors will be underestimated by risk algorithms based on the outcome of receipt of primary joint replacement.
A strength of our study was the comprehensive identification of candidate predictors from a variety of sources including a novel hypothesis-free ‘ReWAS’ study with replication in an independent primary care EHR dataset. This latter technique yielded a small number of candidate predictors not previously reported (eg, arthroscopy). ReWAS also confirmed known prognostic factors or suggested prognostic factors that were most likely proxy markers for known predictors not obtainable from the EHR (eg, analgesic prescriptions as a proxy for pain severity). All such ‘hits’ had to be judged clinically relevant by review panel of clinicians and lay member in order to be included in the modelling stage. Unsurprisingly, many candidate predictors of future primary THR or TKR previously identified in the literature were not routinely available within the primary care record. These included multi-item patient-reported measures of pain severity, structural disease markers from plain X-rays or MRI and measures of occupational and leisure time physical activity. It is not known if their inclusion would significantly improve model performance.

Comparison with other studies

The majority of relevant previous studies have focused on one or more potential causal exposures for future joint replacement. We identified only three small studies that had previously derived and reported a multivariable prediction model for total hip or knee replacement based mainly on patient-reported and imaging variables. Although the overall performance of the prognostic model is of primary importance, the direction and magnitude of association between some of the included predictors and outcome deserves comment. It must be recognised though that these associations are not intended to be, and cannot be interpreted as, valid estimates of causal effect (total, direct or indirect) on primary hip/knee replacement: they are chosen for their informativeness in predicting primary THR/TKR. They may or may not be causal or reversible; all associations were conditioned on having an index consultation for hip or knee osteoarthritis/pain; each coefficient was adjusted for all covariates in the model, but the minimally sufficient set of covariates needed to adjust for confounding would likely differ for each (the ‘table 2 fallacy’). With these concerns in mind, we note that adjusted rates of THR and TKR were lower among moderate/heavy current smokers and higher among those with a previous injury. Prior arthroscopic knee surgery was strongly associated with future TKR, an association which may include a very small direct causal effect but which otherwise we interpret as reflecting a mixture of disease severity, risk of future progression and willingness to undergo a surgical procedure for the knee.

Implications

Our newly developed risk algorithms could have important applications in clinical practice by helping direct annual monitoring, intensive non-surgical care and timely assessment and discussion of the need for surgical referral to those most at risk of progression. The algorithms can specifically identify the individuals who, in the context of current healthcare policies and resources, are at higher risk of future joint replacement, and therefore can be targeted for individual care ranging from earlier surgery to non-invasive care that might postpone the need for surgery. The hypothetical higher risk individual illustrated in online supplementary table S6 and S7, might, for instance, be targeted for a programme of more intensive multimodal therapy including graded supervised exercise and supported weight loss. The algorithm also uses future joint replacement as a proxy for future progression of osteoarthritis, and therefore potentially attempting to identify individuals more broadly who can be targeted for more intensive monitoring and interventions that might prevent such future progressions and severity regardless of whether...
they would actually have had a joint replacement. For each of these clinical activities, a more targeted approach based on risk of progression may help. Monitoring of patients with osteoarthritis for progression of symptoms and impact is regarded as an important aspect of quality of care, but current The National Institute for Health and Care Excellence guidance would result in this being applied to a very large number of patients. Consideration for joint replacement should only be made after proper conservative care. While consistent evidence supports the effectiveness for knee OA of supervised, individually tailored exercise programmes progressed over several visits, many patients do not receive this partly due to limited physiotherapy resource and lack of referral. For many patients, joint replacement will still be the most cost-effective intervention and an earlier recognition of patients’ risk of future joint replacement may facilitate more timely assessment and discussion of appropriateness for referral. However, we caution against over-reliance on these risk algorithms, particularly for individuals whose characteristics mean that they will not be candidates for surgery despite experiencing progressive disease, and against their crude application to ration what are highly cost-effective procedures of primary THR and TKR for osteoarthritis.

Conclusions
We have developed and validated two new risk prediction equations to quantify the absolute risks of primary THR and TKR in patients’ newly presenting with hip pain/OA or knee pain/OA in the primary care setting. The models have the advantage of being based on information routinely available in UK primary care EHR, making them potentially implementable for automatic risk calculation in electronic medical record software. They can be used to identify patients at high risk of end-stage OA for further assessments and intensive non-surgical intervention. The algorithms are readily amenable to further external validation in many developed countries that have routine records available for research. Further research is warranted to evaluate the clinical outcomes and cost effectiveness of using these risk equations in primary care.

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Acknowledgements
The authors would like thank Professor Peter Croft and Professor Elaine Hay for insightful comments on the draft manuscript. This study is based in part on data from the Clinical Practice Research Datalink obtained under license from the UK Medicines and Healthcare Products Regulatory Agency. The interpretation and conclusions contained in this study are those of the authors alone.

Contributors
GMP, KPJ, and DY designed the study. KS and RR designed the validation approach. KS supervised the data analysis. CW as the PPI member attended each stage of the study (study design, reviewed the research protocol and predictors, review and revised the manuscript). DY performed the analysis and drafted the manuscript. JB, JE, KPJ, and GMP defined code lists for predictors and entry criteria, and outcome. JB, JE, CM, VT, VU, DPA, and CW supplemented predictors, reviewed and selected predictors. All authors interpreted the findings. All authors contributed to revision of the paper and have approved the final version. All authors were involved in the interpretation of the data, contributed towards critical revision of the manuscript, and approved the final draft.

Funding
This study was funded by NIHR School for Primary Care Research Funding Round 9 (Project No: 258) and by Public Health England. CDM is funded by the NIHR Collaborations for Leadership in Applied Health Research and Care West Midlands, the NIHR School for Primary Care Research and a NIHR Research Professorship in General Practice (NIHR-RP-2014-04-026). JE is a NIHR Academic Clinical Lecturer. The views expressed in this paper are those of those authors and not necessarily those of the NHS, the NIHR, Public Health England, or the Department of Health. This research is funded by the National Institute for Health Research School for Primary Care Research (NIHR SPHR).

Competing interests
None declared.

Patient consent
Not required.

Ethics approval
This project was approved by the Independent Scientific Advisory Committee (reference number: 15_211) for the CPRD data.

Provenance and peer review
Not commissioned; externally peer reviewed.

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TRANSLATIONAL SCIENCE

Single-cell RNA-seq analysis reveals the progression of human osteoarthritis

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ABSTRACT

Objectives Understanding the molecular mechanisms underlying human cartilage degeneration and regeneration is helpful for improving therapeutic strategies for treating osteoarthritis (OA). Here, we report the molecular programmes and lineage progression patterns controlling human OA pathogenesis using single-cell RNA sequencing (scRNA-seq).

Methods We performed unbiased transcriptome-wide scRNA-seq analysis, computational analysis and histological assays on 1464 chondrocytes from 10 patients with OA undergoing knee arthroplasty surgery. We investigated the relationship between transcriptional programmes of the OA landscape and clinical outcome using severity index and correspondence analysis.

Results We identified seven molecularly defined populations of chondrocytes in the human OA cartilage, including three novel phenotypes with distinct functions. We presented gene expression profiles at different OA stages at single-cell resolution. We found a potential transition among proliferative chondrocytes, prehypertrophic chondrocytes and hypertrophic chondrocytes (HTCs) and defined a new subdivision within HTCs. We revealed novel markers for cartilage progenitor cells (CPCs) and demonstrated a relationship between CPCs and fibrocartilage chondrocytes using computational analysis. Notably, we derived predictive targets with respect to clinical outcomes and clarified the role of different cell types for the early diagnosis and treatment of OA.

Conclusions Our results provide new insights into chondrocyte taxonomy and present potential clues for effective and functional manipulation of human OA cartilage regeneration that could lead to improved health.

INTRODUCTION

Osteoarthritis (OA) is the most common chronic condition associated with ageing and progressive joint dysfunction and the one with the greatest socioeconomic cost. OA is primarily characterised by disordered articular cartilage homeostasis with subsequent inflammation and degradation.1–4 Cartilage is a physiologically non-self-renewing avascular tissue and consists of chondrocytes.5–8 Despite accumulating reports on chondrocytes that have identified strategies to predict and modify OA progression, effective measures of disease-modifying OA diagnoses and outcomes are still lacking. A better understanding of the pathophysiology of inflammation and the mechanisms underlying the role of chondrocytes in the process leading to OA is of critical importance. Nevertheless, intense efforts have focused on the transplantation of tissue-engineered cartilage derived from stem cells and chondrocytes for cartilage regeneration in treating OA.6–8 However, OA cartilage cell-type composition, biochemical markers that can effectively predict OA and the cellular heterogeneity leading to OA progression remain largely unknown.

Articular cartilage is derived from condensed mesenchymal stem cells (MSCs), which subsequently differentiate into chondrocytes.9–10 Following this chondrogenesis, cells produce a collagenous extracellular matrix, a ground substance containing abundant collagen and proteoglycans. Studies have shown that the chondrocyte subtypes in articular cartilage include proliferative chondrocytes (ProCs), prehypertrophic chondrocytes (preHTCs) and hypertrophic chondrocytes (HTCs).11–13 ProCs are mainly found in the proliferative zone of growth plates, preHTCs have the capacity to modulate the onset of hypertrophic differentiation, while HTCs could regulate the mineralisation of the surrounding matrix in cartilage. Recently, senescent cells (SNCs) and cartilage progenitor cells (CPCs) were identified.14–17 SNCs assembled in OA cartilage were found to be cell-cycle-arrested and to exhibit features of a senescence-associated secretory phenotype.14 Selective elimination of SNCs attenuates OA development.14 15 whereas CPCs, which are specialised in their capacity for self-renewal, have the ability to differentiate along multiple lineages, express stem-cell-related surface markers and contribute to the maintenance of OA cartilage repair and homeostasis.15 16 These different chondrocyte subtypes were historically defined by a combination of their developmental origin, localisation, physical properties, morphology and molecular functions. However, because of their phenotypic heterogeneity and the limited number of available markers to identify, isolate and manipulate these cells, how to define the types of articular cartilage chondrocytes in human OA has still not been fully determined. These unknown parameters affect the ontogeny and function of each chondrocyte population in OA pathogenesis.

To study the molecular programmes involved in the relationships among cell populations, we performed single-cell RNA sequencing (scRNA-seq). This allowed us to better elucidate the endogenous heterogeneity of chondrocytes in human OA cartilage, uncover novel phenotypes of OA chondrocytes with defined markers and refine existing classifications. Notably, using...
computational analysis, we identified the relationship between the transcriptional programmes of OA landscapes and clinical outcomes. Therefore, our results offer an unbiased atlas of cartilage chondrocytes and shed light on diagnostic and therapeutic options for human OA.

MATERIALS AND METHODS
Single-cell RNA-seq library construction and sequencing
Sequencing libraries were generated following a modified single-cell tagged reverse transcription (STRT) protocol as previously reported.18–20 Briefly, after chondrocyte isolation, a single chondrocyte was put into the lysis buffer using a micropipette. Reverse transcription reactions were performed using a 25 nt oligo(dT) primer anchored with an 8 nt cell-specific barcode and 8 nt unique molecular identifiers.21–23 First-strand synthesis was performed and second-strand cDNAs were then synthesized, followed by 16 cycles of amplification. The amplified cDNAs of single cells were then pooled together. Biotinylated preindexed primers were applied for further amplification of the products by 4-cycle PCR to introduce biotin tags to the 3’ ends of the amplified cDNAs. Approximately 300 ng cDNA was then sheared to 300 bp using Covaris S2 (Covaris). The 3’ terminals of amplified cDNAs were purified using Dynabeads MyOne Streptavidin C1 beads (Thermo Fisher Scientific). Libraries were constructed using a Kapa Hyper Prep Kit (Kapa Biosystems) and were then submitted to 150 bp paired-end sequencing on an Illumina HiSeq 4000 platform (Novogene).

Correspondence analysis
Correspondence analysis (CA) was performed with the R package ca.24 The input data were from a frequency table (a 49×7 matrix) in which each row corresponded to each sample, and each column corresponded to the frequency of each cluster in the specific sample. We classified samples into two groups: samples with high CA1 coordinates and samples with low CA1 coordinates, using the same method for separating samples described as online supplementary materials and methods. The classification cut-off value for CA1 was 0.27 and the corresponding log rank p value was 1, meaning that CA1 was not significant for classifying samples. We then classified samples based on their CA2 coordinates; the cut-off value was −0.23 significant for classifying samples. We then classified samples with high CA1 coordinates and samples with low CA1 coordinates, using the same method for separating samples described as online supplementary materials and methods. The classification cut-off value for CA1 was 0.27 and the corresponding log rank p value was 1, meaning that CA1 was not significant for classifying samples. We then classified samples based on their CA2 coordinates; the cut-off value was −0.23 and the log rank p value was 0.00026, indicating that CA2 was significant for classifying samples. If the CA2 coordinate of the sample was higher than the cut-off (−0.23), this sample was defined as a high-CA2 sample; otherwise, the sample was defined as a low-CA2 sample.

Other detailed experimental procedures and specific materials are described in online supplementary materials and methods, including patients, isolation of human articular cartilage chondrocytes, RT-qPCR, immunohistochemical assays, processing of single-cell RNA-seq data, identification of cell types, transcription factor (TF) analysis, identification of differentially expressed genes (DEGs) among clusters, cell cycle analysis, identification of favourable and unfavourable genes, and statistical analysis.

RESULTS
Single-cell profiling of human OA cartilage chondrocytes
To define populations and identify genome-wide gene expression patterns, we isolated human OA chondrocytes at different stages obtained from the articular cartilage of 10 patients undergoing knee arthroplasty surgery according to the standard instructions by OARSI and ICRS and performed scRNA-seq using a modified STRT strategy25–27 (figure 1A,B; online Supplementary figure S1 and supplementary table S1). In total, we sequenced 1600 individual chondrocytes and retained 1464 chondrocytes for subsequent analysis after rigorous filtration (online supplementary figure S2A–C and supplementary table S2).

To reveal transcriptional states during direct conversion between stages of OA progression, we analysed OA chondrocytes using principal component analysis (PCA) and identified a strong disease-stage progression along PC2 (figure 1C,D and online supplementary figure S2D). OA chondrocytes at all stages were spread out along PC2 and followed an expected timing, with chondrocytes at the OA early stages (0 and 1) occupying the negative region of the axis and chondrocytes at the OA late stages (3 and 4) occupying the positive region (figure 1D). Since then, genes in PC2 reflected the enrichment of OA chondrocyte disease associated genes. We then analysed the top 50 positively correlated genes and top 50 negatively correlated genes along PC2 and PC1 in these 1464 chondrocytes using hierarchical clustering. We identified four clusters (A, B, C and D) along PC2 and three clusters (1, 2 and 3) along PC1 (figure 1E and online supplementary figure S2E). Cluster 1 highly expressed genes showing a negative correlation along PC1 (ID3, HES1, JUN and others) which are mainly involved in protein binding and RNA metabolic process (online supplementary figure S2E and supplementary table S3), while cluster 2 highly expressed genes revealing a positive correlation along PC1 (KLHL21, SGMS2, ITGASID3 and others) which are associated with angiogenesis and cell motility. Moreover, clusters along PC2 were correlated with OA stages. Cluster A mainly consisted of stage 3 and 4 chondrocytes and highly expressed genes showing a positive correlation along PC2 (TNC, TGFBI, CRTAC1 and others) which are mainly involved in extracellular matrix organisation and collagen catabolism. Cluster B was mainly characterised by stage 0 and 1 chondrocytes and highly expressed genes showing a negative correlation along PC2 (FRZB, C2orf82, TF and others) which are mainly involved in skeletal system development and cellular responses to stress (figure 1E,F and online supplementary table S3), suggesting the early changes that occur during OA pathogenesis.

We also performed an adjacency network analysis and identified the relationships among the OA chondrocytes on the basis of the pairwise correlation between cells, and the results were consistent with the PCA results for PC2 presented above (figure 1C,D and online supplementary figure S2F).

To identify changes in the expression of key genes with OA progression, we performed a volcano plot visualisation of gene expression between the cells in early (stages 0 and 1) and late (stages 3 and 4) stages of OA. We identified a group of stage-specific signature genes that have a potential capacity to promote OA, including SI100A427 and HTRA128, whereas the other set of signature genes showed potential protective characteristics, including FZRB29 and CHRD1L230 (online supplementary figure S2G). To identify the critical candidate TFs involved in modulating OA pathogenesis, we next employed a TF analysis algorithm and found TFs (including DNAJC2, GZF1 and ETS2) that showed higher expression towards the later stages of OA, while other TFs, including SOX4, TRPS1 and EGR2, showed higher expression towards the early stages of OA (figure 1G,H). Taken together, these results reveal the overall pattern of transcriptome states during OA pathogenesis at a single-cell level.

Identification of human OA cartilage chondrocyte populations
To investigate chondrocyte heterogeneity in human OA cartilage, we used selected PC loadings as input and clustered cells with
Figure 1  Single-cell RNA-seq of human OA cartilage chondrocytes. (A) Schematic workflow of the experimental strategy. (B) Representative preoperative and postoperative radiographs of patients with knee OA undergoing arthroplasty. (C) PCA plot of single-cell transcriptomes based on the 500 most variable genes. S0 to S4, stage 0 to 4. (D) Beeswarm plot showing all filtered cells according to their coordinates along PC2 and coloured according to the OA stage of the cartilage sample. The cell density distribution of each stage along PC2 is shown below. (E) Hierarchical clustering of cells (rows) using the 50 most positively correlated and 50 most negatively correlated genes (columns) along PC2. Cells are classified into four clusters (left sidebar). The enriched GO terms for the genes showing the greatest correlation along PC2 are shown below. (F) The expression levels of three representative genes showing positive and negative correlations along PC2. (G) The top 10 candidate transcription factors for early-stage (S0 and S1, yellow) and late-stage (S3 and S4, purple) OA identified by the master regulator analysis algorithm (MARINa). Genes are rank-sorted according to their expression levels on the x-axis for regulators, with the p value depicting the enrichment significance. (H) Boxplots showing the expression levels of early-stage and late-stage OA candidate transcription factors and p values representing the significance of expression levels. GO, gene ontology; PCA, principal component analysis; OA, osteoarthritis.

\(t\)-distributed stochastic neighbour embedding (\(t\)-SNE), resulting in a total of seven putative cell clusters (figure 2A, online supplementary figure S3A,B), including four empirically defined populations: ProCs, preHTCs, HTCs, fibrocartilage chondrocytes (FCs) and three novel populations, named as following: effector chondrocytes (ECs), regulatory chondrocytes (RegCs) and homeostatic chondrocytes (HomCs). ECs preferentially used metabolism, RegCs preferred signalling pathways and HomCs were enriched mainly for modulating cellular homeostasis and highly expressed human circadian clock rhythm markers. Besides, we found that ECs and ProCs were abundant, while FCs were relatively rare (online supplementary figure S3C). To explore the relationship among these chondrocytes, we used the Monocle method based on the single-cell data. We found that the pseudospace trajectory axis derived from Monocle matched well with cell types and the cell arrangement in the pseudospace trajectory corresponded to spatial relationships of the cells, suggesting the pseudospace trajectory demonstrates cells’ similarity in space (figure 2B and online supplementary figure S3D). ECs were distributed in the start of the trajectory, and RegCs and HomCs existed along trajectory. ProCs occupied in the front of preHTCs and HTCs, and FCs were mainly distributed in the end.
We next analysed the OA stage distributions of the cell populations. ECs and RegCs were mainly early-stage OA chondrocytes, while preHTCs, HTCs and FCs were mainly late-stage OA chondrocytes. Moreover, the pairwise correlation analysis revealed close relationships between ECs and ProCs, RegCs and ProCs, preHTCs and FCs as well as HomCs and HTCs (figure 2C and online supplementary figure S3). We found 792 DEGs that best classified chondrocytes into the seven clusters (figure 2D). Representative markers for ECs, RegCs, ProCs, preHTCs, FCs, HTCs and HomCs were revealed (figure 2D, E and online supplementary table S4), representing new combinations of genes to distinguish OA chondrocyte populations. We then performed the immunohistochemistry assay to validate these markers for each chondrocytes population (figure 2F). The results revealed that most of ECs and RegCs were distributed in the superficial zone and ProCs were distributed in the proliferative zone. While

Figure 2 Identification of chondrocyte populations and gene signatures during human OA progression. (A) Visualisation of t-SNE coloured according to cell types for human OA cartilage single-cell transcriptomes. (B) Monocle pseudospace trajectory revealing the OA chondrocyte lineage progression coloured according to cell types. (C) Dot plots showing the stage distribution in each cluster. Heatmap showing the pairwise correlations. (D) Heatmap revealing the scaled expression of differentially expressed genes for each cluster defined in (A). Specific representative genes in each chondrocyte subsets are highlighted along the right margin. The colour scheme is based on z-scores. (E) Dot plots showing the expression of indicated markers for each cell cluster on the t-SNE map. (F) Representative immunohistochemistry assay of indicated genes in cartilage tissues. Scale bar, left, 500 µm; right, 50 µm. The scores of different areas (up, middle, down) in cartilage tissues based on the immunohistochemistry assay are shown. *p<0.05, otherwise, not significant. EC, effector chondrocyte; RegC, regulatory chondrocyte; ProC, proliferative chondrocyte; preHTC, prehypertrophic chondrocyte; FC, fibrocartilage chondrocyte; HTC, hypertrophic chondrocyte; HomC, homeostatic chondrocyte; OA, osteoarthritis; t-SNE, t-distributed stochastic neighbour embedding.
preHTCs and HTCs were distributed in the hypertrophic zone. FCs were mainly late-stage OA chondrocytes and HomCs were distributed in each layer, which were consistent with the findings above. Overall, these results identified a set of novel markers that can be applied in combination to classify chondrocyte phenotypes and reconstructed the cell-type lineage that recapitulates factors contributing to OA progression.

Identification of ECs and RegCs

Two OA chondrocyte clusters, ECs and RegCs, represent newly defined populations that decrease in abundance during OA progression (figure 2A,C). Comparing these two clusters showed that ECs can be distinguished by the signature which includes genes related to alcohol biosynthesis, skeletal system development and cholesterol biosynthesis, including C2orf82, CLEC3A and CYTL1, whereas RegCs were enriched in genes related to responses to endogenous stimuli and cellular responses to oxygen-containing compounds, such as CHI3L2, CRTAC1 and CHI3L1 (figure 3A,B and online supplementary table S3).

To decipher the specified characteristics of these two new populations of OA chondrocytes, we analysed the differences by gene set enrichment analysis (GSEA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Notably, ECs preferentially used metabolic processes consisting of steroid biosynthesis and fatty acid metabolism, whereas RegCs were enriched for signalling pathway regulation, such as Toll-like receptor, mTOR, TGF-β, p53, JAK/STAT, WNT and chemokine signalling (figure 3C). Consistent with the results above, ECs show a catabolic metabolic programme and exhibit a high level of nutrient uptake related to the canonical tricarboxylic acid cycle and a high level of amino acid metabolism (figure 3D). RegCs, by contrast, primarily use a programme of regulation and responses. The GSEA revealed that RegCs possess gene expression related to antigen-processing and antigen-presenting, and we detected the expression of the major histocompatibility complex class of genes. We found that the genes CD74, CD80, CD86 and HLA-DPA1 were expressed at higher levels in a small proportion of RegCs (figure 3E), indicating that these cells might possess immune cell functions during OA progression. Taken together, these data expand our understanding of the novel function of these new chondrocyte subsets in OA.

Determining the relationships among ProCs, preHTCs and HTCs

ProC, preHTC and HTC populations are the three empirically defined populations in human OA articular cartilage, and we next analysed the relationships among ProCs, preHTCs and
HTCs and identified distinctive cluster markers (figure 4A; online supplementary figure S4A and Supplementary file 6), including some TFs (figure 4B and online supplementary figure S4B). ProCs express a unique combination of genes that have the potential to affect RNA metabolic processes and RNA stabilisation; preHTCs distinctively express a biological adhesion and multicellular organismal process gene signature; and HTCs were found to be enriched in the expression of genes related to transmembrane transporter activity and catabolic metabolism (online Supplementary file 6). We next used the Monocle method to investigate the potential transition between cell types to determine a pseudotemporal ordering for delineating differentiation paths. The pseudotime trajectory axis derived from Monocle indicated that preHTCs represent a state between ProCs and HTCs (figure 4C and online supplementary figure S4C,D). Pseudotemporal expression dynamics of specific representative genes also marked the progression from ProCs to preHTCs to HTCs (figure 4D and online supplementary figure S4E). Taken together, these data reveal the relationships among ProCs, preHTCs and HTCs and the potential transition between these cell types.

### Two subpopulations within HTCs

As HTCs were distributed with specific discriminative markers across two subpopulations (figure 2E), we next classified HTCs into transcriptionally distinct clusters, HTC-A and HTC-B, and identified DEGs for these two subclusters (figure 4E,F and online supplementary figure S5A,B). The HTC-A cluster expressed unique markers that were enriched for genes related to cartilage development, connective tissue development and negative growth regulation. In contrast, the HTC-B subset was enriched for genes related to extracellular matrix organisation, ossification and mineralisation (online Supplementary file 7). In addition, the Monocle pseudotime trajectory revealed the progression of the HTC-A and HTC-B subsets.
Figure 5 Identification of FCs and CPCs. (A) Cells are coloured according to the expression levels of the indicated markers on the t-SNE map. (B) Classification of cells categorised as proliferative cells (coloured according to their approximate phase) and quiescent cells (coloured in grey) based on the relative expression of genes associated with the G1/S stage and G2/M stage. (C) Violin plots showing the gene expression of representative candidate marker genes of CPCs. (D) Heatmap showing the scaled expression of differentially expressed genes defining the CPC and FC subsets. The top 20 markers for CPCs and FCs are shown in the right. (E) Heatmap showing the scaled expression of differentially expressed genes defining the CPC and FC subsets. The top 20 markers for CPCs and FCs are shown in the right. (F) Representative immunohistochemical staining of the indicated markers in OA cartilage tissues (stage 0). Scale bar: left, 500 µm; right, 50 µm. CPCs, cartilage progenitor cells; ECs, effector chondrocytes; RegCs, regulatory chondrocytes; ProCs, proliferative chondrocytes; preHTCs, prehypertrophic chondrocytes; FCs, fibrocartilage chondrocytes; HTCs, hypertrophic chondrocytes; HomCs, homeostatic chondrocytes; OA, osteoarthritis; t-SNE, t-distributed stochastic neighbour embedding.

Identification of potential functions of FCs and CPCs
Genes related to one population of late-stage OA chondrocytes were enriched for genes related to collagen fibril organisation, vasculature development, catabolism and cell migration (online supplementary table S4). We validated these cells with fibroblast phenotype markers (COL1A1, COL3A1, COL5A1, S100A4), stem-cell-related surface markers (CD29, CD44, CD73, CD90, CD105), haematopoietic markers (CD34, CD45, CD133) and MSC-specific surface markers (CD106, CD146, ITGA11) (figure 5A and online supplementary figure S5D) and found that these cells expressed high levels of fibroblast-phenotype markers but low levels of MSC-specific surface markers and hematopoietic markers. Therefore, we defined these OA chondrocytes as FCs. To help clarify the process of cartilage regeneration in OA, we next analysed cell proliferation related to developmental programmes and assessed the existence of CPCs in cartilage. Each chondrocyte was scored for the expression of G1/S and G2/M phase signatures. We found a small proportion of chondrocytes that were proliferating and that were actively progressing through the cell cycle in OA cartilage (figure 5B).

We next identified stable markers of CPCs, including BIRC5, CENPU, UNE2C, DHFR and STMN1, which will be useful for investigating the role of CPCs in human OA cartilage regeneration (figure 5C; online supplementary figure S5E and Supplementary file 8).

We then assessed the gene expression and functional characteristics of these two cell types. We found that genes specifically expressed by CPCs, but not by FCs, showed specific features related to the known biological properties of CPCs, including the cell cycle, chromosome organisation, and DNA replication. In contrast, FCs expressed genes enriched for vasculature development and extracellular matrix (ECM) organisation (figure 5D and online Supplementary file 9). Taken together, these data reveal discriminative markers for CPCs and identify FCs, which have implications for cartilage repair in OA.

Characterisation of HomCs
We found that the gene expression of one cluster was enriched mainly for processes related to modulating cellular homeostasis, including regulation of the cell cycle, development, RNA
metabolism and biosynthesis, and the proportion of cells from this cluster was relatively stable at each stage of OA (online supplementary table S4). We refer to this population as HomCs. As the circadian clock system plays an important role in maintaining rhythmic behaviours and daily cycles of metabolism during OA cartilage degeneration,\textsuperscript{31} \textsuperscript{32} we hypothesised that HomCs might exhibit abilities related to the circadian clock system. Interestingly, this population of OA chondrocytes revealed a high level of expression of human circadian clock markers, such as PER1 and SIRT1\textsuperscript{32} (figure 5E). We then investigated the expression of PER1 and SIRT1 in cartilage using immunohistochemistry assays and validated the existence of HomCs in OA cartilage (figure 5F). Therefore, this population of chondrocytes might play an important role in the circadian clock system in OA cartilage, which presents a new dimension for identifying novel therapeutic targets for OA cartilage regeneration.

Clinical outcomes based on the structure of the OA landscape

To investigate the relationships between all types of OA cartilage chondrocytes and the available clinical data, we referred to the new definition for the sample severity index of OA articular cartilage that combines the Hospital for Special Surgery (HSS) knee scoring system and the OA stage of the samples (online supplementary table S1); this definition reflects the severity signature of OA progression. Based on 19,566 human protein-coding genes across the OA chondrocyte types, the patient samples were stratified into two groups: those with expression levels either above (high) or below (low) the determined cut-off values based on each individual gene profile. We identified 336 predictive genes, making them potential candidates for future studies of OA. These predictive genes were classified into two types that contributed to OA progression: 199 predictive genes related to favourable outcomes (favourable genes) and 137 related to unfavourable outcomes (unfavourable genes) (online supplementary figure S6A). Favourable genes and unfavourable genes found to be significant yielded predictive panels for clinical OA outcomes (figure 6A; online supplementary figure S6B, C and Supplementary file 10). Based on average differences in expression, we assessed the distribution of predictive genes in each cell type. We found that the distribution of favourable and unfavourable predictive genes varied among chondrocyte populations in human OA. Favourable genes were mainly expressed in ECs, RegCs and HomCs, while unfavourable genes were expressed in a large proportion of ProCs, preHTCs and FCs (figure 6B), suggesting ECs, RegCs and HomCs might protect cartilage from developing OA, while ProCs, PreHTCs and FCs might promote OA progression.

To evaluate the usefulness of the favourable genes (ADRM1 and HSPA2) and unfavourable genes (RPS29 and COL5A1) in discriminating patients with OA from health controls, we performed RT-qPCR assay to analyse the expression of the genes in cartilage tissues of stage 0, 16 health controls (cartilage from knee without OA) and 16 other controls (cartilage from knee of patients with rheumatoid arthritis). We found that there were no significant differences (online supplementary figure S7A–D). Then we analysed the expression of the candidate biomarkers using RT-qPCR and immunohistochemistry in cartilage samples of patients with OA (figure 6C–F). The results revealed that ADRM1 and HSPA2 were downregulated, while RPS29 and COL5A1 were upregulated in stage 3 cartilage samples (figure 6C–F). We next validated the gene expression in a new cohort of 21 patients with OA using RT-qPCR and found that the results were in line with the predictive findings from the first cohort (online supplementary figure S7E–H). To further evaluate the diagnostic value of the candidate biomarkers, we performed receiver-operating characteristic (ROC) curve analysis in a prospective cohort of 16 health controls and 21 patients with OA. ROC curve analysis indicated that four genes (ADRM1, HSPA2, RPS29 and COL5A1) could serve as valuable biomarkers, with the area under curve being 0.878, 0.896, 0.899 and 0.936, respectively (figure 6G).

To extend the clinical outcome analysis and determine the contribution of these OA chondrocyte populations in clinical therapeutic treatments, we next applied CA based on the cell-type frequency table derived from all OA samples (online supplementary figure S8A). We found that CA component 1 (CA1) mainly captures the tendency of high levels of FCs to co-occur with HTCs, while CA component 2 (CA2) shows a different ordering of OA chondrocyte types and OA samples driven by the high co-occurrence of samples containing HTCs, preHTCs and FCs (online supplementary figure S8B). The CA indicated that these subsets might be associated with clinical OA outcomes. To find the role of these cell types in OA progression, we grouped OA chondrocyte samples based on their CA coordinates (online supplementary figure S8C). We next evaluated CA1 and CA2 to investigate the relationship between CA dimensions and severity index values and found that CA2 was significantly associated with clinical OA outcomes (log rank p value=2.6×10\textsuperscript{−8}). Moreover, we found that higher CA2 values were correlated with worse clinical OA outcomes (online supplementary figure S8D). The CA results show that FCs, PreHTCs and ProCs had high CA2 values, suggesting that these three cell types were correlated with worse clinical outcomes in the OA, which is consistent with the results presented above (figure 6B). Collectively, these data show that the single-cell transcriptional programmes can present potential clues for treating OA.

**DISCUSSION**

Knee replacement surgery is a cost-effective procedure undertaken to relieve pain and restore joint function for symptomatic OA.\textsuperscript{33} An increasingly ageing society, economic pressure and the obesity epidemic all emphasise the demand for new strategies for the diagnosis and treatment of early-stage OA, ultimately to reduce the need for joint replacement surgery.\textsuperscript{34–36} Because the cartilage lacks an intrinsic capacity for self-repair and because there are not enough specific cell markers, a detailed understanding of the internal state of chondrocytes in OA pathogenesis is urgently needed. Here, we investigated OA chondrocytes at different stages at single-cell resolution using comprehensive gene expression profiling. We identified novel cell markers and signatures for verifying each hypothesised chondrocyte cluster. Notably, in addition to the empirically inferred chondrocyte subtypes, we identified new subtypes of chondrocytes, new markers of chondrocyte populations and signalling pathways involved in OA pathogenesis based on scRNA-seq analysis. Importantly, we identified the relationship between transcriptional programmes of the OA progression landscape and clinical outcomes, with the aim of contributing to the early diagnosis and therapeutic treatment of human OA.

Phenotypic changes in chondrocyte behaviour, such as hypertrophy and matrix calcification, can lead to the occurrence of cartilage destruction in OA.\textsuperscript{37} \textsuperscript{38} ProCs are flat and columnar chondrocytes mainly found in the proliferative zone of growth plates.\textsuperscript{39} \textsuperscript{40} The top layer of ProCs have the potential to prevent hypertrophic differentiation and the lower layers close to preHTCs and HTCs allow hypertrophic differentiation.\textsuperscript{40–42}
preHTC populations control the onset of hypertrophic differentiation. HTCs, chondrocytes characterised by their size and absence of cell division, modulate mineralisation of the surrounding matrix. In addition, HTCs attract vascular and bone-cell invasion and then undergo apoptosis and calcium deposition, ultimately triggering the progression of human OA. The lack of effective discriminative markers for ProCs, preHTCs and HTCs leads to an incomplete picture of their functions and roles during OA progression. Our study revealed different functions and specific markers among ProCs, preHTCs and HTCs and the critical TFs involved in each population. Moreover, we demonstrated that preHTCs represent the state between ProCs and HTCs. We also identified HTC-A and HTC-B populations within HTCs that possess different ontologies which has therapeutic implications for OA treatment options.

Metabolism, including anabolic and catabolic activities, is important for the maintenance of optimal cartilage function and the capacity for self-repair. During OA progression,
metabolism is drastically altered and metabolic pathways switch towards glycolysis, which contributes to impaired extracellular matrix synthesis and anabolic processes. Therefore, a deeper mechanistic understanding of metabolic pathways is necessary. In this study, we identified a new population of ECs that shows a high metabolic rate, including processes related to the TCA cycle, glycolysis and lipid and amino acid metabolism, suggesting that ECs are active in energy supply. RegCs are characterised by an antigen-presenting function and B cell and T cell receptor signalling, and we found that a small proportion of RegCs possess a high level of markers specific for the immune system, indicating that these cells might have capacities similar to immune cells. In addition, ECs and RegCs have a high expression level of favourable genes, which was associated with their protective panels in relation to OA clinical outcomes. The characterisation of these two new populations improves our understanding of the role of chondrocytes in OA.

CPCs are characterised by multilineage differentiation ability, function as reparative cells for the maintenance of cartilage homeostasis and possess capacities for responding to injury and migrating.17,32 In early-stage OA, most CPCs were found in the cartilage middle zone and near the fissures.7 The functional roles of CPCs in late-stage OA have not yet been elucidated. Moreover, the definitive identification of CPCs to track cell lineage has remained elusive. Our study identified well-defined markers for CPCs. In addition, we found another cell population, FCs, that was mainly found in the late stages of OA and possesses a high ratio of genes related to unfavourable OA outcomes and a capacity for vascularisation, indicating that FCs promote OA progression, which was supported by the CA result. Therefore, based on their characteristics and functions, comparisons of CPCs and FCs might offer clues for OA cartilage regeneration. During early OA, targeting CPCs to enhance joint resurfacing, extracellular matrix production and their intrinsic chondroprotective ability would preserve cartilage structure and function and could play an important role in OA therapy. In late-stage OA, structural changes in channel formation enable cell passage between cartilage and subchondral bone.5

Recently, multiple reports have revealed that cartilage homeostasis is under a pattern of peripheral circadian clock control.32,33,34 The rhythmic clock-controlled genes include genes involved in cartilage metabolism and remodeling.32 The rhythmic nature of joint cartilage suggests that drug targets might be rhythmically active,35 which should be taken into account in future clinical OA drug trials. In this study, we identified a HomC population that exhibited high expression levels of human circadian clock rhythm markers and favourable genes, which are enriched for cell cycle regulation, metabolic processes and development, suggesting that these cells might act as the main controllers of the circadian clock in OA progression. In addition, we revealed more targets specific to HomCs, which may have new implications for effective OA drug delivery and development.

In conclusion, our single-cell analysis allowed for the separation and identification of admixtures of OA cartilage chondrocyte populations at single-cell resolution and at the whole-transcriptome scale. Our analysis identified paths of transition, discriminative markers and TFs in relation to specific cell subsets and identified close relationships between cell types and clinical outcomes, opening new possibilities for providing important diagnostic and therapeutic strategies for OA-related lifecycle modifications and healthcare.

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microRNA-181a-5p antisense oligonucleotides attenuate osteoarthritis in facet and knee joints

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ABSTRACT

Objectives We recently identified microRNA-181a-5p (miR-181a-5p) as a critical mediator involved in the destruction of lumbar facet joint (FJ) cartilage. In this study, we tested if locked nucleic acid (LNA) miR-181a-5p antisense oligonucleotides (ASO) could be used as a therapeutic to limit articular cartilage degeneration.

Methods We used a variety of experimental models consisting of both human samples and animal models of FJ and knee osteoarthritis (OA) to test the effects of LNA-miR-181a-5p ASO on articular cartilage degeneration. Histopathological analysis including immunohistochemistry and in situ hybridisation were used to detect key OA catabolic markers and microRNA, respectively. Apoptotic/cell death markers were evaluated by flow cytometry. qPCR and immunoblotting were applied to quantify gene and protein expression.

Results miR-181a-5p expression was increased in human FJ OA and knee OA cartilage as well as injury-induced FJ OA (rat) and trauma-induced knee OA (mouse) cartilage compared with control cartilage, correlating with classical OA catabolic markers in human, rat and mouse cartilage. We demonstrated that LNA-miR-181a-5p ASO in rat and mouse chondrocytes reduced the expression of cartilage catabolic and chondrocyte apoptotic/cell death markers in vitro. Treatment of OA-induced rat FJ or mouse knee joints with intra-articular injections of in vivo grade LNA-miR-181a-5p ASO attenuated cartilage destruction, and the expression of catabolic, hypertrophic, apoptotic/cell death and type II collagen breakdown markers. Finally, treatment of LNA-miR-181a-5p ASO in cultures of human knee OA chondrocytes (in vitro) and cartilage explants (ex vivo) further demonstrated its cartilage protective effects.

Conclusions Our data demonstrate, for the first time, that LNA-miR-181a-5p ASO exhibit cartilage-protective effects in FJ and knee OA.

INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis and a leading cause of chronic pain and disability.1 The prevalence of OA is increasing; however, the aetiology of OA is not fully understood. Currently, OA drugs only target joint pain, and there are no approved therapies in clinical practice that modify disease progression. Although OA is a total joint disease affecting all of the joint tissues including articular cartilage, subchondral bone and synovium, the central characteristic is articular cartilage degeneration. Increased expression of cartilage degrading enzymes such as matrix metalloproteinase-13 (MMP13) and a disintegrin and metalloproteinase with thrombospondin motifs-4 and 5 (ADAMTS4 and 5) in articular chondrocytes catabolise the major cartilage extracellular matrix (ECM) components including type II collagen and aggrecan, respectively, leading to cartilage degeneration.3–6 Chondrocyte loss is a hallmark of OA and contributes to the catabolic phenotype as chondrocyte cell death precedes cartilage degeneration.7–9 In addition, chondrocytes in the non-calcified zone of osteoarthritic cartilage undergo hypertrophy, marked by expression of type X collagen (COL10) and accompanying MMPs and ADAMTSs,10,11 which further contributes to cartilage degeneration. With increased chondrocyte catabolic gene expression, hypertrophy and cell death, the remaining cells are unable to produce sufficient, appropriate articular cartilage ECM, leading to cartilage degradation.

MicroRNAs (miRNAs) are an evolutionarily conserved group of small non-coding RNAs that regulate gene expression.12,13 Mature miRNAs are structurally stable and essential regulators of many physiological processes.14 Increasing evidence suggests that through regulation of catabolism, cell death and ECM production, miRNAs play a significant role in OA pathogenesis.15–17 Recent studies suggest that targeting pathologically expressed miRNAs could be a therapeutic option to treat knee OA.18,19 However, there is sparse evidence supporting the targeting of miRNAs as a genuine therapeutic option for OA. Demonstrating that a specific miRNA can be regulated by a drug with high persistence, target affinity and efficacy in animal models of OA and human OA tissues is necessary for translation to clinical application. We recently identified miR-181a-5p as a critical mediator of facet cartilage degeneration during facet joint (FJ) OA.20 Specifically, our in vitro and in vivo studies showed that miR-181a-5p induces articular cartilage degeneration by promoting inflammation, cartilage catabolism and apoptosis/cell death. These observations prompted us to further test if antisense oligonucleotides (ASO) against miR-181a-5p can exhibit cartilage-protective effects in FJ and other joints such as the knee.

Inhibition of miR-181a-5p in human knee OA chondrocytes has been reported as a potential...
therapeutic target in OA, with direct activity against target genes known to suppress apoptosis.\textsuperscript{21,22} Locked nucleic acid (LNA) ASO have been successfully applied in animal studies and some have been tested in human clinical trials.\textsuperscript{23–25} LNA ASO have ribose rings that are “locked” in an optimal conformation for increased target binding affinity, stability and resistance to endonucleases. In the present study, we tested whether LNA-miR-181a-5p ASO exhibited cartilage-protective effects using a combination of human in vitro (chondrocyte culture), human ex vivo (cartilage explant culture), in vivo rat FJ OA and mouse knee OA models. Our findings, for the first time, provide crucial evidence that LNA-miR-181a-5p ASO could be a potential therapy to attenuate cartilage destruction in OA.

RESULTS

MiR-181a-5p expression is increased in degenerated human and rat facet OA cartilage

We first employed in situ hybridisation (ISH) to determine the expression of miR-181a-5p in human FJ OA cartilage. To do this, we investigated FJ OA cartilage exhibiting a moderate to severe degree of cartilage degeneration compared with control FJ cartilage exhibiting no detectable or mild degeneration.\textsuperscript{20} We determined that there was a significant increase in the number of miR-181a-5p-positive chondrocytes in FJ OA cartilage compared with control cartilage (figure 1A–C). Expression of miR-181a-5p was also significantly increased in human FJ OA cartilage compared with control cartilage, as determined by qPCR (figure 1D), validating our previous microarray and qPCR results showing that the expression of miR-181a-5p is markedly elevated with increased severity of facet cartilage degeneration.\textsuperscript{20} In addition to increased expression of miR-181a-5p, we also observed concomitant increased expression of MMP13, COL10, chondrocyte cell death/apoptosis markers poly (ADP-ribose) polymerase p85 (PARP p85) and cleaved caspase 3, and the collagen type II cleavage neoepitope, C1,2C, in FJ OA cartilage compared with control cartilage, as determined by immunohistochemistry (IHC; figure 1E–N; online supplementary figure 1).

To determine if elevated expression of miR-181a-5p and increased cartilage degeneration was conserved in a rat model of FJ OA, we induced FJ OA by needle puncture injury into two levels of the lumbar spine (L4/5 and L5/6).\textsuperscript{26} FJs harvested at 12-week post-needle puncture were analysed by ISH. We found a significant increase in the number of miR-181a-5p-positive chondrocytes in FJ OA cartilage compared with sham-operated (unpunctured) control cartilage (figure 1O–Q). Collectively, these results show that miR-181a-5p expression is elevated in both human and rat degenerated facet cartilage in FJ OA.

MiR-181a-5p expression is increased in degenerated human and mouse knee OA cartilage

The expression of miR-181a-5p in human knee and hip is increased in OA compared with normal chondrocytes.\textsuperscript{21,27} Thus, we investigated if miR-181a-5p was upregulated in degenerated OA cartilage of the knee, one of the most common joints affected by OA. We demonstrated that the number of miR-181a-5p-positive chondrocytes (ISH) as well as the expression of miR-181a-5p (qPCR) was significantly increased in human knee OA cartilage tissue compared with control knee cartilage (figure 2A–D), consistent with expression in human facet chondrocytes.

Next, we investigated the expression of miR-181a-5p in the surgical destabilisation of the medial meniscus (DMM) mouse model of OA. Joint tissues harvested at 10 weeks post-surgery were subjected to ISH to determine the expression of miR-181a-5p. We found a significant increase in miR-181a-5p-positive chondrocytes in mouse knee OA cartilage compared with sham control cartilage (figure 2E–G). These results indicate that, similar to FJ OA cartilage, miR-181a-5p expression is increased in human and mouse knee OA cartilage.

LNA-miR-181a-5p ASO partially rescue interleukin (IL)-1β-induced cartilage catabolic phenotypes in rat FJ and mouse knee chondrocytes

Since we determined that expression of miR-181a-5p is increased in rat FJ and mouse knee OA cartilage compared with control cartilage, we next tested the effect of ASO against miR-181a-5p (LNA-miR-181a-5p ASO), which have perfect sequence complementarity to rat, mouse and human miR-181a-5p. Scramble control oligonucleotides (SCO) with similar sequence length and LNA design to the ASO but lacking homology to any known mouse, rat or human miRNA or mRNA sequence were used as a control.

IL-1β is a major proinflammatory cytokine implicated in OA.\textsuperscript{28} Rat FJ and mouse knee chondrocytes were treated with or without IL-1β in the presence of LNA-miR-181a-5p ASO or SCO (online supplementary figure 2A). The expression of miR-181a-5p was significantly enhanced in response to IL-1β treatment in both rat FJ and mouse knee chondrocytes (online supplementary figure 2B). In the presence of SCO, IL-1β treatment significantly increased the expression of Mmp13 in both rat FJ and mouse knee chondrocytes; however, treatment with LNA-miR-181a-5p ASO significantly attenuated IL-1β-induced Mmp13 (online supplementary figure 2C and D). It should be noted that even though the reduction of Mmp13 expression by LNA-miR-181a-5p ASO was significant, the overall attenuation was only partial and not profound.

We next tested whether LNA-miR-181a-5p ASO could protect chondrocytes from IL-1β-induced cell death. Flow cytometry analysis was performed with rat chondrocytes stained with near-infrared (IR) dead cell stain, which detects live and dead cells based on membrane permeability. Results showed that IL-1β treatment increased cell death in rat FJ chondrocytes while coinreatment with LNA-miR-181a-5p ASO reduced the IL-1β-induced increase in cell death (online supplementary figures 2E,F and 3). To assess cell death via apoptosis in mouse chondrocytes, we used Annexin V and 7-aminoactinomycin D (AAD). Annexin V+/7-AAD− cells are indicative of early apoptotic cells, while Annexin V+/7-AAD+ label late apoptotic or dead cells. Similar to rat chondrocytes, IL-1β-induced mouse chondrocyte apoptosis and cell death was also significantly attenuated by treatment with the LNA-miR-181a-5p ASO (online supplementary figures 2G,H and 4). Collectively, these results indicate that LNA-miR-181a-5p ASO partially reverse IL-1β-induced catabolic and cell death activities in chondrocytes from multiple species (rat and mouse) and joints (facet and knee) in vitro.

IN VIVO GRADE LNA-MIR-181A-5P ASO ATTENUATE THE SEVERITY OF CARTILAGE DEGENERATION IN VIVO USING A RAT FJ MODEL OF OA

Needle puncture of the rat FJ produces OA-like pathologies including significant degeneration of facet cartilage, loss of chondrocyte cellularity and proteoglycan depletion compared with sham surgery (figure 3A & B, online supplementary figure 5) in addition to increased expression of Mmp13 and decreased expression of type II collagen (Col2a1) (online supplementary figure 6A and B). Thus, to determine whether LNA-miR-181a-5p ASO have a protective effect on cartilage degeneration in vivo, we intra-articularly injected in vivo grade LNA-miR-181a-5p ASO into rat FJs.
Figure 1  MiR-181a-5p expression is increased in degenerated facet joint (FJ) cartilage. (A) Representative safranin O/fast green-stained images of human FJ control cartilage (non or mildly degenerated FJ cartilage) and human FJ osteoarthritis (OA) cartilage (degenerated FJ cartilage). (B) Representative images of in situ hybridisation (ISH)-stained human FJ control and FJ OA cartilage probed for miR-181a-5p, U6 (positive control) and scramble (negative) control. (A and B) Scale bars: 100 µm. (C) Quantification of the percentage of cells positive for miR-181a-5p from sections stained by ISH (n=3/group). (D) Expression of miR-181a-5p in human FJ control cartilage and FJ OA cartilage assessed by quantitative real-time PCR (qPCR) (n=5/group). (E–J) Representative immunohistochemistry (IHC) images of FJ control and FJ OA cartilage stained for matrix metalloproteinase-13 (MMP13; E), type X collagen (COL10; F), cell death (apoptosis) markers PARP p85 (G) and cleaved caspase 3 (H), collagen type III cleavage (C1,2C; I) and negative control (J). n=4 FJs/group; scale bar: 100 µm. (K–N) Quantification of the percentage of cells positive for MMP13 (K), COL10 (L), PARP p85 (M) and cleaved caspase 3 (N) from IHC images. (O) Representative images of safranin O/fast green-stained control rat FJ cartilage and OA cartilage. (P) Representative images of ISH-stained rat FJ control and OA tissue sections for miR-181a-5p, U6 and scramble control (n=3/group). (O and P) Scale bars: 100 µm. (Q) Quantification of the percentage of cells positive for miR-181a-5p from sections stained by ISH (n=3/group). (C, D, K–N and Q) Data are presented as scatter dot plots (error bars denote means±SD). Significant differences in the number of positive cells or levels of expression between the control and OA groups were determined using two-tailed Student’s t-tests. *P<0.05; **p<0.01.
Figure 2 MiR-181a-5p expression is increased in human and mouse knee osteoarthritis (OA) cartilage. (A) Representative safranin O/fast green-stained human knee control and OA cartilage. (B) Representative in situ hybridisation (ISH)-stained images of human knee control and OA cartilage probed for miR-181a-5p, U6 and scramble control. (A and B) Scale bars: 100 µm. (C) Quantification of the percentage of cells positive for miR-181a-5p from sections stained by ISH. n=3/group. (D) Expression of miR-181a-5p in human knee control and OA cartilage assessed by quantitative real-time PCR (qPCR). n=5/group. (E) Representative safranin O/fast green stained images of mouse control and destabilisation of the medial meniscus-induced-OA tibias. (F) Representative ISH-stained images of mouse control and DMM-induced OA tibias for miR-181a-5p, U6 and scramble control. (E and F) Scale bars: 100 µm. (G) Quantification of the percentage of cells positive for miR-181a-5p from mouse tibias stained by ISH. n=3/group. (C, D and G) Data are presented as scatter dot plots (error bars denote means±SD). Significant differences in the levels of expression between the control and OA groups were determined using two-tailed Student’s t-tests. *P<0.05.

ASO into rat lumbar FJ following needle puncture injury-induced FJ OA or sham control (unpunctured) surgery. At 3 and 6 weeks post-needle puncture, we injected in vivo grade LNA-miR-181a-5p ASO (right side of FJ; 2 µL of 5 µg/µL per FJ) or in vivo grade SCO (left side of FJ; 2 µL of 5 µg/µL per FJ). At 12 weeks post-needle puncture, FJ tissues were collected and assessed by histology and IHC (figure 3A).

We used ISH to first confirm whether in vivo grade LNA-miR-181a-5p ASO had any effect on the detection of miR-181a-5p in FJ cartilage. We found a significant reduction in the number of miR-181a-5p positive cells in FJs injected with the in vivo grade LNA-miR-181a-5p ASO as compared with those injected with in vivo grade SCO at 12 weeks post-injury (figure 3C and D). Using qPCR analysis of the FJ cartilage, we also detected lower levels of miR-181a-5p at 6 weeks post-needle puncture in joints injected with in vivo grade LNA-miR-181a-5p ASO compared with those injected with in vivo grade SCO (online supplementary figure 7A and B).

The joint protective effects of in vivo grade LNA-miR-181a-5p ASO were evident in our injury-induced FJ OA model.
Figure 3  Intra-articular injection of in vivo grade LNA-miR-181a-5p antisense oligonucleotides (IVG ASO) attenuate facet joint (FJ) cartilage degeneration in a rat model of FJ osteoarthritis (OA). (A) Schematic of the needle puncture (injury)-induced FJ OA model and injection time-course. IVG scramble control oligonucleotides (IVG SCO) or IVG ASO were injected at 3 and 6 weeks post-needle puncture and harvested at 12 weeks. (B) Representative safranin O/fast green-stained tissue sections from sham and injury-induced rat FJs (L4/5). Scale bar: 100 μm. (C) Representative in situ hybridisation (ISH)-stained images probed for miR-181a-5p, U6 (positive control), and scramble (negative) control of rat FJ cartilage with puncture and IVG ASO injection compared with puncture and IVG SCO injection. n=3/group. Scale bars: 100 μm. (D) Quantification of the percentage of cells positive for miR-181a-5p from injury-induced rat FJs injected with IVG SCO or IVG ASO. n=3/group. (E) Representative images of safranin O/fast green-stained tissue sections from injury-induced rat FJs (L4/5 and L5/6) injected with either IVG SCO or IVG ASO. n=10 FJs/group. Scale bars: 100 μm. (F and G) Osteoarthritis Research Society International (OARSI) score (F) and cellularity (G) of sham (n=6), needle puncture without injection (n=6), needle puncture with IVG SCO and needle puncture with IVG ASO tissue sections (n=10/group). (D, F and G) Data are presented as scatter dot plots (error bars denote means±SD). Significant differences in the levels of expression between the groups were determined using two-tailed Student’s t-tests or one-way analysis of variance followed with Tukey’s post-hoc tests. *P<0.05; **p<0.01.

Histopathological analysis of FJ cartilage using safranin O/fast green staining at 12 weeks post-injury showed marked reduction in the degree of FJ cartilage degeneration [as determined by Osteoarthritis Research Society International (OARSI) scoring] associated with reduced proteoglycan loss and increased chondrocyte cellularity in the in vivo grade LNA-miR-181a-5p
In vivo grade LNA-miR-181a-5p ASO attenuate the severity of cartilage degeneration in vivo using the mouse DMM model of OA

After identifying the protective effects of in vivo grade LNA-miR-181a-5p ASO in a rat model of FJ OA, we next examined its ability to attenuate cartilage degeneration in a mouse model of trauma-induced knee OA. We used one of the most validated models of knee OA, which is the mouse DMM model. DMM surgery was performed on the right knees of 12-week-old C57BL/6J male mice, as previously reported. Similar to our in vivo FJ OA study, we intra-articularly injected in vivo grade LNA-miR-181a-5p ASO (3 µL of 1 µg/µL per knee joint) or in vivo grade SCO (3 µL of 1 µg/µL per knee joint) into mouse knee joints at 2 and 4 weeks post-DMM or sham surgery and collected the knee joint tissues at 10 weeks post-surgery (figure 4A).

Similar to the rat FJ cartilage, detection of miR-181a-5p was significantly reduced in cartilage from knee joints injected with the in vivo grade LNA-miR-181a-5p ASO compared with in vivo grade SCO, as assessed by ISH (figure 4B,C). Mouse cartilage at 4 weeks post-surgery (after an injection at 2 weeks; online supplementary figure 8A) also showed reduced detection of miR-181a-5p after joints were injected with the LNA-miR-181a-5p ASO, as assessed by qPCR, when compared with those injected with SCO (online supplementary figure 8B).

We next determined the effect of in vivo grade LNA-miR-181a-5p ASO on the pathogenesis of knee OA. There was no marked difference in the severity of cartilage degeneration between in vivo grade SCO and in vivo grade LNA-miR-181a-5p ASO in the sham surgery groups (figure 4D). In contrast, we found significant attenuation of DMM-induced cartilage degeneration (by OARSI scoring) and a significant reduction in chondrocyte loss in knee joints injected with in vivo grade LNA-miR-181a-5p ASO compared with the in vivo grade SCO-injected knees (figure 4E–G). Overall, in vivo grade LNA-miR-181a-5p ASO exhibit cartilage-protective effects in both facet and knee joints during OA.

We also performed histopathological analysis to assess the severity of synovitis in the knee OA model in response to in vivo grade LNA-miR-181a-5p ASO injection compared with the in vivo grade SCO-injected knees. We did not observe any significant differences in the degree of synovitis between the injection groups (online supplementary figure 9).

In vivo grade LNA-miR-181a-5p ASO attenuate the expression of OA phenotypic makers

Since we observed a reduction in the loss of proteoglycans and chondrocyte cellularity in both rat FJ OA cartilage and mouse knee OA cartilage injected with the in vivo grade LNA-miR-181a-5p ASO, we examined the effect of in vivo grade LNA-miR-181a-5p ASO on phenotypic markers of OA. We found a significant decrease in the percentage of cells positive for MMP13, COL10, PARP p85 and cleaved caspase 3, as well as a reduction in the expression of type II collagen breakdown marker (Cl,2,3C) in the in vivo grade LNA-miR-181a-5p ASO-injected joints compared with in vivo grade SCO-injected joints in both rat FJ and mouse knee joints, as assessed by IHC (figure 5A–T; online supplementary figures 10 and 11). These findings suggest that in vivo grade LNA-miR-181a-5p ASO may impart cartilage-protective effects by reducing chondrocyte hypertrophy, cell death and catabolic activity in vivo.

LNA-miR-181a-5p ASO reduce the expression of catabolic and cell death markers in human articular chondrocytes and cartilage

To evaluate the clinical relevance of LNA-miR-181a-5p ASO, we next investigated whether the LNA-miR-181a-5p ASO could reduce the expression of catabolic and cell death markers in cultured human OA chondrocytes. Chondrocytes were isolated from knee cartilage of patients with OA undergoing total knee replacement (TKR) surgery and treated with in vitro LNA-miR-181a-5p ASO or SCO in the presence or absence of IL-1β. We found that chondrocytes significantly increased the expression of miR-181a-5p in response to IL-1β treatment in the presence of SCO (online supplementary figure 12). In contrast to SCO-treated cultures, treatment with LNA-miR-181a-5p ASO suppressed the IL-1β-induced expression of MMP13 and PARP p85, (figure 6A–C) and partially rescued the expression of COL2A1 in response to IL-1β treatment (figure 6A). Furthermore, flow cytometry analysis showed a significant decrease in IL-1β-induced chondrocyte apoptotic/cell death markers (for Annexin V/7-AAD) in cultures treated with LNA-miR-181a-5p ASO compared with those treated with SCO (online supplementary figure 13).

To test these findings in a system that reflects the chondrocyte microenvironment, we performed ex vivo culture of human knee cartilage obtained from TKR surgery and treated the explants with LNA-miR-181a-5p ASO or SCO for 24 hours (figure 6F). Consistent with our in vitro culture studies using human knee chondrocytes, human knee cartilage explants treated with the LNA-miR-181a-5p ASO exhibited significantly reduced expression of MMP13 and COL10A1 and increased expression of COL2A1 (figure 6G–I), as compared with explant cultures treated with SCO. Overall, these results provide crucial evidence of cartilage protective effects of the LNA-miR-181a-5p ASO in preclinical animal models (in vivo) and human OA cells/tissues (in vitro and ex vivo).

DISCUSSION

In this study, we provide the first evidence that intra-articular injection of in vivo grade LNA-miR-181a-5p ASO can attenuate cartilage degeneration in preclinical models of FJ and knee OA. The in vivo grade LNA-miR-181a-5p ASO consist of a modified oligonucleotide targeting miR-181a-5p. In addition to greater retention are features that make these types of oligonucleotides promising for therapeutic use.

According to ClinicalTrial.gov (https://clinicaltrials.gov/), two clinical trials have tested the therapeutic potential of LNA-miRNAs. Miravirsen, a short LNA-miR-122 ASO for the treatment of hepatitis C, has been studied in a completed phase IIa clinical trial (Clinical trial ID: NCT01200420). TargomiRs, an LNA-miR-16 mimic developed for the treatment of malignant pleural mesothelioma or non-small cell lung cancer, has recently completed phase I clinical trial
Figure 4  Intra-articular injection of in vivo grade LNA-miR-181a-5p antisense oligonucleotides (IVG ASO) attenuate cartilage degeneration in a model of mouse knee osteoarthritis (OA). (A) Schematic of the injection time-course of the mouse destabilisation of the medial meniscus (DMM)-induced knee OA model. IVG scramble control oligonucleotides (IVG SCO) or IVG ASO were injected at 2 and 4 weeks post-surgery and harvested at 10 weeks. (B) Representative in situ hybridisation-stained images of mouse knee cartilage from DMM-surgery injected with IVG SCO or IVG ASO probed for miR-181a-5p, U6 (positive control), and scramble (negative) control. n=3/group. Scale bar: 100 μm. (C) Quantification of the percentage of cells positive for miR-181a-5p from DMM mouse knee joints injected with IVG SCO or IVG ASO. n=3/group. (D) Representative images of safranin O/fast green-stained tissue sections of DMM-induced OA mouse knee joints injected with IVG SCO or IVG ASO. n=5/group. (E and F) Osteoarthritis Research Society International (OARSI) score and cellularity of DMM-induced OA mouse tissue sections injected with IVG SCO or IVG ASO. n=5/group. (C, F and G) Data are presented as scatter dot plots (error bars denote means±SD). Significant differences between groups were determined using two-tailed Student’s t-tests. *P<0.05; **p<0.01.

Clinical trials were performed with systemic drug administration, resulting in some adverse events including loss of consciousness with Miraviren, and cardiomyopathy and cardiac ischaemia with TargomiRs. Adverse events caused by drugs are major concerns in clinical settings. Indeed, there were two phase I clinical trials with MRX34, a liposomal miR-34a mimic, as a potential treatment for advanced solid tumours (Clinical trial ID: NCT01829971) and skin cancer melanoma (Clinical trial ID: NCT02862145). However, these trials were terminated or...
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Figure 5  In vivo injection of in vivo grade LNA-miR-181a-5p antisense oligonucleotides (IVG ASO) decrease expression of cartilage catabolic, hypertrophic, cell death and type II collagen breakdown markers in rat facet joint (FJ) cartilage and mouse knee cartilage. (A–F) Representative images of immunohistochemistry (IHC)-stained tissues of needle puncture (injury)-induced FJs (at 12 weeks post-injury) injected with IVG scramble control oligonucleotides (IVG SCO) or IVG ASO for matrix metallopeptidase 13 (MMP13; A), type X collagen (COL10; B), PARP p85 (C), cleaved caspase 3 (D) and collagen type II cleavage (C1,2C; E) and negative control (F). n=4 animals/group (online supplementary figure 10). Scale bar: 100 μm. (G–J) Quantification of the percentage of cells positive for MMP13 (G), COL10 (H), PARP p85 (I) and cleaved caspase 3 (J) from IHC-stained tissues of injury-induced rat FJs injected with IVG SCO or IVG ASO. n=4/group. (K–P) Representative images of IHC-stained DMM-induced OA mouse knee joint sections (at 10 weeks post-surgery) injected with IVG SCO or IVG ASO for MMP13 (K), COL10 (L), PARP p85 (M), cleaved caspase 3 (N), C1,2C (O) and negative control (P). n=4 animals/group (online supplementary figure 11). Scale bar: 100 μm. (Q–T) Quantification of the percentage of cells positive for MMP13 (Q), COL10 (R), PARP p85 (S) and cleaved caspase 3 (T) from IHC-stained tissues of DMM-induced mouse knee OA joints injected with IVG SCO or IVG ASO. n=4/group. (G–J, Q–T) Data are presented as scatter dot plots (error bars denote means±SD). Significant differences in the quantification of percentage of positive cells were determined using two-tailed Student’s t-tests. *P<0.05; **p<0.01.

withdrawn due to adverse events. Thus, local administration is of interest, if capable of being retained in the target organ, as this would limit systemic effects of the LNA-miRs and potential adverse events of the therapies.

To our knowledge, there are no current or past clinical trials using local injection of in vivo grade LNA-miR ASO. However, previous studies of local delivery using in vivo animal studies have demonstrated encouraging therapeutic potential. For instance, intratracheally instilled in vivo grade LNA-miR-21 ASO remarkably suppressed bleomycin-induced lung fibrosis in mice. Furthermore, delivering in vivo grade LNA-miR-92a ASO to the heart via intracoronary catheter showed a more prominent protective effect for ischaemia/reperfusion injury than intravenous injection in a pig model. Consistent with the positive outcomes using a local mode of administration from these studies, intra-articular injection of in vivo grade LNA-miR-181a-5p ASO in our current study further confirmed the promising potential of in vivo grade LNA-miR ASO delivered by local injection. In vivo treatment of injury-induced rat FJ OA or trauma-induced mouse knee OA joints...
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with intra-articular injections of in vivo grade LNA-miR-181a-5p ASO significantly attenuated cartilage destruction associated with decreased expression of OA catabolic, hyper trophy, apoptotic/cell death and type II collagen breakdown markers. The ability of in vivo grade LNA-miR-181a-5p ASO to reduce catabolic and cell death activity in the cartilage is further supported by our human cartilage explant and chondrocyte culture studies obtained from human FJ and knee OA as well as rat and mouse OA chondrocytes.

This study is not without some limitations. First, minimal off-target effects on unrelated longer RNAs is one of the features of the LNA-miRNA ASO; off-target effects mediating some of the observed study outcomes remains a possibility. Second, although we clearly observed cartilage-protective effects and significant decreases in the expression of catabolic and cell death markers as consequences of in vivo grade LNA-miR-181a-5p ASO treatment, functional assays to directly examine the inhibitory activity of the in vivo grade LNA-miR-181a-5p ASO to miR-181a-5p are needed. However, studies using luciferase activity of direct target genes of miR-181a-5p, including GPD1L and PTEN (both of which suppress apoptosis), indicate that in vitro grade miR-181a-5p ASO can attenuate miR-181a-5p-mediated loss of expression and induction of apoptosis, suggesting that direct inhibition of endogenous miR-181a-5p-target binding is likely contributing to part of the responses seen in our studies. Finally, in vivo grade LNA-miR-181a-5p ASO and ISH probes for miR-181a-5p have some sequence overlap; however, the LNA-modification...
renders the ASO with higher binding affinity and stability to targets. Thus, probes to detect endogenous miR-181a-5p may not be able to compete for targets already bound in a complex with in vivo grade LNA-miR-181a-5p ASO. This suggests that the expression of endogenous miR-181a-5p may not be altered but could instead form a non-functional complex with the in vivo grade LNA-miR-181a-5p ASO and simply be undetectable.

Overall, using two distinct animal OA models in addition to human cells and tissues, we provide the first evidence of cartilage protective effects of LNA-miR-181a-5p ASO. This is the first report showing that LNA-miR-181a-5p ASO attenuate cartilage degeneration during OA across joints (knee and FJ), animal models (rat FJ) OA and mouse knee OA models) and simply be undetectable.

MATERIAL AND METHODS
Detailed experimental procedures are provided in the online supplemental materials and methods.

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Acknowledgements Authors would like to thank Amanda Weston, Kim Perry and Sarah Gabrial for their help collecting facet and knee cartilage. Authors would also like to acknowledge the help of members of the Arthritis Program at the Toronto Western Hospital (Toronto).

Contributors AN was involved in the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content and approved final version of the manuscript. YRR was involved in the design of the study, performed spine surgery in patients with LSS or LDH, provided tissues, performed MRI rading, drafting the article, revising it critically for important intellectual content and approved final version of the manuscript. SN and AS performed animal surgeries to induce osteoarthritic models, inject miRNA antisense oligonucleotide into rat facet joint and mouse knee joints, tissue dissections along with AN, drafting the article, revising it critically for important intellectual content and approved final version of the manuscript. FZ was involved to perform flow cytometry along with AN, drafting the article, revising it critically for important intellectual content and approved final version of the manuscript. ER was involved to perform histology for making sections and safranin O staining, drafting the article, revising it critically for important intellectual content and approved final version of the manuscript. SAA, RK, NH, AVP, NNM, RG and JSR were involved in the interpretation of data, drafting the article, revising it critically for important intellectual content and approved final version of the manuscript. NK was involved in the conception and design of the study, interpretation of data, drafting the article, revising it critically for important intellectual content and approved final version of the manuscript.

Funding This study was supported by grants to MK from the Krembil Foundation, the Canadian Institute of Health Research (FRN: 156299) and Campaign to Cure Arthritis via the Toronto General and Western Foundation, University Health Network, Toronto. AN and SAA are recipients of postdoctoral fellowship funding from the Krembil Research Institute (University Health Network).

Competing interests A US provisional patent application (62/299,305, filed 24 February 2016) and a PCT international patent application (PCT/CA2017/000019, filed 31 January 2017) have been filed in respect of therapeutic and diagnostic uses of miRNA-181a-5p.

Patient consent Obtained.

Ethics approval Human facet and knee cartilage were obtained under the institutional approval.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES


Short-term progression of interstitial lung disease in systemic sclerosis predicts long-term survival in two independent clinical trial cohorts

Elizabeth R Volkmann,1 Donald P Tashkin,1 Myung Sim,1 Ning Li,2 Ellen Goldmuntz,3 Lynette Keyes-Elstein,4 Ashley Pinckney,4 Daniel E Furst,1 Philip J Clements,1 Dinesh Khanna,5 Virginia Steen,6 Dean E Schraufnagel,7 Shiva Arami,7 Vivien Hsu,8 Michael D Roth,1 Robert M Elashoff,2 Keith M Sullivan,9 SLS I and SLS II study groups

ABSTRACT
Objective To assess survival and identify predictors of survival in patients with systemic sclerosis-interstitial lung disease (SSc-ILD) who participated in the Scleroderma Lung Studies (SLS) I and II.

Methods SLS I randomised 158 patients with SSc-ILD to 1 year of oral cyclophosphamide (CYC) vs placebo. SLS II randomised 142 patients to 1 year of oral CYC followed by 1 year of placebo vs 2 years of mycophenolate mofetil. Counting process Cox proportional hazard modelling identified variables associated with long-term mortality in SLS I and II. Internal validation was performed using joint modelling.

Results After a median follow-up of 8 years, 42% of SLS I patients died, and when known the cause of death was most often attributable to SSc. There was no significant difference in the time to death between treatment arms in SLS I or II. Higher baseline skin score, older age, and a decline in the forced vital capacity (FVC) and the diffusing capacity for carbon monoxide (DLCO) over 2 years were independently associated with an increased risk of mortality in SLS I. The Cox model identified the same mortality predictor variables using the SLS II data.

Conclusion In addition to identifying traditional mortality risk factors in SSc (skin score, age), this study demonstrated that a decline in FVC and DLCO over 2 years was a better predictor of mortality than baseline FVC and DLCO. These findings suggest that short-term changes in surrogate measures of SSc-ILD progression may have important effects on long-term outcomes.

INTRODUCTION
Interstitial lung disease (ILD) is the leading cause of death in systemic sclerosis (SSc), accounting for over one-third of SSc-related deaths in a multicentre observational study of over 5000 patients with SSc. In addition, ILD occurs in the majority of patients with SSc and is found in 79% of patients with SSc at autopsy. Immunosuppressant agents, such as mycophenolate mofetil (MMF) and cyclophosphamide (CYC), are currently used to treat SSc-ILD. However, randomised controlled trials (RCTs) have demonstrated that while some patients experience improvement in lung function after treatment with MMF or CYC, other patients experience ILD progression despite treatment with immunosuppression. Moreover, not all patients with ILD develop symptoms or will have progressive disease that leads to death even in the absence of treatment.

The present study sought to develop a mortality prediction model using data from two RCTs for SSc-ILD (Scleroderma Lung Study (SLS) I and II). Using data from RCTs (in contrast to an observational cohort) may minimise confounding due to factors that affect survival, such as timing of treatment initiation, access to healthcare, socioeconomic...
status as well as comorbid conditions. From these two RCTs with rigorous entry criteria, close monitoring of pulmonary function every 3 months for 2 years and standard treatment regimens, the hypothesis was that in this controlled setting of 300 participants with SSC and ILD new predictors of mortality could be discovered.

METHODS
Study participants
All participants enrolled in SLS I6 (NCT01762449; NCT00004563) and SLS II7 (NCT00883129) were eligible to participate in the SLS long-term follow-up study. SLS I and II included adult patients with SSC with evidence of ILD on high-resolution CT (HRCT) with a duration of disease ≤ 7 years from onset of the first non-Raynaud’s symptom of SSC. (Please see online supplementary appendix for complete eligibility criteria.) Only participants who provided informed consent were included in the present analyses.

SLS I and II study design
In SLS I, 158 participants were randomised to receive either oral CYC or matching placebo for 1 year, followed by an additional year of observation off-treatment as previously published.8 In SLS II, 142 patients were randomised to receive either MMF for 2 years or oral CYC for 1 year, followed by an additional year of placebo using a double-dummy design to maintain the blinding as reported.9

SLS I and II assessment measurements
Complete details of SLS I and II assessment measurements are in the online supplementary appendix. Forced vital capacity (FVC) (primary SLS I and II endpoint) and diffusing capacity for carbon monoxide (DLCO) (secondary SLS I and II endpoint) were measured every 3 months during the 24-month study period for both studies.6,7 HRCT thoracic imaging was obtained at baseline and 24 months in SLS II and at baseline and 12 months in SLS I. Quantitative imaging analysis (to quantify the extent of ILD) was performed as previously published and is described in the online supplementary appendix.3,11

Long-term follow-up assessment
During both the SLS I and II study periods, when the statistical centre was informed of a participant’s death, clinical research associates were asked to collect source documentation to determine the cause of death. A mortality and morbidity committee adjudicated the causes of death to determine whether the cause was related to underlying SSC, medication or another cause based on expert consensus. Following the 24-month study periods, patients or designated surrogates were contacted to assess morbidity and mortality outcomes. (Please see online supplementary appendix for further details of the long-term follow-up assessment.)

Statistical analysis
Baseline characteristics
Summary statistics were generated for baseline characteristics from the two cohorts. Group comparisons were performed using two-sample t-tests and \( \chi^2 \) tests.

Primary outcome: survival
The primary outcome was survival. The Kaplan-Meier estimate was used to generate survival curves, and the log-rank test was used to compare survival between groups. If survival status was unknown, survival time was censored at the date when the participant was last known to be alive. Cox proportional hazard models were developed to evaluate the impact of covariates shown previously to be associated with survival, including treatment, baseline Modified Rodnan Skin Score (MRSS), age, sex, race, disease duration, type of SSC (limited or diffuse), serological subtype (Scl-70 antibody-positive, RNA polymerase III antibody-positive), % predicted values for FVC or DLCO, and the radiographic quantitative extent of ILD/fibrosis. Models for FVC and DLCO were first fit using the baseline measure as the covariate of interest, and then in separate models using the longitudinal assessments over 24 months as a time-varying covariate. Final models were validated by fitting joint models for longitudinal and survival data using the SAS macro, JMFit9 (see online supplementary appendix for details on variables’ definitions and selection and joint modelling methods).

All tests were two-sided. All analyses were performed using SAS V9.4.

RESULTS
Participant characteristics
The baseline characteristics of the participants in SLS I and SLS II were fairly similar (table 1). SLS II participants were slightly older and had shorter disease durations compared with SLS I participants. While the FVC%-predicted did not differ between the two cohorts, SLS II participants had slightly more restrictive ventilatory impairment, as reflected by lower total lung capacity, despite less diffusion impairment.

Participant disposition
SLS I
Twelve years after the first patient was randomised in SLS I, 66 of 158 (42%) participants had died (CYC: 38; placebo 28). Among the 37 patients for whom the cause of death was known, 24 deaths (65%) were attributable to underlying SSC, of which 16 (CYC: 8; placebo: 8) were due to respiratory failure (table 2). Two of the deaths (1 CYC, 1 placebo) due to ‘Respiratory Failure’ were not attributed to underlying SSC. Survival status could not be determined in 34 participants. The median follow-up time for all patients in SLS I was 8 years.

SLS II
Eight years after the first patient was randomised in SLS II, 30 of 142 (21%) participants had died (CYC: 16; MMF: 14). Among the 26 patients for whom the cause of death was known, 15 deaths (58%) were attributable to underlying SSC, of which 13 (CYC: 6; MMF: 7) resulted from respiratory failure (table 2). Survival status could not be determined in 12 participants. The median follow-up time for all patients in SLS II was 3.6 years.

CYC does not improve long-term survival compared with placebo in SLS I
During the 24-month study period of SLS I, six participants randomised to CYC and six participants randomised to placebo expired.10 During the 12-year long-term follow-up period, there was no significant difference in the time to death (\( p=0.335 \) by log-rank test; figure 1A) nor the time to death or organ failure (\( p=0.539 \) by log-rank test; figure 1B) for patients randomised to CYC versus placebo in SLS I. Moreover, time to the development of organ failure did not differ between the two study arms (\( p=0.185 \) by log-rank test; online supplementary figure S1), nor did time to the development of malignancy (\( p=0.701 \) by log-rank test; online supplementary figure S2). Types/locations of malignancies in SLS
Table 1  Baseline characteristics of SLS I and SLS II participants

<table>
<thead>
<tr>
<th>Measure</th>
<th>SLS I</th>
<th>SLS II</th>
<th>P values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>n=158</td>
<td>n=142</td>
<td>0.004</td>
</tr>
<tr>
<td>Mean±SD (CYC; placebo)</td>
<td>48.5±12.3 (48.4±12.3; 47.7±12.5)</td>
<td>52.3±9.7 (52.0±9.8; 52.6±9.7)</td>
<td></td>
</tr>
<tr>
<td>Mean±SD (SLS I vs SLS II)</td>
<td>P=0.693</td>
<td>P=0.725</td>
<td></td>
</tr>
<tr>
<td>Gender (female/male) (%)</td>
<td>n=158</td>
<td>n=142</td>
<td>0.477</td>
</tr>
<tr>
<td>Mean±SD (CYC; placebo)</td>
<td>70/30 (76/24; 65/35)</td>
<td>74/26 (78/22; 70/30)</td>
<td></td>
</tr>
<tr>
<td>Mean±SD (SLS I vs SLS II)</td>
<td>P=0.111</td>
<td>P=0.248</td>
<td></td>
</tr>
<tr>
<td>Race (%)</td>
<td>158</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>Mean±SD (CYC; placebo)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean±SD (SLS I vs SLS II)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (%)</td>
<td>67 (67; 66)</td>
<td>68 (66; 71)</td>
<td>0.008</td>
</tr>
<tr>
<td>African–American</td>
<td>16 (15; 18)</td>
<td>23 (26; 20)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>4 (4; 5)</td>
<td>6 (4; 9)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>12 (14; 10)</td>
<td>1 (3; 0)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (0; 1)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Diffuse %/Limited %</td>
<td>59/41 (62/38; 57/43)</td>
<td>59/41 (55/45; 62/38)</td>
<td>0.855</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>3.2±1.1 (3.2±1.3; 3.1±1.8)</td>
<td>2.6±1.8 (2.5±1.8; 2.6±1.7)</td>
<td></td>
</tr>
<tr>
<td>FVC, % predicted</td>
<td>68.1±12.1 (67.6±11.3; 68.6±13.0)</td>
<td>66.5±9.1 (66.5±9.9; 66.5±8.3)</td>
<td>0.194</td>
</tr>
<tr>
<td>FEV1/FVC, %</td>
<td>82.8±8.0 (82.8±5.8; 82.8±7.4)</td>
<td>82.6±5.6 (83.3±5.5; 81.8±5.5)</td>
<td>0.802</td>
</tr>
<tr>
<td>TLC, % reference</td>
<td>69.6±13.1 (69.8±12.9; 69.3±13.3)</td>
<td>65.9±10.9 (65.5±12.0; 66.3±10.0)</td>
<td>0.008</td>
</tr>
<tr>
<td>DLCO, % reference</td>
<td>47.2±14.0 (47.1±13.7; 47.4±14.3)</td>
<td>54.0±12.7 (54.1±14.1; 54.0±11.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BDI (focal score, 0–12)†</td>
<td>5.7±1.8 (5.6±1.7; 5.7±2.0)</td>
<td>7.1±2.2 (7.1±2.3; 7.3±2.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HAQ-DI (score, 1–3)‡</td>
<td>0.83±0.7 (1.0±0.7; 0.7±0.7)</td>
<td>0.72±0.7 (0.7±0.7; 0.7±0.6)</td>
<td>0.176</td>
</tr>
<tr>
<td>VAS – breathlessness (0–100)</td>
<td>28.4±26.1 (27.2±24.6; 29.5±27.6)</td>
<td>24.5±28.1 (24.4±28.9; 24.5±27.4)</td>
<td>0.220</td>
</tr>
<tr>
<td>MRSS (0–51)</td>
<td>14.8±10.9 (15.6±11.4; 14.0±10.5)</td>
<td>14.7±10.5 (14.0±10.6; 15.3±10.4)</td>
<td>0.936</td>
</tr>
<tr>
<td>Lung fibrosis (QLFb) score, whole lung (WL), %</td>
<td>10.2±10.4 (10.3±10.5; 10.1±10.4)</td>
<td>8.6±6.9 (8.9±7.0; 8.3±6.8)</td>
<td>0.148</td>
</tr>
<tr>
<td>Lung fibrosis (QLFb) score, worst zone (ZM), %</td>
<td>26.5±21.9 (28.2±23.4; 25.0±20.5)</td>
<td>22.8±19.6 (22.6±19.3; 23.0±20.2)</td>
<td>0.152</td>
</tr>
<tr>
<td>Quantitative ILD (QILD) score, % WL</td>
<td>35.5±16.9 (35.8±17.1; 35.3±16.9)</td>
<td>29.5±14.0 (31.6±14.4; 27.2±13.2)</td>
<td>0.002</td>
</tr>
<tr>
<td>Quantitative ILD (QILD) score, % ZM</td>
<td>58.1±21.7 (58.2±22.3; 58.0±21.3)</td>
<td>51.2±20.3 (52.3±19.9; 50.0±20.9)</td>
<td>0.009</td>
</tr>
</tbody>
</table>
| *t-tests were used for all comparisons with the exception of gender and diffuse vs limited disease (χ² test).
| †High score denotes worse dyspnoea.
| ‡High score denotes worse function.

BDI, Baseline Dyspnoea Index; CYC, cyclophosphamide; DLCO, single-breath diffusing capacity for carbon monoxide; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; HAQ-DI, Health Assessment Questionnaire for Scleroderma-Disability Index; HRCT, high-resolution CT; MMF, mycophenolate mofetil; MRSS, Modified Rodnan Skin Score; QLFb WL, %, quantitative extent of lung fibrosis (reticulations) in whole lung on high-resolution CT; QLFb ZM, %, quantitative extent of lung fibrosis in the zone of maximal involvement on HRCT; QILD WL, %, quantitative extent of interstitial lung disease (fibrosis+ground glass opacity (GGO)+honeycombing) in whole lung on HRCT; QILD ZM, quantitative extent of interstitial lung disease (fibrosis+GGO+honeycombing) in the zone of maximal involvement on HRCT; SLS, Scleroderma Lung Studies; TLC, total lung capacity; VAS, Visual Analogue Scale.

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Table 2  Long-term morbidity and mortality outcomes of SLS I and II participants

<table>
<thead>
<tr>
<th>Subject status</th>
<th>SLS I CYC (n=79)</th>
<th>Placebo (n=79)</th>
<th>SLS II CYC (n=73)</th>
<th>MMF (n=69)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alive</td>
<td>29 (37%)</td>
<td>29 (37%)</td>
<td>50 (68%)</td>
<td>51 (74%)</td>
</tr>
<tr>
<td>Lost to follow-up*</td>
<td>10 (13%)</td>
<td>22 (28%)</td>
<td>7 (10%)</td>
<td>4 (6%)</td>
</tr>
<tr>
<td>No data available</td>
<td>2 (3%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Deceased</td>
<td>38 (48%)</td>
<td>28 (35%)</td>
<td>16 (22%)</td>
<td>14 (20%)</td>
</tr>
<tr>
<td>Death related to SSc</td>
<td>12</td>
<td>12</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Death unrelated to SSc</td>
<td>6</td>
<td>4</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>Unknown if related to SSc</td>
<td>20</td>
<td>12</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Cause of death†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory failure (ILD)‡</td>
<td>9</td>
<td>9</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Aspiration</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cancer</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Heart failure</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Renal failure</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Complications from lung transplantation</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Complications from hip fracture</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Gastrointestinal tract failure</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sepsis</td>
<td>1</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Seizures</td>
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<tr>
<td>Infection</td>
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<tr>
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<tr>
<td>Unknown</td>
<td>19</td>
<td>11</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Organ failure</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No organ failure</td>
<td>63 (80%)</td>
<td>60 (76%)</td>
<td>58 (79%)</td>
<td>58 (84%)</td>
</tr>
<tr>
<td>Any organ failure</td>
<td>14 (18%)</td>
<td>19 (24%)</td>
<td>8 (11%)</td>
<td>7 (10%)</td>
</tr>
<tr>
<td>No data available</td>
<td>2 (3%)</td>
<td>0</td>
<td>7 (10%)</td>
<td>4 (6%)</td>
</tr>
<tr>
<td>Type of organ failure†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplementary oxygen use</td>
<td>12</td>
<td>17</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Lung transplant§</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Dialysis</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total parenteral nutrition</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cardiac ablation</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Pacemaker</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Malignancy</td>
<td>7</td>
<td>6</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Development of pulmonary hypertension</td>
<td>Not collected</td>
<td>Not collected</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Median follow-up time (IQR), months</td>
<td>97.4 (37.3–121.9)</td>
<td>86.7 (32.3–120.2)</td>
<td>40.78 (24.9–54.5)</td>
<td>42.2 (36.4–58.7)</td>
</tr>
</tbody>
</table>

CYC, cyclophosphamide; ILD, interstitial lung disease; MMF, mycophenolate; SLS, Scleroderma Lung Studies; SSc, systemic sclerosis.

* These patients were not in death registries, but were unreachable.
† Subjects could have more than one cause of death and type of organ failure recorded. In SLS I CYC group, 1 person died of sepsis and respiratory failure, 1 died of heart and renal failure, 1 died of respiratory failure and renal failure, and 1 died of respiratory failure and gastrointestinal failure. In SLS I placebo group, 1 person died of respiratory failure and heart failure, 1 died of respiratory failure and gastrointestinal failure, 1 died of respiratory failure and infection, and 1 died of respiratory failure and seizures.
‡ In the SLS I study, 2 deaths (1 CYC, 1 placebo) due to ‘Respiratory Failure’ were not attributed to underlying SSc based on the case report form.
§ No patients received heart, kidney, liver or bone marrow transplants during the follow-up period.

I included the anus (n=1), colon (n=2), vulvar (n=1), prostate (n=1), sarcoma (n=1) and breast (n=1) within the CYC arm, and colon (n=1), oesophageal (n=1), lung (n=3) and Hodgkin’s lymphoma (n=1) within the placebo arm.

There is no difference in long-term survival between patients randomised to MMF versus CYC in SLS II

Over twice as many deaths occurred in the CYC arm (n=11) compared with the MMF arm (n=5) during the 24-month study period in SLS II (p=0.160 by log-rank test). During the 8-year long-term follow-up period, an additional five deaths occurred in the CYC arm compared with an additional nine deaths in the MMF arm. There was no significant difference in the time to death (p=0.627 by log-rank test; figure 2A) nor the time to death or organ failure (p=0.343 by log-rank test; figure 2B) for patients randomised to CYC versus MMF in SLS II. However, there appeared to be a separation in the survival curves favouring MMF within the first 2 years (figure 2A). There was no significant difference in the time to the development of organ failure between the two groups (p=0.692 by log-rank test; online supplementary figure S3). Two malignancies occurred during the follow-up period (MMF: n=1 thyroid cancer, n=1 papillary urothelial carcinoma; CYC: none).
Systemic sclerosis

Longitudinal assessments of FVC and DLCO predict long-term survival in SLS I and II

SLS I: Cox proportional hazards models and joint models
The basic model developed from the SLS I cohort, as described in the online supplementary methods, consisted of the following covariates: treatment arm (CYC vs placebo), baseline extent of cutaneous sclerosis (MRSS), age at randomisation (years) and sex. Among these variables, increased age and MRSS were associated with increased mortality. The following variables were independently associated with mortality in the final models: (1) baseline FVC%-predicted; (2) longitudinal assessment of the FVC%-predicted measured as a time-varying covariate over 24 months; and (3) longitudinal assessment of the DLCO%-predicted measured as a time-varying covariate over 24 months (table 3). None of the quantitative lung fibrosis/ILD scores was associated with long-term survival when added to the base model. The Akaike information criterion (AIC), which estimates the quality of each model relative to each of the other models, was slightly lower (better) for the models that included the longitudinally measured FVC and DLCO parameters compared with those that included the baseline FVC and DLCO parameters (table 3). Thus, the final SLS I survival models demonstrated that decreased age, decreased extent of cutaneous sclerosis, as well as an improved course of the FVC%-predicted and the DLCO%-predicted over 24 months were associated with better survival outcomes (table 3). As stated in the online supplementary methods, we created separate models for the FVC and DLCO variables to avoid collinearity.

SLS I: joint model validation analysis
Using the same basic model as above (eg, MRSS, age), the longitudinal assessment of the FVC%-predicted was significantly associated with the outcome (table 3). When added to the basic model, the longitudinal assessment of the DLCO%-predicted was also significantly associated with the outcome (table 3). As noted above, the programming for the joint model does not allow for the inclusion of two longitudinally measured covariates simultaneously.

SLS I: exploratory analyses
In an exploratory analysis, we examined whether the change from baseline in the FVC%-predicted and DLCO%-predicted at 12 months predicted survival. When added to the basic model (eg, MRSS, age), none of the change scores was significantly associated with survival. In addition, we explored whether combined, categorical changes in the FVC%-predicted and DLCO%-predicted...
Table 3  Final models for predicting death in SLS I

<table>
<thead>
<tr>
<th></th>
<th>Cox model using baseline FVC as covariate</th>
<th>Cox model using FVC as time-dependent covariate</th>
<th>Joint model using FVC as a time-dependent covariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td><strong>FVC models</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline MRSS</td>
<td>1.03 (1.01 to 1.05)*</td>
<td>1.03 (1.01 to 1.06)**</td>
<td>1.03 (1.01 to 1.06)**</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.06 (1.04 to 1.08)†</td>
<td>1.06 (1.04 to 1.08)†</td>
<td>1.06 (1.03 to 1.08)†</td>
</tr>
<tr>
<td>FVC%-predicted</td>
<td>0.97 (0.95 to 0.99)**</td>
<td>0.96 (0.94 to 0.97)†</td>
<td>0.97 (0.96 to 0.98)†</td>
</tr>
<tr>
<td>AIC</td>
<td>564</td>
<td>546</td>
<td>789</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DLCO models</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline MRSS</td>
<td>1.04 (1.01 to 1.06)†</td>
<td>1.04 (1.02 to 1.07)**</td>
<td>1.04 (1.01 to 1.06)**</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.05 (1.03 to 1.08)†</td>
<td>1.05 (1.02 to 1.07)†</td>
<td>1.05 (1.03 to 1.07)†</td>
</tr>
<tr>
<td>DLCO%-predicted</td>
<td>0.98 (0.96 to 1.00)</td>
<td>0.96 (0.95 to 0.98)†</td>
<td>0.96 (0.94 to 0.98)†</td>
</tr>
<tr>
<td>AIC</td>
<td>569</td>
<td>525</td>
<td>791</td>
</tr>
</tbody>
</table>

AIC, Akaike information criterion; FVC, forced vital capacity; DLCO, single-breath diffusing capacity for carbon monoxide; MRSS, Modified Rodnan Skin Score; SLS, Scleroderma Lung Studies. *P<0.05, **P<0.01, ***P<0.001. †P<0.0001.

at 12 and 24 months predicted survival. None of the categorical declines at 12 months (eg, FVC decline ≥10%; FVC decline ≥15%; DLCO decline ≥15%; FVC decline ≥10% and DLCO decline ≥15%; FVC decline ≥10% or DLCO decline ≥15%) was significantly associated with long-term survival when added to the basic model; however, these individual categorical declines at 24 months were associated with long-term survival when added to the basic model (online supplementary table S1). Too few (patient n=1) experienced an FVC decline 5%–9% and DLCO decline ≥15% to include this covariate in the model.

SLS II: Cox proportional hazards model

The basic model developed from the SLS II cohort consisted of the following covariates: treatment arm (CYC vs MMF), baseline extent of cutaneous sclerosis (MRSS), age at randomisation (years) and sex. Similar to SLS I, increased age and increased MRSS at baseline were associated with increased mortality in SLS II. Baseline FVC%-predicted and baseline DLCO%-predicted were not significantly associated with time to death when added to the basic model that comprised age and MRSS. However, the longitudinal assessment of the FVC%-predicted and the longitudinal assessment of the DLCO%-predicted were each associated with the outcome when added to the basic model (table 4). None of the quantitative lung fibrosis/ILD scores was associated with long-term survival when added to the base model. Therefore, the final SLS II survival models demonstrated that decreased age, decreased extent of cutaneous sclerosis, as well as an improved course of the FVC%-predicted and the DLCO%-predicted over 24 months were associated with better survival outcomes (table 4).

Table 4  Final models for predicting death in SLS II

<table>
<thead>
<tr>
<th></th>
<th>Cox model using baseline FVC as covariate</th>
<th>Cox model using FVC as time-dependent covariate</th>
<th>Joint model using FVC as a time-dependent covariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td><strong>FVC models</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline MRSS</td>
<td>1.49 (0.82 to 2.56)</td>
<td>1.61 (0.93 to 2.77)</td>
<td>1.66 (0.98 to 2.83)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.09 (1.04 to 1.14)***</td>
<td>1.09 (1.04 to 1.14)***</td>
<td>1.08 (1.04, 1.13)***</td>
</tr>
<tr>
<td>FVC%-predicted</td>
<td>1.17 (0.64 to 2.14)</td>
<td>0.48 (0.28 to 0.81)**</td>
<td>0.51 (0.30 to 0.88)*</td>
</tr>
<tr>
<td>AIC</td>
<td>250</td>
<td>217</td>
<td>347</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DLCO models</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline MRSS</td>
<td>1.56 (0.92 to 2.67)</td>
<td>1.86 (1.00 to 3.47)</td>
<td>1.85 (1.07 to 3.19)*</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.08 (1.03 to 1.13)***</td>
<td>1.08 (1.02 to 1.15)**</td>
<td>1.07 (1.03 to 1.12)**</td>
</tr>
<tr>
<td>DLCO%-predicted</td>
<td>0.95 (0.85 to 1.06)</td>
<td>0.83 (0.72 to 0.96)*</td>
<td>0.84 (0.76 to 0.94)**</td>
</tr>
<tr>
<td>AIC</td>
<td>249</td>
<td>150</td>
<td>360</td>
</tr>
</tbody>
</table>

AIC, Akaike information criterion; DLCO, single-breath diffusing capacity for carbon monoxide; FVC, forced vital capacity; MRSS, Modified Rodnan Skin Score. *P<0.01, **P<0.01, ***P<0.001. †P<0.0001.
SLS II: joint model validation analysis
Using the same basic model as above (eg, MRSS, age), the longitu-
dinal assessment of the FVC%-predicted was significantly
associated with the outcome (table 4). When added to the basic
model, the longitudinal assessment of the DLCO%-predicted
was also significantly associated with the outcome (table 4).

SLS II: exploratory analyses
In an exploratory analysis, we examined whether the change
from baseline in the FVC%-predicted and DLCO%-predicted at
12 months predicted survival. When added to the basic model
(eg, MRSS, age), the change in FVC from baseline to 12 months
predicted long-term survival (estimate 0.52 (CI 0.31 to 0.90); p < 0.05),
but not the change in DLCO from baseline to 12
months. In addition, we explored whether combined, categori-

cal changes in the FVC%-predicted and DLCO%-predicted at
12 months predicted survival as described above. We found
that an FVC decline ≥10% at 12 months and FVC decline ≥15% at
12 months were each associated with long-term survival when
added to the base model with the covariates of MRSS and age
(online supplementary table S2). Patients in SLS II had a decline
in DLCO ≥15% at 12 months in SLS II; therefore, we were
unable to analyse any of the the composite categorical decline
variables that included the DLCO.

Use of potential disease-modifying agents
While no data were available regarding the use of potential
disease-modifying agents beyond the 24-month study period in
SLS I, in SLS II these data were collected and demonstrated that
the majority of patients consumed MMF following the 24-month
study period. (Please see online supplementary table S3 for a list
of all potential disease-modifying agents used following ces-
sation of study drug in SLS II.)

DISCUSSION
The present study is the first study to examine mortality
outcomes in patients who participated in two of the largest RCTs
for SSc-ILD. The results presented herein demonstrate that
treatment with 1 year of CYC compared with placebo does not
improve long-term survival outcomes in patients with SSc-ILD.
The findings also demonstrate that there is no difference in long-
term survival between patients randomised to CYC versus MMF
in SLS II.

Both SLS I and II demonstrated that treatment with immuno-
suppression led to short-term improvements in surrogate
measures of SSc-ILD outcomes.\(^4\) However, the present findings
suggest that short-term treatment with CYC and MMF may not
improve long-term outcomes of patients with SSc-ILD. Where
known, the majority of patients in both SLS I and II died of
complications related to SSc, and respiratory failure due to
end-stage lung disease was the leading cause of death. These
findings are consistent with recent reports of mortality outcomes
in observational cohorts.\(^1\)\(^2\)

Relatively few malignancies occurred in either cohort. Further-
more, although studies have demonstrated an increase in haema-
tological malignancies in SSc,\(^14\) only one case of lymphoma
occurred in the placebo arm of SLS I. The paucity of malignan-
cies observed in both cohorts may in part be due to the length
of the follow-up period, especially in SLS II where the median
follow-up was only 4 years, as well as the observation that respi-
atory failure was the leading cause of death.

These findings highlight a need to determine the appropriate
duration of treatment for SSc-ILD. In SLS I, very few patients
reported use of immunosuppression in year 2 of the study despite
the accepted view that ILD progression generally occurs up to 5
years from disease onset in SSc. This may explain why there was
no difference in long-term survival between the two study arms
in SLS I. It is plausible that initiation of a maintenance therapy
regimen after induction therapy may affect long-term survival
outcomes. In SLS II, many patients continued on immunosup-
pression after the trial concluded (MMF was most common).
The use of MMF in the CYC arm may in part explain why the
trend for an MMF-related survival benefit observed in the first 2
years diminished in the subsequent follow-up years.

More research is needed to determine the appropriate length
of treatment for immunosuppression in SSc-ILD. A prior small
retrospective study demonstrated improvement/stability in the
FVC after 18 months of azathioprine (AZA) maintenance
therapy in patients who first completed a 6-month induction
course of intravenous CYC for SSc-ILD.\(^15\) However, without a
control arm, it is impossible to discern whether the observed
improvement/stability in the FVC represents a true treatment
response versus the natural course of ILD in SSc. An additional
study demonstrated that patients who responded favourably to
pulse CYC and were subsequently treated with AZA experienced
a higher rate of improvement or stabilisation in lung function
compared with patients who did not respond to pulse CYC.\(^16\)

The present analysis also revealed significant predictors of
long-term mortality in SSc-ILD. In line with prior observational
studies,\(^17\)\(^19\) increased skin score and increased age were inde-
dependently associated with increased mortality. In contrast to
prior observational studies,\(^18\) 20–22 however, male gender and
African–American race were not associated with an increased
risk of mortality. Regarding gender, the SLS I and II cohorts
comprised predominantly of women; thus, these studies may
be underpowered to detect true gender differences in long-term
survival. In terms of race, our findings could potentially suggest
that in the context of a clinical trial, in which all patients have
equal access to healthcare and uniform follow-up, race does not
play a substantial role in predicting long-term survival.

Consistent with prior observational studies,\(^16\)\(^19\)\(^23\)\(^24\) low base-
line FVC was associated with an increased risk of mortality.
However, the course of the FVC and the DLCO over 24 months
appeared to be more robust predictors of long-term survival in
both SLS I and II than the baseline measurements of these param-
eters when comparing the AIC for the models. The individual
parameter estimates were similar for both the Cox model and
the joint model that we used as a validation approach, suggesting
that the relationship between survival and FVC (or DLCO) is not
biased by non-ignorable missing data.

A recent single-centre observational cohort study of patients
with SSc-ILD also found that pulmonary function trends at 1
and 2 years predicted intermediate to long-term mortality.\(^25\)\(^26\) This
study demonstrated that 1-year categorical trends in the FVC
and DLCO were the most accurate prognostic determinants
of mortality, while at 2 years changes in gas transfer were the most
important predictors of mortality.\(^25\)\(^26\) As this was an observational
study, the authors could not adequately control for treatment
effect and selection bias. We were able to replicate some, but not
all of these findings in the SLS I or II cohorts. Taken together,
the findings of the present study provide further evidence that
trends in pulmonary function may offer more prognostic in-
formation than baseline pulmonary function measurements. This
may in part be due to the fact that substantial variability exists in
a single FVC and DLCO measurement. Repeated measurements
of the FVC/DLCO may yield more clinically meaningful in-
formation regarding ILD progression and survival.


The findings of the present study should be interpreted in the context of specific limitations. There were subtle differences in the baseline characteristics of the SLS I and SLS II cohorts. For instance, the DLCO was lower in SLS I, and this may have been due to less scrupulousness in excluding pulmonary arterial hypertension (PAH). The Baseline Dyspnoea Index (BDI) score was also lower in SLS I, although these differences could be related to using different instruments to administer the BDI in the two studies. The quantitative extent of interstitial lung disease/quantitative extent of lung fibrosis in the zone of maximal involvement on HRCT was higher in SLS II, while the quantitative extent of interstitial lung disease in whole lung on HRCT was higher in SLS I. However, in SLS I, non-volumetric CT scans of 1–2 mm slice thickness were acquired at 10 mm increments, while in SLS II volumetric CT scans of 1–1.5 mm slice thickness were acquired contingously. Overall, the two cohorts were strikingly similar.

A number of SLS I and, to a lesser degree, SLS II participants were lost to follow-up during the course of this longitudinal follow-up study. This can introduce bias, especially in cases where early censoring occurred. The Cox proportional hazards model was used to deal with time to event data in the presence of censoring. Moreover, while a morbidity and mortality committee adjudicated the causes of death during the 24-month study periods, less detailed information was available regarding immunosuppression use. Finally, while PH was identified as the cause of death in only one of the SLS II patients, this comorbidity may have influenced survival rates in both cohorts.

Notable strengths of the present manuscript include the use of two relatively large, well-characterised SSc-ILD cohorts undergoing standard treatment approaches with uniform follow-up measurements over the course of 2 years. These cohorts comprised patients from multiple SSc Centers of Excellence across the USA, augmenting the generalisability of our study to a larger population. Furthermore, we identified the same mortality predictor variables in both cohorts, suggesting that our results are likely reproducible in other similar SSc cohorts. Finally, we used a joint model as a means of internal validation.

In summary, the findings of the present analyses demonstrate that increased baseline skin score, increased baseline age, and the course of the FVC and DLCO over 2 years are important predictors of long-term survival in SSc-ILD. Treatment with immunosuppression may not improve long-term survival in patients with SSc-ILD, in contrast to haematopoietic stem cell transplantation, which seems to offer a more sustained improvement in long-term survival and may especially help those patients who have early, rapidly progressive SSc with organ involvement. Future studies are needed to determine how the duration of immunosuppression affects long-term survival among patients with SSc-ILD. With the emergence of promising new therapies for SSc-ILD (eg, antifibrotics, or combination therapy with antifibrotics and immunosuppression), additional studies are needed to compare how these novel approaches affect survival compared with the current standard of care for SSc-ILD.

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Presented at
This manuscript was based on work previously published at the following conferences: Systemic Sclerosis World Congress 2018 (Volkman ER, Taskin DP, Sim M, et al, The course of the forced vital capacity during treatment for systemic sclerosis-related interstitial lung disease predicts long-term survival in 2 independent cohorts, Journal of Scleroderma and Related Disorders 2018;3(5):69–101) and the American College of Rheumatology Annual Meeting 2017 (Volkman ER, Taskin DP, Sim M, et al, The course of the forced vital capacity during treatment for systemic sclerosis-related interstitial lung disease predicts long-term survival in 2 independent cohorts, Arthritis Rheumatol 2017;69(Suppl 10)).

Acknowledgements Bristol-Myers Squibb supplied cyclophosphamide for use in SLS I and Hoffman-La Roche supplied mycophenolate mofetil for use in SLS II. We thank John Dermond and Grace Ibrahim for their assistance in contacting the participants in SLS I and II, respectively.

Collaborators The following persons and institutions participated in the Scleroderma Lung Study I: University of California at Los Angeles (UCLA), Los Angeles: PJ Clements, DP Taskin, R Elashoff, J Goldin, M Roth, D Furst, K Bulpitt, D Khanna, W-L Chung, S Visacco, M Sterz, L Woolcock, X Yan, J Ho, S Vasumalleshorn, I da Costa; University of Medicine and Dentistry of New Jersey, New Brunswick: JP Seibold, DI Riley, IK Amorosa, VM Hsu, DA McCloskey, JE Wilson; University of Illinois at Chicago, Chicago: J Varga, D Schraunagel, A Wilbur, M Lopata, S Arami, P Cole-Saffold; Boston University, Boston: R Simms, A Theodore, P Clarke, J Korn, K Tobin, M Nute; Medical University of South Carolina, Charleston: R Silver, M Bolster, C Strange, S Schabel, E Smith, J Arnold, K Caldwell, M Bonner; Johns Hopkins School of Medicine, Baltimore: R Wise, F Wigley, B White, L Hummers, M Bohlin, A Polito, G Leatherman, E Forbes, M Daniel; Georgetown University, Washington, DC: V Steen, C Read, C Cooper, S Wheelan, A Carey, A Ortiz; University of Texas at Houston, Houston: M Mayes, E Parsley, S Oldham, T Filemon, S Jordan, M Perry; University of California at San Francisco, San Francisco: K Connolly, J Golden, P Wolters, R Webb, J Davis, C Antolos, C Maynetto; University of Alabama at Birmingham, Birmingham; B Fessler, M Olman, C Sanders, L Heck, T Parkhill; University of Connecticut Health Center, Farmington: N Rothfield, M McCartney, R Cobb, M Aberles, F Ingels, S Pascual Wain State University, Detroit: M Mayes, K Mubarak, J Golub, A Sivilia, Z Injur; R Alexander; Virginia Mason Research Center, Seattle: D Furst, S Springmeyer, S Kirkland, J Miltzor, R Hinke, A Mondt; Data Safety and Monitoring Board: Harvard Medical School, Boston—T Thompson; Veterans Affairs Medical Center, Brown University, Providence, Rhode Island—S Rounds; Cedars Sinai—UCLA, Los Angeles—M Weinstein; Clinical Trials Surveys, Baltimore—B Thompson; Mortality and Morbidity Review Committee: UCLA, Los Angeles—H Paulus, S Levy; Johns Hopkins University, Baltimore—D Martin. The following persons and institutions participated in the Scleroderma Lung Study II: University of Boston, Boston: AC Theodore, RW Simms, E Kissin, FY Cheong; Georgetown University, Washington, DC: JD Steen, CA Read Jr, C Fridley, M Zulmatakhvi; Johns Hopkins University, Baltimore: RA Wise, FM Wigley, L Hummers, G Leatherman; Medical University of South Carolina, Charleston: RM Silver, C Strange, FN Hant, J Ham, K Gibson, D Rosson; University of California, Los Angeles (UCLA), Los Angeles: DP Taskin, RM Elashoff, MD Roth, PJ Clements, D Furst, S Kafaja, E Kleea, D Elashoff, J Goldin, E Arloia, G Marlis, J Mason-Berry; P Saffold, M Rodriguez, L Guzman, J Brook; University of California, San Francisco (UCSF), San Francisco: J Golden, MK Connolly, A Elter, D Leong, M Lalosh, J Obata; University of Illinois, Chicago: S Volkov, D Schraunagel, S Arami, D Franklin; Northwestern University, Chicago: J Varga, J Dematte, M Hinrichs, C Deluca, H Donnelly, C Marin; University of Medicine and Dentistry of New Jersey, New Brunswick—DI Riley, VM Hsu, DA McCloskey; University of Michigan, Ann Arbor: K Phillips, D Khanna, FJ Martinez, E Schiup, J Konkle; University of Texas, Houston: M Mayes, B Patel, S Assais, F Tan; National Jewish Health, Denver: A Fischer, J Swigis, R Meehan, K Brown, T Warren, M Morrison; University of Utah, Salt Lake City: MB Scholand, T Frecht, P Carey, M Villegas; University of Minnesota, Minneapolis: J Molitoris, P Carlson.

Contributors All coauthors meet the criteria for authorship.
Funding  This work was supported in part by the NIH/NIAID: R01-AI05419 (KMS) and R01-AI05419 (KMS); the Scleroderma Foundation (ERV); NIH/NIMH: R01 AR 070470 and K24 AR 063120 (DK); and NHLBI/NHL: R01 HL089758 (DPT), R01 HL089901 (RME), U01 HL 60587 (DPT) and U01 HL 60606 (RME).

Competing interests None declared.

Patient consent Not required.

Ethics approval UCLA IRB and the institutional review board of each site approved the studies.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

**In vivo** pathogenicity of IgG from patients with anti-SRP or anti-HMGCR autoantibodies in immune-mediated necrotising myopathy

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**ABSTRACT**

**Objectives** In autoimmunity, autoantibodies (aAb) may be simple biomarkers of disease or true pathogenic effectors. A form of idiopathic inflammatory myopathy associated with anti-signal recognition particle (SRP) or anti-3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) aAb has been individualised and is referred to as immune-mediated necrotising myopathy (IMNM). The level of aAb correlates with IMNM activity and disease may respond to immunosuppression, suggesting that they are pathogenic. We aimed to evaluate the pathogenicity of IgG from patients with anti-SRP or anti-HMGCR aAb in vivo by developing the first mouse model of IMNM.

**Methods** IgG from patients suffering from anti-SRP or anti-HMGCR associated IMNM were passively transferred to wild-type, Rag2-/- or complement C3-/- mice. Muscle deficiency was evaluated by muscle strength on electromyostimulation and grip test. Histological analyses were performed after haematoxylin/eosin staining or by immunofluorescence or immunohistochemistry analysis. Antibody levels were quantified by addressable laser bead assay (ALBA).

**Results** Passive transfer of IgG from patients suffering from IMNM to C57BL/6 or Rag2-/- mice provoked muscle deficiency. Pathogenicity of aAb was reduced in C3-/- mice while increased by supplementation with human complement. Breakage of tolerance by active immunisation with SRP or HMGCR provoked disease.

**Conclusion** This study demonstrates that patient-derived anti-SRP and anti-HMGCR IgG are pathogenic towards muscle in vivo through a complement-mediated mechanism, definitively establishing the autoimmune character of IMNM. These data support the use of plasma exchanges and argue for evaluating complement-targeting therapies in IMNM.

**Key messages**

What is already known about this subject?

- Immune-mediated necrotising myopathy (IMNM) is a severe form of myopathy characterised by the presence of necrotic and atrophic muscle fibres associated with sarcolemmal complement C5b-9 deposits and mild inflammatory infiltrates.

What does this study add?

- For the first time, in vivo, we demonstrate the pathogenic role of IgG from anti-SRP or anti-HMGCR autoantibody positive IMNM patients.

How might this impact on clinical practice or future developments?

- These results support the use of therapies targeting anti-SRP and anti-HMGCR autoantibody production and/or targeting antibody effect or functions such as complement.

**INTRODUCTION**

Immune-mediated necrotising myopathies (IMNM), formerly also referred to as necrotising autoimmune myopathies, are particular forms of idiopathic inflammatory myopathies (IIM) which encompass a group of severe acquired muscle disorders including dermatomyositis, inclusion body myositis, polymyositis and overlap myositis. IMNM have been clearly distinguished from other forms of IIM based on histological patterns, that is, presence of myofibre necrosis, complement deposits on myofibres and paucity or absence of inflammatory cells.1-3 This paucity of inflammatory infiltrates and presence of complement deposits on myofibres is suggestive of an autoantibody (aAb)-mediated pathophysiology rather than T-cell-dependent cytotoxicity presumably involved in other forms of IIM. IMNM were also recognised in the 2017 classification criteria for myositis.4 IMNM are typically associated with the presence of aAb directed against two distinct antigens, namely signal recognition particle (SRP)7 or 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR).3 These two aAb are present in two-thirds of patients with IMNM,4 while approximately one-third remains seronegative as of today. IMNM are associated with a spectrum of clinical presentations. Anti-SRP+ IMNM ranges from severe and rapidly progressive disease7-10 to slowly progressive forms that may mimic limb
girdle muscular dystrophy and delay treatment onset. Anti-HMGCR IMNM is also severe, although less than its anti-SRP counterpart, and may occur during or after statin exposure. Extramuscular manifestations are typically infrequent and mild when present. SRP is a ribonucleoprotein complex involved in guiding nascent polypeptides into the endoplasmic reticulum. Whereas anti-SRP aAb from patients may recognise each of the different SRP components, the signal peptide-binding 54 kDa subunit (SRP54) is the major target since reactivity to SRP54 is almost always present in anti-SRP positive sera; it is therefore used in diagnostic immunoassays. HMGCR is an enzyme located at the membrane of the endoplasmic reticulum. It is responsible for the production of mevalonate, a rate-limiting step of cholesterol biosynthesis. HMGCR can be pharmacologically inhibited by statins which are used to reduce cholesterol levels and the risk of heart disease. Anti-HMGCR aAb were discovered in statin-exposed patients with IMNM using immunoprecipitation, but were also found in a large proportion of non-exposed patients. The reactivity of anti-HMGCR aAb is directed against the C-terminal catalytic domain. Since the expression of SRP and HMGCR is ubiquitous rather than muscle specific, the pathogenic role of aAb directed against these molecules in IMNM has remained elusive and the autoimmune character of IMNM has not been formally established as of today. However, anti-SRP aAb from patients have been shown to inhibit the in vitro translocation of secretory proteins into the endoplasmic reticulum. In a longitudinal follow-up study, we showed that the levels of anti-SRP54 aAb in patients are closely correlated with disease activity. Along those lines, plasma exchanges and B-cell targeting therapies can be efficient in treatment-resistant IMNM. Regarding anti-HMGCR IMNM, cross-sectional studies have suggested that aAb levels are also associated with disease severity. Two in vitro studies recently supported the hypothesis of a pathogenic role of these aAb. Anti-SRP54 serum plus complement was shown to reduce cell survival of myoblast cultures. We also showed that addition of anti-SRP or anti-HMGCR aAb impaired myoblast fusion and induced atrophy of myotubes in vitro. Together, these observations suggest that anti-SRP and anti-HMGCR aAb may be useful biomarkers of disease and may also be direct pathogenic players in IMNM.

To obtain more insight into the pathogenic mechanisms that cause IMNM and formally establish their autoimmune character, we evaluated the pathogenicity of IgG from patients with anti-SRP and anti-HMGCR aAb in mice in vivo.

METHODS
Human samples
All patient samples were collected after written informed consent was obtained. Plasma positive for anti-SRP and anti-HMGCR aAb were obtained from patients (see online supplementary methods 1) who fulfilled the consensus criteria for IMNM with the presence of anti-SRP or anti-HMGCR aAb titrated by addressable laser bead immuno-assay (ALBIA) that we described previously (see online supplementary figure S1).

Mice
Eight-week-old female C57BL/6 mice were purchased from Charles River, France. Rag2 deficient (Rag2−) and complement component 3 deficient (C3−) C57BL/6 mice were purchased from Jackson Laboratories. The protocol for animal experimentation was approved by an institutional ethics committee (Comité régional d’Ethique en Expérimentation Animale, Mont Saint Aignan, France) under the reference N/33-11-12/56/11-15, and 201508071625487 for complement-related experiments.

Statistical analysis
Data were compared by t test, and the Mann-Whitney test or Wilcoxon test, as appropriate, using Prism 5 software (GraphPad). P<0.05 was considered statistically significant.

RESULTS
Reactivity of human autoantibodies against their cognate murine targets
The C-terminal domain of human HMGCR shares more than 98% homology with mouse HMGCR, whereas SRP54 is 100% conserved between human and mouse. We first determined whether anti-SRP and anti-HMGCR aAb from patients could react with their cognate antigen in mouse. The presence of immunoreactive SRF in mouse muscle was confirmed by immunostaining with a commercial anti-SRP antibody (figure 1A,B). Plasma from anti-SRP+ patients recognised their target in mouse muscle (figure 1C). Both commercial anti-SRP Ab and patient plasma yielded a peripheral reinforcement, resulting in a sarcolemmal or subsarcolemmal pattern. This aspect was similar for different anti-SRP+ samples tested (data not shown), and with IgG purified from patient suffering from anti-SRP associated IMNM (figure 1D). As expected, immuno-affinity purified anti-SRP specific aAb reacted against muscle SRP, with disappearance of signal in a competition experiment in which the aAb was preincubated with recombinant SRP54 (figure 1E,F).

Immuno-affinity purified anti-SRP specific aAb recognised the recombinant SRP54 protein on western blot (figure 1G, left panel). Similarly, anti-HMGCR+ IgG from patients recognised the recombinant murine HMGCR (mHMGCR) on western blot (figure 1G, right panel) as well as using ALBIA (figure 1H).

Together, these results show that anti-SRP and anti-HMGCR aAb from patients recognise their cognate antigenic target in mouse.

In vivo pathogenicity of IgG with anti-SRP and anti-HMGCR reactivity
We investigated the effects of IgG from patients with anti-SRP and anti-HMGCR aAb on mouse muscle after their passive transfer in vivo. For this, C57BL/6 mice were injected daily with plasma or purified IgG from patients suffering from anti-SRP or anti-HMGCR associated IMNM (figure 2A). To limit xenoinmunisation against human proteins, animals were transiently immunosuppressed with a single dose of cyclophosphamide. Injection of plasma from two different patients suffering from anti-SRP+ IMNM provoked a reduction in grip strength (figure 2B). Similar results were obtained when using NOD mice as recipients of anti-SRP IgG, indicating that the pathogenicity is not dependent on the genetic background (see online supplementary figure S3).

To confirm that the effect of anti-SRP+ plasma was due to patients’ aAb, the same experiment was carried out with purified IgG or IgG-depleted flow-through, from anti-SRP+ or anti-HMGCR+ plasmas. After injection, anti-SRP and anti-HMGCR aAb from patients circulated at high levels in the blood of injected mice (see online supplementary figure S4A,B). As compared with control IgG, injection of IgG from both anti-SRP+ and anti-HMGCR+ plasma provoked a significant decrease in grip
Myositis

strength which tended to be more pronounced in mice receiving the former than the latter (figure 2C,E). The IgG-depleted fraction of the same plasma had no significant effect, indicating that pathogenicity is indeed mediated by IgG. To confirm these results, we used another muscle assessment technique which consisted in measuring gastrocnemius strength on electrostimulation of the sciatic nerve (referred to as muscle strength). Using this method, the muscle strength of mice injected with anti-HMGCR+ and anti-SRP+ IgG also decreased as compared with mice injected with IgG-depleted plasma or control IgG (figure 2D,F). As an additional control, injection of a human IgG1 mAb recognising the human HMGCR but not the murine HMGCR was inefficient (see online supplementary figure S2B,C).

Histological analysis revealed a statistically significant increase in the number of necrotic myofibres (p=0.014) 8 days after administration of IgG purified from anti-HMGCR+ plasma (figure 2H). Immunohistochemical staining of F4/80, a specific marker for macrophages, revealed myophagocytosis of necrotic muscle fibres (figure 2G, lower right panel).

When the duration of experimentation was extended, the muscle strength of mice injected with purified IgG from anti-SRP+ plasma returned to normal values at around day 14 (data not shown). This lack of a long-lasting effect was likely due to the xenogeneic response elicited against human IgG that unavoidably appeared despite the short cyclophosphamide regimen. Indeed, injection of plasma or purified IgG elicited production of anti-human IgG Ab but not when mice received an IgG-depleted fraction (see online supplementary figure S4C). Cyclophosphamide had an immunosuppressive effect since anti-human IgG levels were much higher in mice not receiving this regimen. To completely avoid xenoinmunisation, we performed passive transfer experiments using genetically-modified Rag2-/- immunodeficient mice. After injection of purified IgG from patients suffering from anti-SRP or anti-HMGCR associated IMNM to Rag2-/- mice (figure 3A), the results obtained at day 8 were similar to those observed in normal C57BL/6 recipients. As expected, muscle deficiency was maintained beyond day 7 in these conditions and was more pronounced in mice receiving anti-SRP+ IgG than anti-HMGCR+ IgG (figure 3B,C). Myofibre necrosis and C5b-9 deposits were present (figure 3D,E).

Pathogenicity after breakage of tolerance against SRP and HMGCR

Immunisation with recombinant SRP induced significant levels of anti-SRP Ab from day 28 (figure 4A). In contrast to OVA control immunisation, this required additional antigenic boosts (figure 4B). As compared with mice immunised with OVA,
Figure 2  Patient’s IgG containing anti-SRP and anti-HMGCR aAbs are pathogenic in mice. (A) Experimental setting: C57BL/6 mice were transiently immunosuppressed by a single injection of cyclophosphamide (CYC) and received daily injections of (B) plasma from two patients positive for anti-SRP aAbs (P1 or P2) (n=5), (C–F) purified IgG or IgG-depleted plasma from patients positive for anti-SRP (P3) or anti-HMGCR aAbs (P4) (n=6–8). Muscle strength was evaluated by (B,C,E) the variation in grip test performance between day 0 and day 7 (Δ Grip strength), and by (D,F) measurement of gastrocnemius contraction on electrostimulation (Muscle strength). (B–F) All data are presented as mean±SD; *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney two-tailed test. (G) Immunohistological analysis of mouse muscle at day 8. H&E staining of muscle cryosection from a mouse injected with IgG from control, patients positive for anti-SRP or anti-HMGCR aAbs. Arrowhead shows example of necrotic myofibre. Immunohistological staining (lower right panel) of F4/80+ macrophages, showing myophagocytosis of a necrotic muscle fibre. (H) Quantification of necrotic muscle fibres in tibialis, triceps and gastrocnemius muscles from four mice injected with purified IgG from a patient positive for anti-HMGCR aAb (P5). All data are presented as mean±SD; *p<0.05 by t test. HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; SRP, signal recognition particle.
breakage of tolerance against SRP induced significant loss of grip strength starting from day 38 (figure 4C) and muscle strength deficiency (figure 4D). Immunisation with recombinant human HMGCR induced the production of anti-HMGCR Ab in the serum of mice (figure 4E) and subsequent muscle strength deficiency as compared with OVA-immunised animals (figure 4I), in as little as 14 days.

Involvement of complement in pathogenicity
To determine how anti-SRP and anti-HMGCR aAb exert their pathogenic effect, we assessed the involvement of the complement system. For this, mice deficient in C3 complement fragment (C3−) were subjected to a passive transfer, as above. When compared with wild type (C3+), C3− mice receiving anti-SRP+ or anti-HMGCR+ IgG had a less pronounced deficiency in muscle strength, indicating that pathogenicity is dependent on the presence of complement (figure 5A,B).

Yet, mice are known to have rather low complement activity (CH50) as compared with humans. Moreover, in other animal models of autoimmune pathologies, the pathogenicity of human IgG on the target mouse tissue may require the addition of human complement since human IgG do not efficiently activate mouse complement. In a similar way in our model, human complement supplementation of mice receiving anti-SRP+ and anti-HMGCR+ IgG was more potent to induce a decrease of muscle strength as compared with IgG alone (figure 5D).

**DISCUSSION**
This is the first report that IgG from patients with anti-SRP and anti-HMGCR aAb demonstrate pathogenic properties
Figure 4  Immunisation with recombinant SRP (red) or recombinant human HMGCR (blue) provokes a muscle deficiency. Mice were immunised with SRP (n=8), HMGCR (n=10) or ovalbumin (OVA) as control (n=8 or 10), in the presence of adjuvant. (A) Anti-SRP and (E,H) anti-HMGCR Ab levels in mouse serum were determined by addressable laser bead immunoassay (ALBIA). (B,F,G) Anti-OVA antibody levels were determined by ELISA. Muscle strength was evaluated by (C) grip strength and by (D,I) measurement of gastrocnemius contraction on electrostimulation. All data are presented as mean±SD; **p<0.01 and compared by (A,B,C,E,F,G,H) Wilcoxon or (D,I) Mann-Whitney two-tailed test. Results shown in (E–I) are representative of two independent experiments. HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; SRP, signal recognition particle.

in vivo in mice. IMNM have recently been recognised as an individual subgroup of idiopathic inflammatory myopathy in which aAb directed against SRP and HMGCR were found in 65%–72% of patients. Although anti-SRP aAb were described 30 years ago, anti-HMGCR aAb were not identified until 2010. Until now, their role in IMNM pathogenesis...
as cause or epiphenomenon of disease has remained unknown. Indeed, they may represent mere biomarkers generated by a breakage of tolerance after autoantigens are released by myofibre necrosis. Alternatively, they could be key pathogenic players that cause disease. Solving this issue has important therapeutic consequences.

We show herein that anti-SRP+ plasma is able to induce muscle deficiency in mice. To prevent possible non-specific effects of components other than anti-SRP aAb that might be present in the plasma of patients, we purified IgG before injection. Using daily IgG injections, we show that the deficiency is indeed caused by the IgG fraction of plasma. In addition, we also show that anti-HMGCR+ plasma is able to induce muscle deficiency by IgG fraction. In both cases, IgG-depleted plasma did not affect muscle strength. Muscle deficiency was evidenced by two independent methods of evaluation, that is, grip test and muscle strength upon nerve electrostimulation. Using these methods, loss of muscle strength tended to be more severe in anti-SRP+ than in anti-HMGCR+ IgG injected mice. This is consistent with observations in humans, with a higher severity in patients

Figure 5  Involvement of the complement system in IgG pathogenicity of IMNM. Complement C3-deficient (C3-) or wild-type C57BL/6 (C3wt) mice (n=5–8) were transiently immunosuppressed by a single injection of cyclophosphamide (CYC) and received daily injections of purified IgG from (A) patient P3 suffering from anti-SRP associated IMNM or (B) patient P4 suffering from anti-HMGCR associated IMNM. Muscle strength was evaluated by measurement of gastrocnemius contraction strength at day 8 on electrostimulation. (C) Besides, C57BL/6 mice were injected daily with IgG positive for anti-SRP or anti-HMGCR aAbs in conjunction with or without human complement (C57BL/6 mice were injected daily with IgG positive for anti-SRP or anti-HMGCR aAbs in conjunction with or without human complement (C') (n=8). (D) Muscle strength was evaluated at day 8 by measurement of gastrocnemius contraction strength on electrostimulation. All data are presented as mean±SD *p<0.05, **p<0.01, ***p<0.001 by Mann-Whitney two-tailed test. Results shown in (B,D) are representative of two independent experiments. HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; SRP, signal recognition particle.
suffering from patients with anti-SRP reactivity. Despite being named its necrotising character, the extent of myofibre necrosis in IMNM remains in the percent range for patients with anti-SRP aAbs and is not different between patients with anti-Jo-1 and anti-HMGCR aAbs patients for instance. Consistently, myofibre necrosis was modest but present after transfer of anti-SRP" or anti-HMGCR" IgG (figure 2).

Interestingly, no cellular inflammatory infiltrate was evidenced, in accordance with observations in patients with IMNM in whom muscle biopsies show little if any inflammation.9 10 11 Our observation that experimental disease develops similarly in immunocompetent and immunodeficient Rag2-2 mice confirms the dispensable role of the adaptive immune system in driving the pathogenicity of aAb once produced. Further, because the absence of T-cells and B-cells prevents the xenogeneic response the pathogenicity of aAb once produced. Yet, one of the most distinguishing histological features of IMNM is represented by complement deposits on non-necrotic myofibres.2 4 5 Anti-SRP and anti-HMGCR aAb may recognise ectopically expressed cellular targets at the surface of myofibres and trigger complement-mediated pathogenic effects. We previously observed that more than 80% of patients suffering from anti-SRP associated IMNM have IgG1 subclass anti-SRP aAb, an isotype capable of activating complement, whereas non-complement-activating IgG4 were significantly less frequent.23 27 Also, 100% of patients with anti-HMGCR reactivity have IgG1.28 For anti-SRP aAb, one in vitro study suggested a pathogenic effect on myoblasts with involvement of complement.29 We recently reported the accessibility of SRP and HMGCR at the surface of muscle cells in vitro and evidence of activation of classical pathway of complement cascade, accompanied by deposition of sarcolemmal immunoglobulins on biopsies from patients suffering from anti-SRP and anti-HMGCR associated IMNM.29 30 Therefore, the major effector mechanism in IMNM is presumably the complement-mediated pathogenicity of aAb.

In this line of thinking, the absence of complement activation cascade abolished the pathogenic effect of anti-SRP" and anti-HMGCR" IgG in passively transferred C3-deficient mice. Besides, it is known that mice have lower complement activity than humans.34 We hypothesised that supplementing mice with human complement could increase the pathogenic potency of anti-SRP and anti-HMGCR aAbs, as already shown for other aAbs in neuromyelitis optica or Miller Fisher syndrome.34 35 Indeed, muscle deficiency was more pronounced when anti-SRP" or anti-HMGCR" IgG were given in conjunction with human complement (figure 5D).

Together, our findings establish the pathogenic role of IgG from patients with anti-SRP and anti-HMGCR aAb in vivo by a mechanism partly involving complement. These results support the use of plasma exchange and B cell targeting therapies in anti-SRP and anti-HMGCR associated IMNM and argue for the evaluation of complement-targeting therapies.

Acknowledgements The authors would like to thank Roger Albesa, Damien Amelin, Audrey Aussy, Magalie Bénard, Ebba Brakenholm, Jean-Claude Do Rego, Anais Dumesnil, Arnaud Ferry, Fabienne Jouën, Jérémie Martinet, Eckart Mummet, Antoine Obry, Christophe Pitot, Reymond Public, Gaitan Ricu, Jean-Philippe Simon, Werner Stenzel, Serena Viapian, for their help and/or helpful discussion during this study. We are grateful to Nikki Sabourin-Gibbs, Rouen University Hospital, for her help in editing the manuscript.

Contributors CB, LD and OBo designed, performed and interpreted experiments presented in this manuscript. They also wrote and edited most of the manuscript content. HC, CA, GB, LJ and RZ performed experiments presented in this manuscript. YA, LA-D, NG, MM and OBe interpreted the experiments. OBo and DL are also the principal investigators of the study.

Funding The work presented in this study was supported in part by Association Française contre les Myopathies (16624), CSL Behring Foundation (France), the European Union and the Normandie Regional Council with the European Regional Development Fund (ERDF). Europe gets involved in Normandie with the ERDF.

Competing interests OBo is member of the Scientific Committee of CSL Behring Foundation (France) and received consulting fees from Inova Diagnostics.

Patient consent Not required.

Ethics approval The study was conducted on approval of the Ile-de-France III ethics committee. All patient consents were obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.
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Three-year cost-effectiveness analysis of the DRESS study: protocolised tapering is key

The DRESS (Dose REduction Strategy of Subcutaneous TNF inhibitors) study previously showed clinical non-inferiority and superior cost-effectiveness of disease activity-guided tapering of tumour necrosis factor inhibitors (TNFi) (dose reduction, DR group) over full dose continuation (usual care, UC group) in patients with rheumatoid arthritis (RA) with low disease activity.1,2 The safety and efficacy of this strategy were maintained up to 3 years, with a large reduction in TNFi use.3 During the extension phase, the majority of the UC group attempted dose reduction. This prevented a valid comparison of disease activity-guided tapering with full dose continuation over the entire study period but presented an opportunity to make the following comparisons:

1. Tapering long-term results (in the DR group 18–36 months) versus short-term results (in the DR group 0–18 months).
2. Tapering at the rheumatologist’s discretion (in the UC group 18–36 months) compared with full dose continuation (in the UC group 0–18 months).
3. Tapering at the rheumatologist’s discretion (in the UC group 18–36 months) compared with protocolised tapering (in the DR group 0–18 months).

We previously reported the main results of the DRESS extension study (Dutch trial registration number NTR3216), an open-label, non-inferiority, randomised controlled trial in which patients with RA with low disease activity on a stable TNFi dose (adalimumab or etanercept) were randomised 2:1 to disease activity-guided tapering or full dose continuation. In the first 18 months in the DR group, the TNFi dose was reduced stepwise until flare or TNFi discontinuation. In the extension phase, both groups were treated according to a treat-to-target protocol: tapering was recommended in case of a stable low disease activity, at the discretion of the rheumatologist in both groups.1,2 Quality-adjusted life years (QALYs) were determined by trapezoid method based on the EuroQol-5D5L measured quality of life. Since medication costs were the main cost drivers in the DRESS study, only medication costs were recorded from 18 to 36 months. Because comparisons within one group are paired observations, we bootstrapped within-patient differences in QALY and costs instead of group-level differences for these comparisons for a more efficient analysis.

The results from 1000 bootstrapped replications concerning mean QALYs and total medication costs for the three comparisons are presented in figure 1 and table 1. As shown, for the DR group, costs are slightly but non-significantly higher after 18 months (higher in 86.3% of replications), with QALY being lower (66.2%, higher in 33.8% of replications, 0.007 (95% CI 0.039 to 0.026) higher QALY for 0–18 months). Tapering at the rheumatologist’s discretion is associated with lower cost (100% of replications) and slightly lower QALY (98.5%) compared with full dose continuation, but also with higher cost (99.7% of replications) and non-significantly lower QALYs compared with protocolised tapering (in 90.2%). These results are not explained by differing disease activity at the start of tapering, as DAS28-CRP was higher and the proportion of DAS28-CRP remission was lower in those starting protocolised reduction (2.17 vs 2.01, and 67% vs 71%, respectively). Also, bias due to selective dropout is unlikely (dropout <5%).

In conclusion, the cost-effectiveness of protocolised tapering was maintained from 18 to 36 months, although medication costs rose slightly (but non-significantly), possibly because a subset of patients returned to a higher dose during follow-up. Tapering at the rheumatologist’s discretion was less cost-saving than protocolised tapering and resulted in higher QALY loss with full dose continuation.

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Table 1 Summary of results for each comparison

<table>
<thead>
<tr>
<th>Comparison</th>
<th>QALY</th>
<th>Difference in QALY</th>
<th>Medication costs</th>
<th>Difference in medication costs (€)</th>
<th>iNMB (€)</th>
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</thead>
<tbody>
<tr>
<td>Protocolised tapering 18–36 months versus protocolised tapering 0–18 months</td>
<td>1.230 (1.189 to 1.272)</td>
<td>−0.007</td>
<td>12 282 (10 775 to 13 866)</td>
<td>578</td>
<td>−1104</td>
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<tr>
<td>Unprotocolised tapering 18–36 months versus usual care 0–18 months</td>
<td>1.208 (1.152 to 1.272)</td>
<td>−0.047</td>
<td>15 717 (13 783 to 17 757)</td>
<td>−5941</td>
<td>2151</td>
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<tr>
<td>Unprotocolised tapering 0–18 months</td>
<td>1.237 (1.207 to 1.268)</td>
<td>−0.028</td>
<td>15 717 (13 783 to 17 757)</td>
<td>4013</td>
<td>−6309</td>
</tr>
</tbody>
</table>

Positive QALY or iNMB and negative cost differences favour the group listed first. All figures are presented as mean (95% percentile-based CI). iNMB, incremental net monetary benefit based on a willingness to pay 80 000 per QALY.4 QALY, Quality-adjusted life years.
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Handling editor Josef S Smolen

Contributors NvH, AAdB, AvdM, WK, FHJvdH, RFvV, NdB and HWJB were involved in the study design. NvH, AAdB, AvdM and FHJvdH were involved in the data collection. NdB and WK performed the data analyses. All authors were involved in writing, revision and final approval of the manuscript. NdB is the study guarantor.

Competing interests RFvV: grants from AbbVie, Amgen, BMS, GSK, Pfizer, Roche and UCB, and personal fees from AbbVie, Biotest, BMS, Celgene, Crescendo, GSK, Janssen, Lilly, Merck, Novartis, Pfizer, Roche, UCB and Vertex, outside the submitted work. HWJB: grants and personal fees from Pfizer and AbbVie, during the conduct of the study, and grants and personal fees from Roche, Bristol-Myers Squibb and Union Chimique Belge, outside the submitted work. AAdB: Congress invitations from Roche and AbbVie and an expert witness fee from Amgen.

Patient consent Not required.

Ethics approval CMO Regio Arnhem-Nijmegen: NL37704.091.11.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement The authors commit to making the relevant anonymised patient-level data available on reasonable request.

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Received 9 April 2018
Revised 28 June 2018
Accepted 1 August 2018
Published Online First 27 August 2018


REFERENCES
Adding baseline protein biomarkers to clinical predictors does not enhance prediction of treatment response to a methotrexate strategy in early rheumatoid arthritis

Recently, we identified baseline higher disease activity score assessing 28 joints, current smoking and no alcohol consumption as predictors of inadequate response (IR) to methotrexate (MTX), used with or without other conventional synthetic disease modifying anti-rheumatic drugs, here designated as ‘MTX+’, in new-onset rheumatoid arthritis (RA).1 For those with a predicted IR to ‘MTX+’, a more intensive treatment strategy could be initiated, if prediction would be reliable. Therefore, we investigated, within the same patient population, protein biomarkers for additive predictive value to these clinical predictors.

A model was developed using data from patients with RA in the U-Act-Early trial (ClinicalTrials.gov number NCT01034137) treated with a step-up MTX strategy (n=106) and was validated in patients who received MTX therapy (n=80) in the treatment in the Rotterdam Early Arthritis Cohort trial (tREACH, ISRCTN26791028).1,3 IR to ‘MTX+’ therapy was defined as the need to initiate a biological within the first treatment year. In baseline serum, 85 proteins were analysed in 2014 using multiplex Luminex profiling; seven candidate proteins, identified by partial least square discriminant analyses, were remeasured in 2018.

Clinical baseline characteristics of the patients in both studies are shown in table 1. Of the proteins identified in the development cohort, in the validation sample, the best, although not statistically significantly predicting biomarker, was vascular cell adhesion protein 1 (VCAM-1), OR 1.36, 95% CI 0.84 to 2.35; p=0.19. The area under the receiver operator characteristics curve (AUROC) of the combined model (ie, clinical model plus VCAM-1) in the development sample was 0.80 (95% CI 0.71 to 0.88), compared with 0.75, 95% CI 0.65 to 0.84 of the clinical model, p=0.051; no significant improvement in discriminative ability (figure 1). Also, when not taking ESR into account, as it may be considered a biomarker, adding VCAM-1 not significantly increased prediction (p=0.07). The protein model with VCAM-1 (AUROC 0.67, 95% CI 0.57 to 0.77) performed significantly worse than the clinical model (p=0.010). In the validation sample, for the combined

### Table 1  Baseline clinical characteristics of the two study samples

<table>
<thead>
<tr>
<th></th>
<th>U-Act-Early, n=106 (development sample)</th>
<th>tREACH, n=80 (validation sample)</th>
<th>P values</th>
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</thead>
<tbody>
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<td>Age, mean (SD) years</td>
<td>52 (14)</td>
<td>53 (14)</td>
<td>0.56</td>
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<td>BMI, mean (SD) kg/m²</td>
<td>26 (4)</td>
<td>27 (5)</td>
<td>0.47</td>
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<tr>
<td>Current smoking, n (%)</td>
<td>31 (29)</td>
<td>23 (29)</td>
<td>0.033</td>
</tr>
<tr>
<td>Female gender, n (%)</td>
<td>69 (65)</td>
<td>57 (71)</td>
<td>0.37</td>
</tr>
<tr>
<td>Alcohol consumption (≥1 unit per week), n (%)</td>
<td>64 (60)</td>
<td>60 (75)</td>
<td>0.002</td>
</tr>
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<td>Symptom duration, median (IQR) days</td>
<td>27 (15–46)</td>
<td>141 (94–187)</td>
<td>&lt;0.001</td>
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<tr>
<td>Anti-CCP positive, n (%)</td>
<td>84 (79)</td>
<td>65 (81)</td>
<td>0.73</td>
</tr>
<tr>
<td>RF positive, n (%)</td>
<td>86 (81)</td>
<td>64 (80)</td>
<td>0.85</td>
</tr>
<tr>
<td>SJC28, median (IQR)*</td>
<td>6 (3–10)</td>
<td>5 (2–8)</td>
<td>0.20</td>
</tr>
<tr>
<td>TJC28, median (IQR)*</td>
<td>7 (4–10)</td>
<td>5 (2–9)</td>
<td>0.005</td>
</tr>
<tr>
<td>ESR, median (IQR) mm/hour</td>
<td>25 (12–45)</td>
<td>23 (14–40)</td>
<td>0.70</td>
</tr>
<tr>
<td>CRP, median (IQR) mg/L</td>
<td>9 (3–21)</td>
<td>11 (5–26)</td>
<td>0.63</td>
</tr>
<tr>
<td>DAS28, mean (SD)‡</td>
<td>5.1 (1.2)</td>
<td>4.7 (1.3)</td>
<td>0.037</td>
</tr>
<tr>
<td>HAQ, median (IQR)§</td>
<td>1.0 (0.6–1.4)</td>
<td>1.0 (0.4–1.5)</td>
<td>0.44</td>
</tr>
<tr>
<td>Sharp/van der Heijde score, median (IQR)¶</td>
<td>0 (0–1)</td>
<td>1 (0–3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Erosion score, median (IQR)**</td>
<td>0 (0–0)</td>
<td>1 (0–1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>JSN score, median (IQR)††</td>
<td>0 (0–0)</td>
<td>0 (0–2)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Range 0–28; 28=maximum.  
†Range <10 mg/L.  
‡Range 0–9.4; 9.4=maximum.  
§Range 0–3; 3=maximum.  
¶Range 0–280; 280=maximum.  
**Range 0–168; 168=maximum.  
BMI, body mass index; CCP, cyclic citrullinated peptide; CRP, C-reactive protein; DAS28, disease activity score assessing 28 joints; ESR, erythrocyte sedimentation rate; HAQ, health assessment questionnaire; JSN, joint space narrowing; RF, rheumatoid factor; SJC, swollen joint count; TJC, tender joint count.

model, the AUROC was 0.67 (95% CI 0.55 to 0.80), not significantly different from those of the clinical model (0.68, 95% CI 0.55 to 0.80; p=0.94) and protein model (0.58, 95% CI 0.46 to 0.71; p=0.06). In the combined model, similar predictive accuracy was found for other candidate proteins, and a combination of the best performing biomarkers did not enhance prediction (online supplementary tables 1 and 2).

To evaluate stability of VCAM-1 over time, we compared median (IQR) concentrations within the 2014 versus 2018 immunoassay in the development sample (figure 1). They were 272 (226-341) and 181 (152-229) pg/mL (×1000), respectively: a clear decrease over time (proportional difference: 33%), although serum had been stored frozen below the eutectic point of enzymatic activity (≤−25°C) of blood plasma. This decrease is most likely due to molecular breakdown, indicating compromised usability over time of absolute VCAM-1 concentrations on the individual level. However, when analysing the concentrations on group level, a significant correlation (0.73, 95% CI 0.62 to 0.81; p<0.001) was found between both assays. Similar results were found for the other biomarkers (online supplementary table 3). By normalising (ie, computing z-scores) the data, the effect of generally decreased concentrations is eliminated and protein biomarkers may still be of use in relative prediction on the group level.

In summary, in baseline serum of the same patients analysed for clinical predictors, no valid predictive protein biomarker was found. VCAM-1, the best performing biomarker, did not increase the predictive value of the clinical predictors for the need for adding a biological to ‘MTX+’ therapy in patients with early RA. Clinical applicability of protein biomarkers seems limited, as absolute concentrations are prone to be influenced by numerous factors, such as storage time. Nevertheless, on the group level, protein biomarkers could be of interest for research purposes.

Figure 1  Identifying protein biomarkers for predicting IR to ‘MTX+’ therapy. ROC curves in the (A) development and (B) validation sample showing the predictive accuracy of the different models. The clinical model contains baseline DAS28, current smoking and alcohol consumption as predictors, and the protein model contains VCAM-1 as predictor; the combined model contains the predictors of both the clinical and protein model. Scatterplots of (C) absolute concentrations, in pg/mL (×10 000) (D), normalised (z-scores, that is, scores expressed as SD units from the mean) VCAM-1 concentrations within the first versus second immunoassay. Negative z-scores indicate lower than average concentration of the sample. Dotted diagonal line in the scatterplots represents perfect correlation between both assay’s and the cyan line the regression line. AUROC, area under the receiver operator characteristic curve; DAS28, disease activity score assessing 28 joints; IR, inadequate response, MTX, methotrexate; ROC, receiver operator characteristic; VCAM-1, vascular cell adhesion protein 1.

Table 3  AUROC values for the clinical model, protein model, and combined model, for the development and validation sample.

<table>
<thead>
<tr>
<th>Model</th>
<th>Development sample</th>
<th>Validation sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUROC (95% CI)</td>
<td>AUROC (95% CI)</td>
</tr>
<tr>
<td>Clinical</td>
<td>0.75 (0.65-0.84)</td>
<td>0.68 (0.55-0.80)</td>
</tr>
<tr>
<td>Protein</td>
<td>0.67 (0.67-0.77)</td>
<td>0.58 (0.46-0.71)</td>
</tr>
<tr>
<td>Combined</td>
<td>0.80 (0.71-0.88)</td>
<td>0.67 (0.55-0.80)</td>
</tr>
</tbody>
</table>

Xavier M Teitsma, 1 Johannes W G Jacobs, 1 Pascal H P de Jong, 2 Johanna M W Hazes, 2 Angelique E A M Weel, 2,3 Paco M J Welsing, 1 Attila Pethö-Schramm, 4 Michelle E A Borm, 5 Jacob M van Laar, 1 Johannes W J Bijlsma, 1 Floris P J G Lafeber 1

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Handling editor Prof Josef S Smolen

Acknowledgements The authors especially would like to thank the participating patients in both the U-Act-Early and tREACH trials for their cooperation and willingness to contribute as well as all rheumatologists, study nurses, laboratory personnel, co-investigators and others who were involved and also all the participating hospitals in which patients were recruited and treated.

Contributors All authors were involved with drafting the article or revising it critically and approved the final draft to be published and agree to be accountable for all aspects. Study conception or design: XMT, JWGJ, PMJW, PHPdJ, FPJGL, JWJB. Acquisition of data: XMT, JWGJ, PMJW, PHPdJ, AP-S and MEAB. Analysis or interpretation of data: all authors.

Funding The U-Act-Early trial was funded by Roche Nederland BV and the work within the tREACH trial was supported by an unrestricted grant from Pfizer.

Competing interests The department of the authors who included patients (JWGJ and JWJB) in the U-Act-Early trial received reimbursements from Roche Nederland BV. JWJB reported grants and fees from Roche, AbbVie, Bristol-Myers Squibb, Merck Sharp & Dohme, Pfizer and UCB. JWJB received fees from Arthrogen, MSD, Pfizer, Eli Lilly and BMS and research grants from AstraZeneca and Roche-Genentech. FPJGL reports grants from Roche. AP-S is an employee of F Hoffmann-La Roche, and MEAB is an employee of Roche Nederland BV.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

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Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2018-213767).


Received 14 May 2018
Revised 20 June 2018
Accepted 8 August 2018
Published Online First 29 August 2018


REFERENCES
Evaluation of the predictive accuracy of MRI-detected erosions in hand and foot joints in patients with undifferentiated arthritis

Radiographic erosions are a clear hallmark of rheumatoid arthritis (RA). The European League Against Rheumatism (EULAR) definition of radiographic erosive disease has a high specificity, and its fulfilment alone is sufficient to classify RA. However, the sensitivity of radiography to detect erosions early in the disease is low. Other imaging techniques, such as MRI, are more sensitive to detect erosions than radiography and are therefore recommended by a EULAR imaging task force. To determine the specificity of MRI-detected erosions, we recently compared erosions in the metacarpophalangeal (MCP) and metatarsophalangeal (MTP) joints (scored according to the RA MRI Scoring System (RAMRIS)) of patients presenting with RA with those of symptom-free persons and patients presenting with arthritides other than RA. MRI-detected erosions were present in all groups; therefore, the specificity of the presence of any MRI-detected erosions was low. By evaluating different erosion features, a few features were identified as specific for RA; these were severe erosions (grade ≥2, defined as >10% of bone eroded), erosions in MTP5 and erosions in MTP1 in persons aged <40. A subsequent and clinically relevant question is whether MRI-detected erosions in patients presenting with undifferentiated arthritis (UA) are valuable in predicting future progression to RA. This was explored to a limited extent in our previous study but as the number of patients with UA was limited (n=192), the predictive value of the different ‘RA-specific erosions’ could not be studied. In addition, the outcome was fulfilment of classification criteria but start of disease-modifying antirheumatic drugs (DMARDs) was not considered, while DMARD treatment might have hampered progression to fulfilment of RA classification criteria. Finally, MRI-detected erosions were only evaluated in the MCP and MTP joints and not in wrist joints, while erosions in the wrist are prevalent. To evaluate the predictive accuracy of MRI-detected erosions more thoroughly, we performed cross-sectional comparisons between patients with early RA and patients with other arthritides to search for erosion features of wrist joints which are RA specific.

All studied patients were consecutively included in the Leiden Early Arthritis Clinic cohort. Inclusion required the presence of clinically confirmed inflammatory arthritis and symptom duration less than 2 years. At baseline 1.5T MRI of the second to fifth MCP, wrist and first to fifth MTP joints was performed as described. Erosions were scored on a scale 0–10 according to the RAMRIS system. Wrist erosions specific for RA were assessed by performing cross-sectional comparisons of MRI-detected erosions in the wrist in 238 patients with RA and 351 patients with other arthritides who were included between 2010 and 2014; the number, location and severity of erosions as well as concomitant bone marrow oedema (BME) were evaluated. Thereafter, the predictive value of MRI-detected erosions in MCP, wrist and MTP joints in 286 patients with UA (using the 2010 criteria to classify RA), included between 2010 and 2016, was evaluated. The predictive accuracy of the presence of any MRI-detected erosions, defined as score ≥1 by both readers, as well as of the presence of RA-specific erosions (as defined previously for MCP and MTP joints or as studied here for the wrist) was assessed. Ninety-four per cent of the 286 2010 UA patients were anticitrullinated protein antibody (ACPAs)-negative (online Supplementary table 1), which is in line with other descriptions of the population of 2010 UA patients. Patients were followed for 1 year on RA development, defined as fulfilling the 2010 criteria or the start of DMARDs because of a clinical diagnosis of RA. The latter was added as ACPA-negative patients need >10 involved joints to fulfil the 2010 criteria which could be hampered by DMARD treatment. One hundred and twenty-eight (45%) patients with UA developed the outcome, of which 111 had a
Although MRI is sensitive to detect the presence of erosions, the clinical diagnosis of RA and started DMARDs and 17 fulfilled the 2010 criteria.

First, we searched for MRI-detected wrist erosions that were specific for RA. The median total number of erosions in the wrist was 1.0 (IQR 0–3.0) for patients with RA and 1.0 (IQR 0–2.0) for patients with other arthritides (Mann-Whitney U test: p=0.82). Severe erosions, defined as grade ≥2, were infrequent and present at a similar rate in patients with RA and patients with other arthritides (5% and 6%, respectively; online Supplementary table 2). With respect to the location, erosions were most frequently observed in the capitate, triquetrum, lunate and scaphoid, especially at increasing age of onset; however, the frequency was not different in patients with RA and patients with other arthritides (online Supplementary table 2). Finally, the combined presence of erosions with BME within the same bone was evaluated. This combination was more prevalent with increasing age of onset, but frequencies were comparable in both groups (3% of both patients with RA and patients with other arthritides, online Supplementary table 2). Altogether, no RA-specific features of MRI-detected erosions located in the wrist could be identified.

Next, the predictive value of MRI-detected erosions was evaluated in patients with UA. Any MRI-detected MCP and MTP erosions were present in 49% of the 286 patients with UA and were not predictive for RA development (OR 1.2, 95%CI 0.8 to 2.0, PPV 48%, table 1). RA-specific erosions were present in only 7% of the 2010 UA patients and were also not associated with development of RA (OR 0.6, 95%CI 0.2 to 1.5, PPV 33%). Similar findings were obtained for the individual RA-specific erosions (table 1). Any MRI-detected wrist erosions were present in 61% of the patients with UA and also not predictive for RA development (OR 1.5, 95%CI 0.9 to 2.4, PPV 49%). Sensitivity analyses stratified for the outcome (DMARD-start or only 2010 criteria positive) revealed similar results (data not shown).

This is the largest longitudinal dataset on MRI-detected erosions in hand and foot joints in UA to date. In all analyses, MRI-detected erosions were not associated with an increased risk on RA. Although MRI is sensitive to detect the presence of erosions, the present data suggest that evaluation of MRI-detected erosions in UA is not relevant for the early detection of RA.

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Received 30 May 2018
Revised 17 July 2018
Accepted 19 July 2018
Published Online First 31 July 2018

REFERENCES

Table 1 Predictive values of MRI-detected erosions within 2010 UA patients for the development of RA

<table>
<thead>
<tr>
<th>Patients with UA with erosion feature, n (%)</th>
<th>PPV (95% CI)</th>
<th>NPV (95% CI)</th>
<th>OR (95% CI)</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any MRI-detected erosion in MCP and/or MTP joint</td>
<td>141 (49)</td>
<td>48% (39 to 56)</td>
<td>58% (50 to 66)</td>
<td>1.2 (0.8 to 2.0)</td>
<td>52% (44 to 61)</td>
</tr>
<tr>
<td>Grade 2 erosion in MCP- and/or MTP-joint</td>
<td>21 (7)</td>
<td>33% (17 to 55)</td>
<td>61% (52 to 70)</td>
<td>1.3 (0.5 to 3.2)</td>
<td>66% (58 to 74)</td>
</tr>
<tr>
<td>Erosion in MTP1 and aged &lt;40</td>
<td>16 (6)</td>
<td>44% (23 to 67)</td>
<td>55% (49 to 61)</td>
<td>1.0 (0.3 to 2.6)</td>
<td>5% (3 to 11)</td>
</tr>
<tr>
<td>Erosion in MTP5</td>
<td>5 (2)</td>
<td>31% (12 to 77)</td>
<td>54% (48 to 60)</td>
<td>0.8 (0.1 to 5.0)</td>
<td>2% (0 to 6)</td>
</tr>
</tbody>
</table>

The prior risk for development of RA and/or DMARD-use within 1 year was 45%. Any MRI-detected erosion was defined as score ≥1 by both readers according to the RA MRI Scoring System. Any RA-specific erosion was defined on MRI as the presence of a grade ≥2 erosion in an MCP and/or MTP joint, an erosion in MTP5 and/or an erosion in MTP1 in the age group <40 years, as described earlier.

DMARD, disease-modifying antirheumatic drug; NPV, negative predictive value; PPV, positive predictive value; RA, rheumatoid arthritis.

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Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2018-213851).

Elective approval Local Medical Ethics Committee, which is named ‘Commissie Medische Ethiek’.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement Data can be requested from the corresponding author.

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Letters

Secretory form of rheumatoid arthritis–associated autoantibodies in serum are mainly of the IgM isotype, suggesting a continuous reactivation of autoantibody responses at mucosal surfaces

Several lines of evidence obtained in recent years indicate a role of mucosal surfaces in the development of autoimmune responses associated with rheumatoid arthritis (RA). Therefore, more attention is going to the influence of the microbiome in RA development. Secretory antibodies are typically produced at mucosal surfaces and transported through epithelial cells for secretion at the luminal site. Importantly, secretory antibodies comprise both IgM and IgA, and both isotypes can harbour a J-chain that binds to the polymeric Ig receptor (pIgR).1 Following transport through epithelial cells, the antibodies are enzymatically released at the luminal site by cleavage of the pIgR leaving a fragment of the receptor, the secretory component (SC) bound to the immunoglobulin. SC-containing antibodies can however also be detected systemically in the circulation, although the mechanism by which these SC-containing antibodies arise in serum is still unclear.5 Interestingly, antigen-specific secretory antibodies are present in serum after mucosal immunisation,1

![Figure 1](https://example.com/figure1.png)

**Figure 1** Secretory anti-CarP, ACPA and RF are increased in patients with RA. ELISAs were performed to detect secretory anti-CarP antibodies (A), secretory ACPA (B) and secretory RF (C) in sera of 207 HC and 363 patients with RA. Next to this total secretory IgA (D), total IgA (E), AU secretory IgA per milligram total IgA (F), total secretory IgM (G), total IgM (H) and AU secretory IgM per milligram total IgM (I) were measured in all HC and patients with RA. Plates were coated with Ca-FCS (A), CCP2 (B) and human IgG (C) and after serum incubation bound antibodies were detected using goat–anti-human secretory component (Nordic Mubio). For the detection of total secretory IgA and IgM, plates were coated with mouse–anti-human secretory component (SPM217) and after serum incubation bound antibodies were detected using goat–anti-human IgM (Millipore) or IgA (Novex). Total IgA and IgM was measured using the Bethyl kit (manufacturer's protocol). The 97th percentile in HC was used as cut-off for the presence of secretory autoantibodies. The dotted line represents the cut-off and the red line the median. The green lines represent the ‘normal’ range for total IgA and IgM in serum. The specific secretory antibody reactivity is depicted in arbitrary units per millilitre and total IgA and IgM in milligrams per millilitre. Please note, as levels are depicted in AU, no direct comparisons in absolute antibody levels can be made. The number of samples tested and the percentage positivity are shown below the graphs. Mann-Whitney U tests were carried out to determine differences in antibody levels between patients with RA and HC. The Pearson χ² test was used to determine differences in positivity between patients with RA and HC. ACPA, anti-citrullinated protein antibody; anti-CarP antibody, anti-carbamylated protein antibody; AU, arbitrary units; SC, secretory component. Mann-Whitney U test *p=0.05–0.002, **p=0.002–0.0002, ***p=0.0002–0.0001, ****p<0.0001.
Secretory anti-CarP, ACPA and RF are predominantly of the IgM isotype. Serum samples of nine patients with rheumatoid arthritis were depleted for IgM (A–F) or IgA (G–L) and the presence of secretory anti-CarP antibodies, secretory ACPA and secretory RF (D–F, J–L) as well as total IgM, IgA and IgG (A–C, G–I) was analysed in the start and depleted material. Plates were coated with Ca-fetal calfs serum (FCS) (D, J), CCP2 (E, K) and human IgG (F, L), and after serum incubation, bound antibodies were detected using goat–anti-human secretory component (Nordic Mubio). Total IgM, IgA and IgG was measured using the Bethyl kit (manufacturer’s protocol). Every colour represents a patient. ACPA, anti-citrullinated protein antibody; anti-CarP antibody, anti-carbamylated protein antibody; AU arbitrary units; RF, rheumatoid factor.

The prevalence and levels of SC-anti-CarP, SC-ACPA and SC-RF are increased in patients with RA compared with HC (prevalence: p<0.0001, levels: p<0.0001) (figure 1A–C). Levels of SC-total-IgA and SC-total-IgM as well as total IgA and total IgM were increased in patients with RA compared with HC (p<0.0001 for all) (figure 1D,E,G,H). The increase of SC-total-IgM appeared RA specific as no increase was observed in disease controls (online supplementary figure S1). Intriguingly and unexpectedly, we observed that the SC autoantibodies were predominantly present as IgM and not of the IgA isotype as mostly assumed (figure 2). Indeed, the presence of SC-containing autoantibodies correlates best with SC-total-IgM (online supplementary figure S2), also pointing towards their presence in the IgM isotype. The increased representation of SC-IgM autoantibodies is not a general feature of SC immunoglobulins present in RA, but likely more specific for autoantibodies as correction for total levels of IgM normalised the observed increase in SC-total-IgM (figure 1F,I). Importantly, the avidity of anti-CarP IgA and IgM, ACPA IgA and IgM and RF IgA and IgM are similar (online supplementary figure S3), indicating that the observed
differences are not explained by ‘technical issues’ related to the sensitivity of the methods used to detect SC-IgA versus SC-IgM autoantibodies.

Overall, SC-anti-CarP, SC-ACPA and SC-RF are present in and specific for RA and consist predominantly of the IgM isotype. These findings are of importance as this indicates that especially autoreactive IgM-expressing B cells represent the most prominent B-cell subset that is reactivated at mucosal areas, possibly (re)activating and keeping the immune response ongoing. This (re)activation could involve both naive and pre-existing memory IgM B cells or even (gut) IgM+ plasma cells directed against commensals, possibly pointing to a role of the microbiome in steering RA-specific autoimmune responses.

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Handling editor Josef S Smolen

Acknowledgements We thank E W N Levarht for her advice and technical assistance and help with the collection of the materials. We thank E de Moel for her help with some statistical analysis and C van Kooten for his help and advice in interpreting the data.

Contributors MAMvD set up the study design and performed the experiments, as well as the analysis, interpretation of the data, drafting the article and approval of the final manuscript. DvdW and REMT contributed to the interpretation of the data, revising the manuscript and approval of the final manuscript. LAT contributed to study design, interpretation of the data, revising the manuscript and approval of the final manuscript.

Funding This work was supported by the Dutch Arthritis Foundation (14-2-402) and the IMI JU–funded project BeTheCure (115142-2). DvdW is supported by a ZON-MW Veni grant, REMT by a ZON-MW Vici grant and LAT is supported by a ZON-MW Vidi grant (91712334).

Competing interests REMT and LAT are listed as inventors in a patent application regarding the detection of anti-Carp antibodies for RA.

Patient consent Not required.

Ethics approval Ethical committee of the LUMC.

Provenance and peer review Not commissioned; externally peer reviewed.

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► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/annrheumdis-2018-213724).


Received 7 May 2018
Revised 20 July 2018
Accepted 22 July 2018
Published Online First 14 August 2018

REFERENCES
Two distinct clinical phenotypes of pulmonary arterial hypertension secondary to systemic lupus erythematosus

Pulmonary arterial hypertension (PAH) is a severe complication of systemic lupus erythematosus (SLE), and SLE-PAH is the most common connective tissue disease (CTD)-associated PAH in Chinese patients. The prognosis of SLE-PAH is poor, with 3-year survival rates varying from 45% to 88%. Due to the complexity and heterogeneity of the underlying disease, it is necessary to further differentiate among SLE-PAH patterns to better understand the disease and optimise its management.

Between 2011 and 2016, a derivation cohort (Shanghai Ren Ji Hospital, n=108) and a validation cohort (Guangdong General Hospital, n=87) of patients with SLE-PAH from two medical centres were included. Patients with SLE-PAH were diagnosed based on right heart catheterisation or echocardiography (peak tricuspid regurgitation velocity >3.4 m/s), and those with left heart disease, pulmonary thrombosis and lung diseases were excluded. Based on the baseline clinical manifestations and laboratory findings at the time of the diagnosis of PAH, two distinct clusters were identified and validated by multiple correspondence analysis and k-means clustering. Cluster 1 had systemic manifestations and high SLE disease activity, including pericarditis, rash, arthritis, nephritis and neuropsychiatric lupus, while cluster 2 tended to have low disease activity but purer PAH. According to their characteristics, cluster 1 and cluster 2 were named the vasculitic subtype and the vasculopathic subtype, respectively. Kaplan-Meier survival analysis revealed that patients with the vasculitic subtype had a significantly higher 3-year mortality rate than those with the vasculopathic subtype (34.5%–40.2% vs 13.0%–18.6%, p<0.05; HR 2.84–3.15). The difference in survival still existed after adjusting for treatment variations, including corticosteroids, immunosuppressants and PAH-targeted vasodilators, by propensity score matching (figure 1), which further underscores the importance of two clinical phenotypes. To identify predictors of the high-risk vasculitic subtype, multivariate logistic regression was performed and optimal cut-offs were obtained by receiver operating characteristic curve. The time interval between the diagnoses of SLE and PAH (<2 years, p<0.0001) and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI >9, p=0.001) score were identified as two independent predictors in both
cohort. A prediction model combining these two factors was further developed and a weighted score ≥2 yielded a sensitivity of 98.5% and a specificity of 74.4% (area under the curve 0.94, p<0.0001) in discriminating the vasculitic subtypes (table 1).

To the best of our knowledge, this is the first study aiming to distinguish between different clinical phenotypes of SLE-PAH in two independent cohorts. A simple and reliable prediction model was developed, which yielded a high predictive performance for the high-risk vasculitic subtype. Of the two predictors identified in this study, SLEDAI scores are widely used to evaluate SLE disease activity, and the categories of ≤4, 5–9 and >9 are clinically meaningful to represent low, moderate and high overall SLE activity, respectively. Interestingly, early onset of PAH after the diagnosis of SLE was a newly identified predictor of the vasculitic subtype in our study. It has been reported that patients with a simultaneous diagnosis of PAH and CTD are more likely to have active underlying disease and are prone to respond to intensive immunosuppressive therapy. It is noteworthy that this dichotomisation and nomenclature was based on clinical rather than pathological characteristics. Nevertheless, the existence of distinct clinical phenotypes of SLE-PAH hypothetically suggests different underlying pathophysiological mechanisms, that is, autoimmune-mediated processes versus non-inflammatory vascular remodelling. However, the clustering of patients with SLE-PAH found in this retrospective study needs further confirmation in prospective studies. The next key question that remains unanswered is how to balance the utility of immunosuppressants and PAH-targeted drugs in patients with different phenotypes. The results of our study may help facilitate the individualisation of disease management and guide future clinical trial design in patients with SLE-PAH.

Table 1 Parameters of the prediction model in the derivation, validation and pooled cohorts

<table>
<thead>
<tr>
<th>Weighted score cut-off values</th>
<th>Derivation cohort (n=108)</th>
<th>Validation cohort (n=87)</th>
<th>Pooled cohort (n=195)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≥1</td>
<td>≥2</td>
<td>≥3</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>100</td>
<td>97.3</td>
<td>51.4</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>36.6</td>
<td>81.7</td>
<td>100</td>
</tr>
<tr>
<td>PPV (%)</td>
<td>45.1</td>
<td>70.6</td>
<td>100</td>
</tr>
<tr>
<td>NPV (%)</td>
<td>100</td>
<td>98.2</td>
<td>79.8</td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>0.95 (0.91 to 0.99)</td>
<td>0.94 (0.89 to 0.99)</td>
<td>0.94 (0.90 to 0.97)</td>
</tr>
</tbody>
</table>

Prediction model and scoring system

<table>
<thead>
<tr>
<th>Time interval between SLE and PAH</th>
<th>Derivation cohort</th>
<th>Validation cohort</th>
<th>Pooled cohort</th>
</tr>
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<tbody>
<tr>
<td>&lt;2 years</td>
<td>1</td>
<td>SLEDAI</td>
<td>&gt;9</td>
</tr>
<tr>
<td>≥2 years</td>
<td>0</td>
<td></td>
<td>5–9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;5</td>
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</table>

AUC, area under the curve; NPV, negative predictive value; PAH, pulmonary arterial hypertension; PPV, positive predictive value; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

Figure 1 Survival curves for patients with systemic lupus erythematosus-pulmonary arterial hypertension in two distinct clusters from two independent cohorts (A and B) and the pooled cohort (C). Pooled cohort (C) was derived by propensity score matching according to different treatments.

A summary of the table:

- For the derivation cohort, the sensitivity was 100% for the weighted score ≥1, 97.3% for ≥2, and 51.4% for ≥3.
- The specificity was 36.6% for ≥1, 81.7% for ≥2, and 100% for ≥3.
- The PPV was 45.1% for ≥1, 70.6% for ≥2, and 100% for ≥3.
- The NPV was 100% for ≥1, 98.2% for ≥2, and 79.8% for ≥3.
- The AUC was 0.95 (95% CI: 0.91 to 0.99).

The study findings support the development of a prediction model that can help identify patients at high risk of the vasculitic subtype of SLE-PAH. This model could aid in guiding treatment decisions and clinical trials.
Letters

Competing interests SY has received research funding from the National Key Research and Development Program of China. FS has received research funding from Ren Ji Hospital South Campus, School of Medicine, Shanghai Jiao Tong University. Other coauthors have nothing to disclose.

Ethics approval The ethical approval was waived for this study due to its retrospective design.

Provenance and peer review Not commissioned; externally peer reviewed.
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References
Pre-existing antiacetylcholine receptor autoantibodies and B cell lymphopaenia are associated with the development of myositis in patients with thymoma treated with avelumab, an immune checkpoint inhibitor targeting programmed death-ligand 1

Immune checkpoint inhibitors enhance the immune response against tumours but may also trigger immune-related adverse events (IRAEs). Myositis is a rare IRAE. For example, creatine kinase (CK) elevations occurred in just 0.3% of those treated with avelumab, an antiprogrammed death-ligand 1 antibody.1

Thymomas are the most common anterior mediastinal masses in adults. Since effective systemic therapies for thymic epithelial tumours are lacking, we included seven patients with recurrent thymoma and one patient with recurrent thymic carcinoma in a phase I trial of avelumab (NCT01772004). Details regarding this trial have been published separately.2

Myasthenia gravis and myositis occur in up to 30% and 5% of patients with thymoma, respectively.3 Although no patient had a history of autoimmunity or weakness and each had normal baseline CK levels, four patients developed weakness and elevated CK levels, ranging from 762 IU/L to 16 037 IU/L, within 5 weeks of avelumab administration (see online supplementary text and table 1). CK levels normalised in patients within weeks of stopping avelumab and starting immunosuppressive therapy. Of note, one patient with myositis also had myocarditis and one patient without myositis developed autoimmune enteritis.

We tested for thymoma-associated autoantibodies in sera collected before and after avelumab treatment (table 1). Four patients had pre-existing muscle acetylcholine receptor (mAChR) autoantibodies and each developed CK elevations. No patient without mAChR autoantibodies developed myositis (100% vs 0%; p=0.029). Myositis and myasthenia have been reported to occur together as an IRAE.4 Although we cannot exclude

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serum CK (IU/L)</th>
<th>Anti-AChR (nmol/L)</th>
<th>Anti-STR (dilutions)</th>
<th>Anti-VGKC (nmol/L)</th>
<th>Anti-GAD65 (nmol/L)</th>
<th>Anti-a3 (nmol/L)</th>
<th>Anti-CRMP5</th>
<th>Anti-AMPA</th>
<th>Anti-GABAB</th>
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<td>55</td>
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</tr>
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<td>#3 Pre</td>
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<td>#6 Pre</td>
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<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
</tbody>
</table>

Note that serum CK levels included in this table are those obtained at the time sera were collected for autoantibody testing and may not reflect peak CK levels for a given patient. Autoantibody testing was performed at the Neuroimmunology Laboratory, Mayo Clinic, Rochester, Minnesota.

α3, ganglionic α3 acetylcholine receptor; AChR, acetylcholine receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Caspr2, contactin-associated protein-like 2; CK, creatine kinase; CRMP5, collapsin response-mediator protein-5; GABAR, gamma-aminobutyric acid receptor; GAD65, glutamic acid decarboxylase E5; LGI1, leucine-rich glioma-inactivated 1; Neg, negative; NMDA, N-methyl-D-aspartate receptor; NT, not tested; Pos, positive; STR, striational; VGKC, voltage-gated potassium channel.
the possibility that our patients could have had both myositis and myasthenia, electrophysiological studies revealed evidence of a neuromuscular junction defect in just one patient. Three patients had both mACHr and striational autoantibodies. Voltage-gated potassium channel autoantibodies were found in two patients and one of them developed myositis; neither patient developed manifestations of potassium-channel autoimmunity. Since approximately 70% of patients with myositis have a myositis-specific autoantibody (MSA), we screened preavelumab and postavelumab serum samples for 16 MSAs using Autoimmune Inflammatory Myopathies line blots (EUROIMMUN). Although no patient had an MSA on this panel, we cannot exclude the possibility that they may have had an unidentified, potentially pathogenic, autoantibody.

Flow cytometry performed on peripheral blood mononuclear cells collected prior to avelumab therapy revealed that patients who developed myositis had low B cell frequencies (Figure 1, online supplementary tables 2 and 3). A single patient without myositis, but who developed enteritis, also had low B cell levels. Taken together, patients with thymoma who developed myositis or enteritis had lower B cell frequencies (0.19%, 0.12%–0.73%; median, IQR) than patients with thymoma who did not (12.37%, 5.14%–16.5%), those with non-thymic malignancies (8.3%, 2.4%–11.7%) or healthy controls (16.3%, 11.9%–17.6%).

These observations suggest that testing for mACHr autoantibodies and/or B cell levels may identify patients with thymoma most at risk for developing myositis with avelumab. Since mACHr autoantibodies cause myasthenia but not myositis or elevated CK levels, and because mACHr autoantibody levels did not increase with myositis, we conclude that they are most likely a marker of pre-existing autoimmunity rather than the direct cause of muscle damage. B cell lymphopaenia, which occurs in half of patients

**Figure 1**  Immune cell subsets in patients with thymoma prior to treatment with avelumab. Flow cytometry was performed on peripheral blood mononuclear cells (PBMCs) prior to avelumab treatment. Cell types were defined as follows: regulatory T cells are CD4+CD25+FoxP3+CD127−, natural killer cells are CD56+CD3−, and myeloid derived suppressor cells are CD11b+HLA-DRlow/−CD33+. The median and IQR are indicated by long and short horizontal bars, respectively. The patient without myositis who developed autoimmune enteritis is noted with an open square.
with thymoma, has not been described in myositis. Interestingly, a recent study reported that declining B cells preceded the development of IRAEs in patients with melanoma following combination CTLA4 and PD1 checkpoint blockade; however, unlike the patients described here, these patients had normal B cell levels prior to checkpoint blockade. It remains unclear why declining B cell levels would be associated with IRAEs, including myositis.

Additional studies are needed to confirm these findings and to determine whether pre-existing autoantibodies or immune cell subset dysregulation predicts which non-thymic tumour patients are at increased risk for IRAEs.

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Contributors ALM, AR, RND and JLG designed the study, collected the data, analysed the results and wrote significant portions of the manuscript. TL, AZ, SJP, JS and JLG designed aspects of the study, collected the data and analysed the results. All authors revised the manuscript critically for important intellectual content and approved the final version.

Funding This work was financially supported by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases and the National Cancer Institute of the National Institutes of Health. LC-R is funded in part by the Donald B and Dorothy L Stabler Foundation.

Competing interests None declared.

Patient consent Not required.

Ethics approval NIH IRB.

Provenance and peer review Not commissioned; externally peer reviewed.

Conflict of interest None.


Received 15 May 2018
Revised 20 July 2018
Accepted 30 July 2018
Published Online First 5 September 2018

REFERENCES
Depression and anxiety associate with less remission after 1 year in rheumatoid arthritis

Depression and anxiety have been considered to influence disease activity, and with great interest we read the recently published report by Michelsen et al. In this large, prospective, multicentre observational study, depression and anxiety reduced the likelihood of joint remission based on composite scores, in rheumatoid arthritis (RA) after 3 and 6 months. Differences were predominantly caused by subjective markers of disease activity rather than by C reactive protein or erythrocyte sedimentation rate. The study cannot prove causality; however, their findings imply that baseline depression/anxiety can impair the fulfilment of remission criteria during follow-up, influencing important treatment decisions.

As replication is a keystone in research, we aimed to validate their findings in an independent cohort, the Leiden Early Arthritis Clinic (EAC), to assess generalisability of the results. The EAC is a population-based inception cohort of patients with newly diagnosed arthritis that started in 1993; from 2010 onwards patients completed the Short Form-36 (SF-36) at baseline. We studied patients included between 2010 and 2014 who fulfilled the 2010 criteria for RA (n=343) and selected patients who completed the SF-36 (n=293). Patients with RA were treated according to the insight of the treating rheumatologist: standard therapy regimen consists of early initiation with methotrexate; in case of failure a second synthetic disease-modifying antirheumatic drug (DMARD) was prescribed and in case of failure a biologic DMARD was allowed. Outcome of joint remission was 44-joint Disease Activity Score (DAS44 ≤2.4) after 1 year. Similar as Michelsen et al we identified depression/anxiety by the SF-36 mental health subscale (MH ≤56) and SF-36 mental component summary (MCS ≤38).

Baseline characteristics are shown in table 1. The percentage of depressed/anxious patients with RA was 20% according to the SF-36 MCS ≤38 and 23% according to the SF-36 MH ≤56. Anxious/depressed patients were significantly younger and had a higher patient global assessment (table 1). Anxiety and depression were negatively associated with achieving DAS remission after 1 year, analysed with logistic regression models corrected for age, gender and symptom duration (OR, 0.21, 95% CI 0.09 to 0.46 for MCS; OR, 0.24, 95% CI 0.11 to 0.51 for MH; P<0.001; figure 1). Analyses with additional correction for baseline DAS showed similar results (MCS P<0.001; MH P=0.001).

Thus, our study on the association of baseline anxiety and depression with remission after 1 year validated the findings from Michelsen et al. We observed higher percentages of patients with RA in DAS remission, which could be caused by the longer duration of treatment (evaluation of remission at 1 year, instead of 3 and 6 months by Michelsen et al).

Concluding, baseline depression and anxiety are associated with a lower chance to achieve DAS remission, which was mostly reflected by associations with subjective features of disease activity. Also our study cannot prove causality, although the association between the mental state and DAS components suggests that efforts to improve the psychological well-being early in the disease course may prevent higher DAS scores later on. This could potentially prevent increased medical costs due to more intensified treatment strategies.

Table 1 Baseline characteristics of patients with rheumatoid arthritis with versus without baseline depression/anxiety according to the MCS ≤38 or MH ≤56

<table>
<thead>
<tr>
<th></th>
<th>All patients (n=293)</th>
<th>Depressed/anxious (n=81)</th>
<th>Not depressed/anxious (n=212)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>57 (15)</td>
<td>54 (15)</td>
<td>58 (14)</td>
<td>0.02</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>193 (66)</td>
<td>58 (72)</td>
<td>135 (64)</td>
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<tr>
<td>Symptom duration months, median (IQR)</td>
<td>3 (1–8)</td>
<td>3 (1–7)</td>
<td>3 (1–8)</td>
<td>0.72</td>
</tr>
<tr>
<td>Currently smoking, n (%)</td>
<td>65 (23)</td>
<td>25 (33)</td>
<td>40 (20)</td>
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</tr>
<tr>
<td>ACPR-positive, n (%)</td>
<td>162 (55)</td>
<td>43 (53)</td>
<td>119 (56)</td>
<td>0.64</td>
</tr>
<tr>
<td>ESR (mm/hour), median (IQR)</td>
<td>28 (14–41)</td>
<td>28 (14–42)</td>
<td>28 (14–41)</td>
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<td>CRP (mg/L), median (IQR)</td>
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<td>7 (3–26)</td>
<td>10 (3–20)</td>
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<tr>
<td>EGA, mean (SD)</td>
<td>49 (20)</td>
<td>49 (24)</td>
<td>49 (19)</td>
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<tr>
<td>PGA, mean (SD)</td>
<td>45 (27)</td>
<td>54 (27)</td>
<td>42 (26)</td>
<td>0.001</td>
</tr>
<tr>
<td>Pain, mean (SD)</td>
<td>60 (25)</td>
<td>63 (24)</td>
<td>58 (25)</td>
<td>0.92</td>
</tr>
<tr>
<td>68-TJC, median (IQR)</td>
<td>10 (5–17)</td>
<td>11 (6–19)</td>
<td>10 (5–16)</td>
<td>0.18</td>
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<tr>
<td>66-SJC, median (IQR)</td>
<td>5 (2–11)</td>
<td>5 (2–10)</td>
<td>6 (2–11)</td>
<td>0.14</td>
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<tr>
<td>DAS44, mean (SD)</td>
<td>2.9 (0.8)</td>
<td>3.0 (0.8)</td>
<td>2.9 (0.8)</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Pain measured by a 0–100 VAS.
68-TJC, 68 tender joint counts; 66-SJC, 66 swollen joint counts; ACPR, anticitrullinated peptide antibody; CRP, C reactive protein; DAS44, 44-joint Disease Activity Score; EGA, evaluator’s global assessment by a 0–100 VAS; ESR, erythrocyte sedimentation rate; MCS, mental component summary; MH, mental health subscale; PGA, patient’s global assessment by a 0–100 VAS; VAS, Visual Analogue Scale.

Figure 1 Percentages of patients with RA in remission at 1 year (DAS44 ≤2.4) who did or did not have depression/anxiety at the time of diagnosis. DAS44, 44-joint Disease Activity Score; RA, rheumatoid arthritis; SF-36 MCS, Medical Outcomes Survey Short Form-36 mental component summary; SF-36 MH, Medical Outcomes Survey Short Form-36 mental health subscale.

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Contributors ACB, TWJh and AvdH-vM made a substantial contribution to the acquisition, analysis and interpretation of the data. All authors made a substantial contribution to the conception and design of the work, ACB and AvdH-vM drafted the manuscript. TWJh revised the manuscript critically for important intellectual content. All authors approved the final version of the manuscript.

Funding The research leading to these results has received funding from a Vidi grant of the Netherlands Organisation for Health Research and Development and from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (starting grant, agreement no 714312). 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.
Correspondence

Patient consent Obtained.

Ethics approval Medisch Ethische Commissie van het Leiden University Medical Center.

Provenance and peer review Not commissioned; internally peer reviewed.
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Received 18 December 2017
Accepted 23 December 2017
Published Online First 8 January 2018

REFERENCES
Response to: ‘Depression and anxiety associate with less remission after 1 year in rheumatoid arthritis’ by Boer et al

We thank Boer et al for their interesting report validating our findings of depression and anxiety as strong negative predictors of remission in rheumatoid arthritis (RA).

Depression and anxiety are frequent disorders among patients with inflammatory arthritides, and emphasis on these conditions may be important in a treat-to-target strategy, not only in the shared decision-making of a treatment target between patient and rheumatologist, but also in the decision of type of treatment target. That is, as baseline depression and anxiety are found to be associated with more subjectively weighted measures, but not acute phase reactants and swollen joint count during follow-up, alternative (composite) measures of disease activity as well as target values should be considered, in accordance with recommendation number 5 in the treat-to-target recommendations.

Boer et al used Disease Activity Score 44 remission, and we used Disease Activity Score 28, Simplified Disease Activity Index, American College of Rheumatology/European League Against Rheumatism Boolean and modified Disease Activity index for Psoriatic Arthritis remission (patients with psoriatic arthritis were also included in our study), with similar findings of baseline depression and anxiety as strong negative predictors of remission. Subjective weighted measures (patients’ global assessment, tender joint count, pain) are included in all these composite scores and may cause misinterpretation of RA disease activity also due to impaired pain perception in patients with depression and anxiety.

We fully agree with Boer et al that depression and anxiety may influence important treatment decisions in RA and that it is of importance to take this into consideration to prevent increased medical costs as well as patient burden due to unnecessarily intensified treatment regimens.

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Competing interests None declared.

Provenance and peer review Commissioned; internally peer reviewed.

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To cite Michelsen B, Kvien TK. Ann Rheum Dis 2019;78 e2.

Received 8 January 2018
Accepted 9 January 2018
Published Online First 22 January 2018

http://dx.doi.org/10.1136/annrheumdis-2017-212867

REFERENCES
Remission or low disease activity as a target in systemic lupus erythematosus

We read the report by Zen et al1 on the impact of lupus low disease activity state (LLDAS) on damage accrual with great interest and would like to congratulate the authors on their work. In this single-centre Caucasian cohort, followed for 7 years, only 38 of their 293 patients (11.3%) failed to achieve LLDAS for at least 1 year; moreover, of the 255 patients who achieved LLDAS for at least 1 year, 246 (96.5%) also satisfied the definition of remission for the same length of time. And, in 214 (83.9%) patients, the duration of remission was the same as that of LLDAS. Being in LLDAS for at least 2 years was associated with a reduced risk of damage; but, when remission was included in the multivariable model, remission for at least 2 years was associated with a reduced risk of damage whereas LLDAS was not. Based on these findings, the authors suggest that the effects of LLDAS could be overlapping with those of remission. However, the prevalence of systemic lupus erythematosus (SLE) in non-Caucasian populations is higher and the disease is more severe with patients having, in general, less favourable outcomes; this limits the applicability of Zen et al’s results to patients with different characteristics. In fact, our previously published data from the multiethnic, multinational inception Latin American cohort2 differs quite a bit from Zen et al’s data. Remission was a relatively rare event with only 273 of 1350 patients (20.2%) achieving it at least once during their follow-up, and another 192 patients (14.2%) achieving low disease activity state (LDAS) while 885 (65.6%) did not achieve either status, with a follow-up of 2.4 years. Since we have a larger number of patients with pure LDAS, it was possible to evaluate the real impact of this status on SLE prognosis. Additionally, since we evaluated these statuses as intervals, we could independently ascertain the impact of remission and LDAS; we found that both statuses were protective of new damage occurrence whereas remission was protective of severe new damage, defined as an increase of at least 3 points in the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) Damage Index. Moreover, remission and LDAS were protective of both, new and severe new damage excluding those items clearly related to glucocorticoid use suggesting that the effect of LDAS and remission on damage accrual is primarily related to the level of disease activity. Although the definitions of LLDAS and LDAS used by Zen et al and by us are not exactly the same, they nevertheless do not explain by themselves the differences between our publications (both definitions required a Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) ≤4, and similar treatment; the definition used by Zen et al included no new lupus disease activity compared with the previous assessment, Physician Global Assessment ≤1 which ours did not require).

Studies from other countries have reported an incidence of remission and LLDAS more comparable to our’s than to Zen et al’s suggesting than in disadvantaged SLE populations, LLDAS may be an alternative, yet not the ideal, treatment target.3

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Contributors All authors were involved in drafting or critically revising this manuscript for important intellectual content, and all authors approved the final version to be published.

Competing interests None declared.

Provenance and peer review Not commissioned; internally peer reviewed.

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Received 19 December 2017
Accepted 27 December 2017
Published Online First 8 January 2018

http://dx.doi.org/10.1136/annrheumdis-2017-212911


REFERENCES

1Our data were not cited by Zen et al as the dates of both the publications, theirs and ours, were quite close in time.
Response to: ‘Remission or low disease activity as a target in systemic lupus erythematosus’ by Ugarte-Gil et al

We appreciated the comments by Ugarte-Gil and co-authors on our report dealing with lupus low disease activity status (LLDAS) in Caucasian patients.

We agree that Caucasian patients with systemic lupus erythematosus (SLE) have a better prognosis compared with non-Caucasian ones, but, in our opinion, race does not fully elucidate the different results in terms of prevalence of low disease activity and remission obtained in the studies by Zen et al and Ugarte-Gil et al.

The different design of the studies is more relevant than race in explaining the divergent results. Indeed, the Grupo Latino Americano De Estudio del Lupus (GLADEL) study analysed an inception cohort of patients shortly after the disease onset (median disease duration 0.3 years), and the authors assessed remission and low disease activity status (LDAS) during the first years of follow-up. By contrast, we analysed a non-inception cohort of patients with SLE with a mean disease duration of 11 years. It is well known that SLE is more active in the first years after diagnosis: the relapsing–remitting profile decreases and the long quiescent profile increases over the disease course as shown by Győri et al. Thus, the inclusion of patients with a different disease duration could account for the different proportion of low disease activity and remission observed in the two cohorts. Notably, we did not exclude patients with a recent diagnosis of SLE, thus our cohort is representative of what can be observed in a ‘real-life’ lupus clinic.

The GLADEL study design looks like that of the Wilhelm’s study, where the first remission period achieved by patients in the John Hopkins Lupus Cohort was considered, which could be responsible for the low prevalence of remission found in this study, as we recently underlined in a letter to the editor of Annals of Rheumatic Diseases.

In addition, the different duration of the follow-up in our study compared with that of Ugarte-Gil et al (7 years vs a median of 2.6 years, respectively) could have contributed to the higher frequency of low disease activity and remission observed in our cohort, since the longer the observation time, the higher the probability of detecting the occurrence of low disease activity or remission.

We also considered the longest period of remission or LLDAS achieved during the follow-up by each patient, and not the sum of intervals spent in remission or LDAS in the entire cohort, as in the GLADEL study.

We would like to highlight that our results are really in keeping with the findings of other recent studies on remission and LLDAS in different ethnic groups, which used a study design similar to ours. Mok et al found prolonged remissions in 35.3% of Chinese patients with a disease duration of ≥7 years. Similarly, Tsang-A-Sjoe et al observed a prolonged remission in 32.5% and a LLDAS lasting ≥50% of observational time in 64.5% of patients in a multiethnic cohort followed up for a median time of 5 years.

Since the GLADEL cohort includes patients followed for more than 10 years, the analysis of the prevalence of remission and LLDAS (or LDAS) in any single patient during the whole follow-up would be of great interest.

Ugarte-Gil et al considered a very large cohort of patients, which allows the independent evaluation of the impact of remission and LDAS on damage and found that both statuses were protective. Unfortunately, our cohort is smaller than the GLADEL cohort, preventing a separate analysis of patients in LLDAS but not in remission. However, it has to be pointed out that even in our multivariate analysis, LLDAS was an independent protective factor against new damage. Only when the remission status was added in the model, LLDAS did not show any additional protective effect against damage progression over remission.

Thus, in our opinion, remission remains the optimal target in the management of SLE; when remission cannot be achieved, low disease activity could be considered an acceptable alternative target.

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Handling editor: Josef S Smolen

Funding: This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests: None declared.

Provenance and peer review: Commissioned; internally peer reviewed.

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Received 3 January 2018
Accepted 3 January 2018
Published online First 17 January 2018

REFERENCES
‘Neuropsychiatric lupus or not? Cerebral hypoperfusion by perfusion-weighted MRI in normal-appearing white matter in primary neuropsychiatric lupus erythematosus’ by Papadaki et al

I read with interest the article by Papadaki et al1 relating to the role of cerebral hypoperfusion in neuropsychiatric lupus. While I applaud the quality of work and the intricate interpretation of enhanced MR imaging, the authors came to the wrong conclusions.

First of all, the definitions (of which I was a co-author) for evaluating a diverse group of patients as having central nervous system systemic lupus erythematosus (SLE) are outdated and not evidence based.2 The best objective measure for a central nervous system inflammatory process is a lumbar puncture. Pleocytosis, increased protein levels, increased IgG synthesis rates, oligoclonal bands or antineuronal antibodies are the only objective metrics available to make this diagnosis outside of obvious neuroimaging abnormalities or a brain biopsy. None of the patients in the paper was reported to have a spinal tap.

Second, the publication was not the first to evaluate the role of hypoperfusion in neuroimaging in neuropsychiatric lupus as claimed. The authors correctly note that vasospasm in the watershed regions plays a vasomotor role in other disorders (see refs, 39–41) but apparently were unaware of previous work in this area germane to lupus.

Our group clearly demonstrated that the majority of lupus patients with neuropsychiatric lupus had single photon emission computerized tomography (SPECT) imaging abnormalities consistent with hypoperfusion in the watershed regions.3 In other words, most patients had vasomotor instability on an autonomic basis (‘Raynaud’s of the brain’) in, for example, the frontal–parietal interface where the vasculature is very small, numerous and prone to spasm. While some of these patients have an inflammatory process, the majority develop ‘lupus fog’ as a consequence of intermittent hypoperfusion. This should not be considered to be neuropsychiatric lupus and is managed with cognitive behavioural therapy, anxiety reduction measures, biofeedback and approaches that target the dysautonomia of lupus.4

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Handling editor Josef S Smolen

Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Provenance and peer review Not commissioned; internally peer reviewed.

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To cite Wallace DJ. Ann Rheum Dis 2019;78:e5.

Received 2 January 2018
Accepted 2 January 2018
Published Online First 17 January 2018

REFERENCES
Response to: ‘Neuropsychiatric lupus or not? Cerebral hypoperfusion by perfusion-weighted MRI in normal-appearing white matter in primary neuropsychiatric lupus erythematosus’ by Papadaki et al1 by Wallace

We thank Dr Wallace1 for his interest in our paper entitled ‘Neuropsychiatric lupus or not? Cerebral hypoperfusion by perfusion-weighted MRI in normal appearing white matter in primary neuropsychiatric lupus erythematosus’ by Papadaki et al1 and for giving us the opportunity to clarify aspects of our work. Our response to the points raised, follow next:

1. American College of Rheumatology (ACR) nomenclature. We do agree that this nomenclature, although useful, is indeed outdated and may need refinement and updating. In our study, the attribution of primary NPSLE was done by physician judgement and according to the European League Against Rheumatism (EULAR) recommendations3 after taking into consideration the ACR nomenclature and Italian Study Group attribution model.4

2. Lumbar puncture for the diagnosis of primary neuropsychiatric lupus. The modest sensitivity and specificity of cerebrospinal fluid (CSF) studies for diagnosis of Neuropsychiatric Systemic Lupus Erythematosus (NPSLE) led to the EULAR recommendation statement that its value is predominantly to exclude other non-lupus related causes of neuropsychiatric disease, such as infections or demyelinating disease. This recommendation was supported by data from the Italian Study Group, where abnormal CSF is not considered as a favouring factor for attribution to lupus in most neuropsychiatric manifestations.

3. Hypoperfusion in systemic lupus erythematosus (SLE). We never claimed that our study is the first to evaluate the presence of hypoperfusion in neuroimaging in NPSLE. Notwithstanding, our study is the first Dynamic Susceptibility Contrast-MRI perfusion study in SLE that included patients with secondary NPSLE as a separate clinical group. In this study, we differentiate between patients with primary NPSLE and those with neuropsychiatric symptoms not attributed to the disease by adopting a normalised left sensorial centre cerebral blood flow (CBF) cut-off value of 0.77.

4. Hypoperfusion in the watershed areas of the brain in SLE and vasospasm/autonomic dysfunction. Our results of widespread hypoperfusion in normal-appearing white matter of patients with NPSLE, which is more pronounced in the sensorial centre, are in agreement with hypoperfusion in the watershed regions of the frontal lobes in 81% of lupus patients with neuropsychiatric symptoms found by Driver et al5. These changes are likely consistent with the diffuse chronic ischaemia and neuronal loss due to widespread vasculopathy in these patients. Although a role of cerebral vasospastic phenomena in the development of some neuropsychiatric syndromes (including cognitive dysfunction) in patients with SLE and Raynaud’s phenomenon has been proposed,6 autonomic nervous system involvement in patients with SLE varies widely among studies, is often asymptomatic and its prevalence does not correlate with clinical neuropsychiatric manifestations.7

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Received 15 January 2018
Revised 17 January 2018
Accepted 19 January 2018
Published Online First 23 January 2018

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Handling editor Josef S Smolen

Competing interests None declared.

Provenance and peer review Commissioned; internally peer reviewed.

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Detection of myositis-specific antibodies

It was with much interest that we read the recent European League Against Rheumatism/American College of Rheumatology classification criteria for idiopathic inflammatory myopathies.1 These criteria include Jo-1 autoantibodies, and the authors discussed that future updates of the criteria should also include the more recently identified myositis-specific autoantibodies.1, 2 The interest in autoantibodies for classification is also illustrated by a recent proposal for a new clinicoserological classification of adult autoimmune myositis, which is based on the association of autoantibodies with distinct clinical phenotypes.3, 4 For example, antibodies to synthetases (eg, Jo-1, PL-7 and PL-12) define the antisynthetase syndrome, anti-MDA-5 antibodies are associated with myositis with overlap features such as interstitial lung disease, anti-TIF-1γ and anti-NXP-2 define a subgroup of dermatomyositis and anti-SRP and anti-HMGCR are associated with necrotising autoimmune myositis.2

As autoantibodies play a role in the newly proposed classifications,1, 2 it is expected that measurement of myositis-specific autoantibodies will be increasingly introduced in clinical practice. Most of the myositis-specific autoantibodies have been identified by immunoprecipitation. Alternative, easy-to-use commercial line/dot immunoassays are available. However, such assays are not standardised and may suffer from low specificity.3 Therefore, these assays need to be further validated.

We evaluated a cohort of 144 patients with inflammatory myopathy (IIM) and 240 controls (blood donors, chronic inflammatory demyelinating polyneuropathy, rheumatoid arthritis, systemic sclerosis, Sjögren’s syndrome and systemic lupus erythematosus; 40 of each) for myositis-specific autoantibodies using assays from Alphadia (myositis 12 IgG dot for Bludiver) (Mons, Belgium), Euroimmun (Euroline Autoimmune Inflammatory Myopathies) (Lübeck, Germany) and Trinity Biotech (ImmcoStripes Myositis Advanced LIA) (Buffalo, New York, USA).

The results are shown in table 1. We observed differences in specificity (reactivity in controls) between the manufacturers and between individual antibodies. For example, 2.9% and 2.4% of controls tested positive for anti-Jo-1 by Euroimmun and Trinity, respectively, compared with 0.4% by Alphadia. Overall, Euroimmun and Trinity showed more reactivity in controls than Alphadia, except for anti-SAE for which Euroimmun showed less reactivity in controls. Differences in reactivities between manufacturers were also observed in myositis patients, with the most pronounced difference for anti-TIF-1γ (2.1% with Alphadia versus 12.4% with Euroimmun and 11% with Trinity). It should be noted that even for an established marker such as anti-Jo-1 antibodies, differences between manufacturers were observed in patients with IIM. The likelihood ratio (LR) (prevalence of antibodies in patients divided by prevalence of antibodies in controls) gives a good estimate of how the test result affects the post-test probability (LR = 10 indicates a clinical significant difference in pretest to posttest probability). The LRs are shown in table 1 and further illustrate differences between individual antibodies and between manufacturers.

Table 2 shows the corresponding phenotype of myositis-specific antibodies in patients with IIM. The association between antisynthetase antibodies and interstitial lung disease, arthritis and Raynaud’s phenomenon was highly significant for all assays. The association of TIF-1γ antibodies and dermatomyositis was high for two of the three assays tested. The association of other antibodies with certain phenotypes (eg, association of TIF-1γ and NXP-2 with malignancy, of Mi-2 with dermatomyositis, NXP-2 with calcinosis and MDA-5 with amyopathic IIM) were weaker and differed between the assays (table 2), indicating that the assays did not perform similarly.

Taken together, as myositis-specific autoantibodies are included in classification criteria, it is important that clinicians and laboratory professionals are aware of the performance characteristics of the assays used to detect such antibodies. Initiatives to harmonise assays across manufacturers are needed.

Jean-Baptiste Vulsteke,1 Ellen De Langhe,2, 3 Kristl G Claey’s,4, 5, 6 Doreen Dillaerts,7 Koen Poens,8, 9 Jan Lenaerts,2 René Westhoven’s,2, 3 Philip Van Damme,4, 5, 6 Daniel Blockmans,8 Petra De Haes,2, 3 Xavier Bossuyt,3, 9

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Acknowledgements We would like to thank Alphadia, Euroimmun and Trinity for providing the reagents to perform this study. PVD holds a senior clinical investigatorship of FWO-Vlaanderen.

Contributors EDL, J-BV, KGC, KP and XB designed the study. J-BV, DD, EDL and XB analysed the data. DD performed the autoantibody assays. EDL, KGC, PDH, JL, PVD, RW and DB take care of the patients included in the study and revised the manuscript. J-BV, EDL and XB drafted the manuscript.

Funding This study was funded by Alphadia, D-tek, Trinity – Immco and Euroimmun.

Competing interests None declared.

Patient consent Retrospective study using leftover samples.

Ethics approval Local Ethics Committee.

Provenance and peer review Not commissioned; internally peer reviewed.

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J-BV and EDL contributed equally.
### Table 1  Myositis-specific and myositis-associated antibodies in 144 patients with myositis and in 240 controls

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For each antibody, the number of positive samples (and percentage) is given for myositis patients and controls as well as the number of positive samples with a value higher than 3 times the cut-off (for patients with myositis). The number of concordant (con) results is given as follows: on the line that describes the Alphadia results, concordance between all three assays (first number), between Alphadia and Euroimmun (second number) and between Alphadia and Trinity (third number) is given. On the line that describes the Euroimmun results, concordance between Euroimmun and Trinity is given. The likelihood ratio (LR) is given as well as the association between each of the antibodies and IIM (evaluated by χ² testing or Fisher’s exact test (if cell size was <10) using Analyse-it for Excel). Patients with IIM (female/male: 80/64) (median age at diagnosis 53 years; age range 3–84 years) included dermatomyositis (n=57), polymyositis (n=48), antisynthetase syndrome (n=15), necrotising myositis (n=6), clinically amyopathic dermatomyositis (n=7), sporadic inclusion body myositis, overlap and undifferentiated myositis (n=9) and undefined (n=2). Diagnosis was based on a combination of clinically significant muscle weakness, elevated creatine kinase levels, electromyography, muscle biopsy (available in 90 of 144 patients) and/or skin manifestations. Demographic data (female/male, median age (age range)) of the controls were 149/91, 57 years (16–85 years); 172/23, 56 years (18–69 years) for the blood donors; 82/32, 56 years (32–75 years) for the chronic inflammatory demyelinating polyneuropathy (CIDP); 30/10, 57 years (26–75 years) for rheumatoid arthritis (RA); 25/15, 61 years (41–81 years) for systemic sclerosis (SSc); 35/5, 52 years (16–85 years) for Sjögren’s syndrome (SS); and 34/6, 47 years (25–84 years) for systemic lupus erythematosus (SLE). IIM, inflammatory myopathy.
## Table 2  Association of myositis-specific antibodies with clinical phenotype in patients with IIM

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The association was evaluated by χ² testing or Fisher’s exact test (if cell size was <10) using Analyse-it for Excel.

IIM, inflammatory myopathy.

*Correspondence*

REFERENCES


Response to: ‘Detection of myositis-specific antibodies’ by Vulsteke et al

It is with great interest we read the letter titled ‘Detection of myositis-specific antibodies’ by Dr Vulsteke et al published in the Annals of the Rheumatic Diseases. The authors analysed the presence of myositis-specific autoantibodies in patients with idiopathic inflammatory myopathies (IIM) and in controls consisting of patients with other inflammatory conditions and blood donors. These analyses were performed using three different assays. In addition, the authors studied the association of autoantibody positivity from the different assays with specific clinical phenotypes in patients with IIM. In conclusion, differences in specificities between assay manufacturers and between individual antibodies were found. The authors point to the fact that autoantibody data, namely anti-Jo-1 autoantibody positivity, were included in the 2017 European League Against Rheumatism/American College of Rheumatology classification criteria for adult and juvenile IIM and their major subgroups, and that users should be aware of the characteristics of the autoantibody assays used in clinical settings. The authors also emphasise the need for initiatives to harmonise assays across manufacturers, and we welcome their contribution in this field.

In the original publication we acknowledged the limitation of the low frequency of autoantibody data recorded in the data set, due to not yet identified antibodies and lack of available antibody assays at the start of the study. Our study required that antibody tests were performed in serum using standardised and validated tests. There were no requirements on the format of the assays, but in a survey performed among the study participants we found that ELISA was the most often used assay for anti-Jo-1 antibody detection (44%), followed by line blot (26%) and immunoprecipitation (15%). One important consideration for the classification criteria work was to include all medical disciplines involved in myositis research and care, as well as to obtain a broad geographical coverage of participating clinics. A stricter requirement of a specific antibody assay would not have been applicable to clinical practice and would have limited the available data even further. Although classification criteria should have high specificity, they also need high sensitivity, as well as be clinically applicable and practical.

We encourage a future revision of the new classification criteria with inclusion of more autoantibody data. Our commitment to this is reflected by the large international interdisciplinary collaboration, the Global Myositis Network MyoNet, which we have initiated. The network includes clinicians and researchers with interest in myositis, and one important topic on the research agenda is the standardisation and harmonisation of sample collection and analyses, including autoantibody assays. A global longitudinal registry for data on patients with myositis, the EuroMyositis database, is accompanying the network including more than 20 centres worldwide. At present, more than 4500 patients are registered. Large emphasis has been placed on systematic collection of autoantibody data, using validated and standardised procedures. We believe that these data will be of great value for a future revision of the classification criteria.

We thank Vulsteke and colleagues for their important contribution to the research of myositis-specific antibodies and for increasing the knowledge and awareness of the importance of appropriate assays and interpretation of results. The results from their cohort underline the possibility of different performance between different assays, which emphasise the need for further validation studies of commercially available assays using large cohorts including patients with a broad spectrum of clinical phenotypes and including the ‘golden standard’ assay immunoprecipitation for comparison. Such a study is ongoing within the MyoNet and EuroMyositis collaboration, and will be important for future updates of classification criteria for IIM.

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Funding This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Provenance and peer review Commissioned; internally peer reviewed.


Received 17 January 2018
Accepted 17 January 2018
Published Online First 30 January 2018

http://dx.doi.org/10.1136/annrheumdis-2018-212915


REFERENCES
Efficacy and safety of tocilizumab in patients with refractory Takayasu arteritis

We have read with interest the article by Nakaoka and colleagues on the efficacy and safety of tocilizumab in patients with refractory Takayasu arteritis. The authors have presented a randomised controlled trial in which they suggest a preference for tocilizumab over placebo for time to relapse of Takayasu arteritis, without any new safety concerns. We would like to draw attention to a few points in this regard.

First, Takayasu arteritis and giant cell arteritis share some similarities in their clinical, radiological and histological presentations, representing a spectrum of the same disease. Tocilizumab, which is used to treat giant cell arteritis, demonstrates an impressive glucocorticoid-sparing effect, which is important because relapses are common during steroid tapering, and it often necessitates the administration of high cumulative doses of glucocorticoids, which could cause significant toxicity. Treatment for Takayasu arteritis is still challenging because glucocorticoids are associated with significant adverse effects. Thus, the use of tocilizumab as a glucocorticoid-sparing agent is appealing. In the context that Takayasu arteritis and giant cell arteritis may be different phenotypes of a single disease, the steroid-tapering effect of tocilizumab is expected in the treatment for Takayasu arteritis. However, this study did not examine the effect of tocilizumab on the tapering of glucocorticoid agents in patients with Takayasu arteritis, because the study was designed with mandatory glucocorticoid tapering.

Second, biologic agents are generally used as second-line treatment. Tocilizumab is usually used in patients with large-vessel vasculitis refractory to immunosuppressants, because of its high cost and potential toxicity. Methotrexate (MTX) and mycophenolate mofetil (MMF) are effective means of inducing remission and minimising glucocorticoid therapy and toxicity in Takayasu arteritis. MTX and MMF lower the risk of relapse and reduce exposure to glucocorticoids; thus, MTX and MMF are therapeutic options in addition to the standard-of-care treatment with glucocorticoids for Takayasu arteritis. MTX and MMF might be particularly useful in treating patients at high risk of developing glucocorticoid-related adverse effects. However, the efficacy and safety of tocilizumab compared with these immunosuppressive drugs in patients with Takayasu arteritis were not investigated.

Currently, there are no data to show the superiority of tocilizumab over immunosuppressants. Further studies are warranted to determine the benefits of tocilizumab in terms of its glucocorticoid-sparing effect, remission and reduction in relapse, compared with MTX or MMF therapy.

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Handling editor Josef S Smolen

Competing interests None declared.

Provenance and peer review Not commissioned; internally peer reviewed.

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To cite Lee YH, Song GG. Ann Rheum Dis 2019;78:e9.

Received 13 December 2017
Accepted 16 December 2017
Published Online First 29 December 2017

REFERENCES
Response to: ‘Efficacy and safety of tocilizumab in patients with refractory Takayasu arteritis’ by Lee and Song

I thank Lee and Song1 for their thoughtful comments on our recent publication.2 The authors state that our study did not examine the steroid-sparing effect of tocilizumab because of mandatory glucocorticoid tapering. Given that the dose reduction rate of glucocorticoid significantly correlates with the relapse rate,3 this randomised, double-blind, placebo-controlled study was designed with mandatory glucocorticoid tapering to investigate whether tocilizumab treatment enables glucocorticoid tapering without relapse of Takayasu arteritis. Although the primary endpoint, time to relapse of Takayasu arteritis, was not met in our study, the results suggested the effect of tocilizumab as a promising steroid-sparing agent. Moreover, the steroid-sparing effect of tocilizumab was observed during an open-label extension period of the study in which the glucocorticoid dose was tapered based on the disease activity of the patient.4 The final results from the long-term extension will be reported in future publications.

I concur with Lee and Song that methotrexate and mycophenolate mofetil are commonly used therapeutic agents in patients with refractory Takayasu arteritis. As shown in online supplementary figure S1, 25 of 36 (69.4%) patients in our study had previously received disease-modifying antirheumatic drugs or immunosuppressants. Our study, however, was designed to compare tocilizumab with placebo because the use of immunosuppressive agents had not been found to have a consistent clinical benefit or steroid-sparing effect in a prospective comparative study.3 The findings in our randomised controlled study support the use of tocilizumab as one of the treatment options for patients with Takayasu arteritis who are resistant to glucocorticoid therapy and in whom glucocorticoid withdrawal is difficult. Further discussion of an evidence-based treatment algorithm for patients with Takayasu arteritis is warranted in the future.

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Handling editor Josef S Smolen

Contributor YN wrote the response to the eLetter.

Funding This study was funded by Chugai Pharmaceutical Co. Funding for manuscript preparation was provided by F. Hoffmann-La Roche.

Competing interests YN reports personal fees from Chugai as a consultant of the sponsor-initiated clinical trial (Chugai Pharmaceutical Co.) using tocilizumab for Takayasu arteritis; grants and personal fees from Chugai; grants and personal fees from Astellas, Pfizer, and MSD outside the submitted work; grants from Takeda, Otsuka, Bayer outside the submitted work; and personal fees from Daiichi Sankyo and Kowa Pharmaceutical Co. outside the submitted work.

Provenance and peer review Commissioned; internally peer reviewed.

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To cite Nakaoka Y. Ann Rheum Dis 2019;78:e10.

Received 10 January 2018
Revised 29 January 2018
Accepted 30 January 2018
Published Online First 10 February 2018

REFERENCE


http://dx.doi.org/10.1136/annrheumdis-2017-212838