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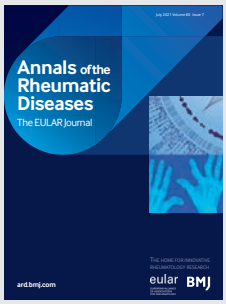




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## Epidemiology



## Letters



## Electronic pages

# Tenascin-C, a novel target to inhibit new bone formation in axial spondyloarthritis, linked with inflammation, mechanical strain and tissue damage

Margot Van Mechelen [1,2] Rik Lories [1,2]

Axial spondyloarthritis is a chronic inflammatory musculoskeletal disease hallmarked by the paradoxical co-occurrence of inflammation, trabecular bone loss in the vertebrae and new bone formation with syndesmophyte growth potentially leading to spinal fusion or ankylosis. All these features can contribute to the burden of disease: pain, fatigue and loss of mobility and function.[1] State of the art effective treatment strategies such as tumour necrosis factor (TNF) and interleukin (IL)-17 inhibitors focus on barring inflammation, but whether these approaches suffice to halt the bone remodelling aspects of the disease that determine the ultimate prognosis of patients is still debated.[1] Long-term studies indicate that sustained suppression of inflammation impacts structural disease progression.[2] Yet, the high individual variability in the ankylosis process suggests that there is an unmet need for early direct intervention in high risk or rapidly progressing patients.[3] Anatomy and imaging studies demonstrate that spinal ankylosis originates from pathological changes in the enthesis, the insertion sites of ligaments and tendons onto the bone. Former studies highlighted how growth factor pathways that are essential in skeletal development and growth, are inappropriately reactivated in the ankylosis process.[4 5] However, targeting signalling systems such as the bone morphogenetic protein and Wnt cascades comes with important considerations about impact on other tissues and organs.

More attractive therapeutic targets may be found in earlier and specific disease processes, an area that remains largely unexplored. In this journal, Li *et al* identify tenascin-C (TNC) as a key driver of new bone formation originating from the enthesis, and mechanistic experiments intriguingly position this extracellular matrix molecule as a converging node between inflammation, mechanical strain, tissue damage and new bone formation.[6] TNC is a glycoprotein with a number of remarkable features as extensively discussed by Midwood *et al*.[7] The founding father of the tenascin family, TNC is abundantly found in extra-cellular matrix during development. The molecule's name is based on its abundance in tendons (ten-) from embryos or *nasci*, Latin for 'to be born'. An alternative early name was cytotactin, now reflected in the C epithet and defining its function as a cell adhesion molecule. TNC has a multimodular structure allowing it to bind a wide variety of ligands both on the cell surface as well as in the extracellular matrix.[7] During development it is typically found at sites of motile cells, during branching processes and in tissues associated with locomotion: bone, tendons and ligaments. In adult life, its minute levels increase on injury and inflammation, suggesting a role in coordinating repair. Applying the concept that ankylosis in spondyloarthritis is an inadequate repair or remodelling response to counter damage or mechanical instability,[8] would suggest that TNC levels may rise in disease-affected entheses but direct evidence for a presence and role of TNC in this particular disease context was missing.

In a whole transcriptome analysis approach using an amazing collection of spinal ligament tissues from axial spondyloarthritis patients and from controls with primarily orthopaedic issues, TNC and genes associated with increased levels of TNC were found to be upregulated in diseased tissue compared with the controls.[6] Subsequently, the authors demonstrate how absence of the *Tnc* gene in genetically modified mice as well as anti-TNC antibody treatment inhibit the development of joint ankylosis in dedicated animal models of arthritis. They further unravel the underlying molecular mechanism by extensive *in vitro* and *in vivo* work: presence of TNC decreases the adhesion force of the extracellular matrix. This likely means that the mechanical interactions between the extracellular matrix and the cells within it are altered. In a connective tissue built to withstand mechanical force such as the enthesis, this will affect mechanosensing by the cell and mechano-transduction onto and into the cell. Optimal sensing and transduction of mechanical forces can be considered part of the homeostatic response. Hence, these changes, linked to the presence of TNC, will alter entheseal cell biology.

Effectively, by decreasing the adhesion force, the Hippo/YAP signalling pathway is activated, leading in its turn to increased chondrogenesis, a critical early step in the process of endochondral ossification that is leading to new bone formation and ankylosis. As proof of concept, selective targeting of the Hippo/YAP pathway abrogates new bone formation in murine arthritis. Single cell sequencing data further reveal that TNC is predominantly secreted by fibroblasts in the enthesis. In line with earlier observations that TNC expression is induced by inflammation, TNFα, IL-17A and IL-22 are increasing TNC levels in human fibroblasts isolated from ligamentous tissue. Hence, TNC can be linked to the concepts of abnormal mechanical stress, inflammation and a molecular shift within the fibroblasts towards chondrogenesis.

Whereas the paper by Li *et al* offers an exciting view into a novel mechanism that likely contributes to the ankylosis process in axial spondyloarthritis, it also triggers a number of new questions and topics for further research. The preclinical data using anti-TNC antibodies are impressive and suggest that targeting an extra-cellular matrix molecule within a connective tissue such as the enthesis by antibodies is possible. However, it is still unclear whether such an approach would work in patients and whether associated toxicity would be acceptable. Although mice with a genetic deletion of *Tnc* are born without striking abnormalities, Li and colleagues report an observed altered neurological behaviour in adult mice, confirming earlier data.[9] This and other potential effects such as defects in the wound healing process would require careful attention.[10] Thus, whether TNC is a better target than bone morphogenetic

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proteins and Wnt proteins remains to be demonstrated.

TNC is a complex multimodular protein suggesting that different domains within the molecule may have different functions.[7] Hence, specific targeting of different domains could be further evaluated in a pre-clinical setting to assess the efficacy and safety of different in depth targeted approaches, potentially identifying antibodies that selectively inhibit the change in adhesion force. TNC has the ability to form multimers including hexamers. It remains unclear under what form of TNC has the observed effect in the *in vitro* and *in vivo* models discussed by Li *et al*, another important consideration when developing a targeted strategy in humans. Similarly, there are a number of different splice variants of *TNC*[7] and it remains unclear which form(s) play the observed key role in the models of axial spondyloarthritis.

Furthermore, for a deeper understanding of what drives the bone formation process, other triggers, beyond inflammation, should be considered as being able to trigger upregulation of TNC. Such triggers could include mechanical tissue damage or some biomechanical forces, in particular in a genetically susceptible individual. Although the current results, with human data derived from the axial skeleton, and murine data from the peripheral skeleton, point towards one overarching principle, some nuances are likely to depend on the anatomic location and function of the ligamentous tissue.[11] In addition, understanding exactly how TNC is regulated, may identify alternative targeted approaches, bypassing the need to completely interrupt TNC signalling and diminishing safety concerns related to other tissues and organs.

Lastly, the question remains which patients will benefit from targeting TNC. The multiple effective anti-inflammatory agents that are currently available seem to have the potential to halt new bone formation when given early and in a sustained way.[12–14] Despite all recent advances in training of physicians and in imaging, a diagnostic delay remains a concern for patients with axial spondyloarthritis.[15] A TNC-targeting approach might offer an escape route for those patients in whom disease activity has been already too high for too long, or in whom the process of bone remodelling already has started and where solely stopping inflammation will not suffice anymore. Of note, TNC has been suggested as a biomarker for axial spondyloarthritis in different studies, although that the effect is not specific as increased levels have also been seen in patients with rheumatoid arthritis.[16–18] The current data suggest that the value of TNC levels as predictive factor for radiographic progression need to be urgently studied.

In summary, the discovery that targeting TNC in mouse models of disease inhibits the progression of new bone formation is novel and important. Translation of this concept into clinical practice comes with challenges, but appears to be worth ample consideration and active investigation.

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# Rheumatology in 2049: the age of *all data*

Johanna Mucke , Philipp Sewerin , Matthias Schneider

If you think about the unthinkable long enough it becomes quite reasonable.

Josephine Tey (1896–1952)

British author

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Have you ever thought about what our life as specialists for rheumatic diseases will look like in 2049? The amount of data gathered from us and our patients is increasing exponentially, and eventually, these data will be used to improve and facilitate patient's care. We, that means primarily rheumatologists and—as the responsibilities will also change with *all data*—all other health professionals involved in the care of patients with rheumatic diseases, should know what to expect and actively contribute to this process.

## WORKING ROUTINE IN 2049

It is 10:00 on a sunny 21 July in 2049 and you are currently at your most favourite place in the world: a small cottage in the mountains, a vineyard in France or your house with a breath-taking view of the sea. You just finished your morning round on your virtual ward and a glimpse at your computer shows that 99% of the 50 000 customers in your virtual practice do not have any complaints and are enjoying their life without health-related limitations. In fact, most of them have never even had any symptoms as they were diagnosed before disease manifestation and preventive measures have successfully been applied. Like every morning, the system reports a few patients that deviate from their normal status. In some patients, the system has already adapted or changed therapy or has given behavioural advice. Most patients do not need any further adjustments. The system has identified two customers who need personal assessment in your virtual clinic, and therefore, an appointment has already been made. Other patients are still on your agenda for the virtual expert meeting this afternoon, as they do not fit into the known disease entities or treatment standards. Your avatar will present these complex cases to the other members of your expert board and together with already established artificial intelligence (AI)-algorithms you will find the best solution. In rare situations, it is still necessary to see the patients in person since the sensitive and trained sensors that track the patients' condition sometimes miss a rare manifestation that is unknown to them, but relevant for making the diagnosis.

In the age of virtual patient contact, you can work from anywhere you like. However, we believe that there is more to rheumatology care in 2049 than just operating from your favourite place. There are plenty of good reasons to proactively shape our future and we would like to get you on board to discuss in which direction our medicine should evolve in the future and to reflect on your dreams, hopes and fears.

## COPING WITH INCREASING AMOUNT OF DATA NOWADAYS

In only a few years of time, medical problem-solving has evolved quickly and changed drastically: we have a continuously increasing amount of data at our immediate disposal due to the exponential growth in medical knowledge, abundant data acquisition and the easy data availability. In 1950, medical knowledge was doubled within 50 years.[1] Today, it takes only 73 days to double. At the time a medical student graduates, he or she acquired about 6% of all medical knowledge.[1] Therefore, it is already impossible to keep up to date even in the rather small field of rheumatology. We are already taking the opportunity to use devices to handle new knowledge: a computer like Watson already has all data and facts from PubMed available for medical decisions.[2]

## THE VITREOUS PATIENT

Big data is expected to advance personalised medicine not only in terms of diagnostics but also by improving the care of every individual patient. Terms that are frequently used in this context are the 'omics' like genomics, epigenomics or proteomics. Nevertheless, *all data* is more than the scientific data. *All data* means data provided by our patient: information regarding his or her medical history provided by health records, body sensors and home camera systems as well as information about all individuals from the entire world that have similar or the same complaints.[3–5] In fact, *all data* means to have access to *all data* of every individual. Extended health-related information such as sleep quality and activity but also non-health-related data that is, nutritional and environmental information, consumption habits and internet activities. First consultations would be much more effective and efficient as all information would already be available to the physician without important information being lost. Every patient has given written and informed consent to the distribution of their anonymised data and all regulatory questions have been solved. In most cases, our clinical examination is replaced by modern technology as most clinical conditions can be detected by body sensors, automated ultrasound systems, whole body MRIs and skin robots that capture plenty of body parameters. However, a big challenge will be to also integrate interpersonal information such as emotions and expectations into *all data*, which are and will be a substantial part of rheumatology care.

## REDUCTION OF INFORMATION VERSUS INTEGRATION OF *ALL DATA*

Our perception is characterised by the fact that we constantly try to reduce information diversity by comparing and matching as much information as possible.[6] In clinical practice, it is then often few seemingly inappropriate pieces of information that help us progress or we simply recognise patterns, often based on personal experiences and focus. Once a diagnosis has been made, we often stick with it as long as possible until we are forced to change it.

With *all data* the challenge will be to use the additional information both in the treatment of each individual and, of course, the entire field of rheumatology and beyond, which requires the connection of *all data* and its interpretation. Imagine that in the future, all our patient's test results are combined into one outcome and we do not know the individual results. This system of data and their interpretation resembles a so-called complex system[7] which is a network of many components that may interact with each other and evoke a complex collective behaviour, discerning information processing and adaption through learning and development.[8] We will be unable to fully capture the validation and interpretation of any result and we need to rely on support from AI.

One scenario could be that we as physicians make the diagnosis and report it back to the system, which, in turn, can verify or question the diagnosis and accurately predict the patient's prognosis. The same may apply to treatment decisions. Situations could occur in which the system disagrees with our diagnosis. In another scenario, the system makes suggestions and we check these for plausibility. The system would then serve as guidance and rather facilitate than dictate decisions. These situations need to be carefully reflected by considering both ethical and legal consequences that would rise from ignoring the system's recommendation which is based on an infinite pool of data and algorithms. All intermediate results, for example, highly complex information from 'omics', will be controlled by experts, and a holistic interpretation of all areas requires all professions together or one specialist who relies on the analyses of the others. AI will take the expert's part and constantly optimise itself and will support us by recognising and assigning specific patterns. Even today, a standardised, transparent and rigorous report procedure for AI interventions in clinical research is recommended.[9] In the scenario outlined at the beginning, therapy will be adjusted by the system. This treatment decision will be based on a correction or shift in data leading to a different outcome of the algorithm. This clinical shift might not be noticed by the patient itself—the mere fact that a better therapeutic option exists, leads to the adjustment. In case of clinical symptoms, the system will react directly, initiate further diagnostic testing if necessary and adjust therapy in accordance with the ideal personalised approach. Each patient would receive the best treatment at the earliest possible time, even before symptoms occur. As it is today, our goal will be to achieve the best results for the patient.

Given that knowing *all data* and its connections is impossible for an individual person and even for an expert group, a selection of the most important aspects could be provided by the system on a dashboard as it has already been successfully realised with literature (Blinkist[10]). Should further information be required at some point, one can look deeper into the specific data by selecting it on the dashboard. The system would provide the current state of data and the specific data used to solve the respective case. This way, complex analytical processes could be broken into smaller segments, which are, in the sense of understandable AI, easier to understand and modify and facilitate the comprehension and accessibility of AI-processes for the user.

## WHAT DEFINES US AS RHEUMATOLOGISTS IN THE FUTURE?

It is on us to define our role and grade of participation in this scenario. As all data is expected to highly improve patient care, ignoring this development cannot be the solution to this challenge. To this day, in addition to the empathy for patients and our communicational skills, it is our knowledge, our insights and our (clinical) experience that define us as rheumatologists. In the setting of all data, information gained from these skills and experiences is likely to get another relevance for decision-making in precise medicine. We have to ensure that relevant information and interactions are not lost when we base our decisions on knowledge not acquired and reflected by us personally but by an impersonal, alien-like 'expert system'.

Given that it knows all data, we cannot win a knowledge battle against the system. However, rheumatologists should control the interpretation of data, or at least know which ratings they are based on and how they are generated. Being a relevant part of this process of diagnosis and treatment decision, our choices will turn into further information in the system, and algorithms will then be adjusted accordingly. We will remain the gold standard for some time until the system gains more sensitivity and specificity and will take our place by training itself. With time, the system will move away from the dichotomous evaluation of 'normal' and 'abnormal' and will be able to class everything in continuum from normal to altered by developing new 'normalities' of human beings that differ from the mean value of the general population. At this point, the system will live up to the diversity of all human beings and their health-related characteristics and challenges and the aim will be to target outcomes such as well-being and vitality instead of correcting anomalies. Each individual will be considered 'normal' in his own cohort of human beings that might be spread all over the world. This will not only change the approach to disease but allows for rare manifestations and characteristics to find a comparative population. This will significantly shape our specialty, as rare diseases will subjectively occur more frequent. Nonetheless, this development will force us to move away from the standard we have believed and confided in. Pattern recognition will become finer and the grading continues to grow as computing power and data volumes increase.

At this point, we could (1) accept less, but digitisable data and hence the omission of data or (2) get involved so that relevant data sources are still available to us. For this, we do not only have to take an active part, but we must be allowed to take decisions. We should be active participants in knowledge management and develop an ethically valuable technology for our customers. Therefore, it is crucial that we understand as much of the system as possible, an aspect that should be included in the curriculum. *All data* management must be part of the curricular education of every medical student.

We can integrate the system as an extension of our senses, like a blind person learning to see with a retinal implant.

The legal aspect is highly relevant as well: the rights to the system have to be placed in the right hands and every patient should be the personal data owner. The system should be able to carry itself, develop further and the profit should primarily lie with the customer as it is being developed in the European commission funded DECODE project.[11]

**Box 1 Questions to be addressed regarding *all data***

- How do we want to actively shape the *all data* development and who do we want to be in the future?
- What will be the value of our skills as rheumatologists, such as physical examination, communicational skills and empathy in the era of *all data*?
- How will *all data* impact the medical training in general?
- What will a rheumatologist need to know and understand of all data?
- To which extent should we allow that 'knowing' and possibly 'intelligent' machines take over our genuine power and our tasks?
- Will we furthermore be responsible for making diagnoses and treatment decisions?
- Will subspecialties as rheumatology still be necessary or will the knowledge and the expertise within specialties be replaced by *all data*?
- Will artificial intelligence take the expert's part and constantly review and optimise itself or does it only help us by recognising and assigning specific patterns, which the expert must interpret himself?
- Which data will be integrated into the system, how will its quality be guaranteed, and measurement errors identified?
- Will the peer-review system, as we know from scientific journals, be transferred into system and the journals disappear as the medium for data communication?
- Who will guarantee data security?
- How will computer viruses and hacker attacks be warded off?

With *all data* being available, our misjudgements and mistakes inevitably become apparent and part of the system. Hence, they may be corrected by the system, but we should take them as a possibility for our personal and the system's development. And surely, we will have to learn to handle mistakes differently: neither correcting or justifying mistakes nor reducing our actions and decisions to things we are fully convinced to be capable of, will be the way to success. *All data* is a challenge with many opportunities to improve healthcare of patients with rare diseases and with many unasked and unanswered questions to reflect on (box 1).

It is on us to shape the new developments and their implementation in our field in order to realise our visions and derive the greatest benefit for our patients and for us from the Big Data Age.

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# Tissue physiology revolving around the clock: circadian rhythms as exemplified by the intervertebral disc

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## ABSTRACT

Circadian clocks in the brain and peripheral tissues temporally coordinate local physiology to align with the 24 hours rhythmic environment through light/darkness, rest/activity and feeding/fasting cycles. Circadian disruptions (during ageing, shift work and jet-lag) have been proposed as a risk factor for degeneration and disease of tissues, including the musculoskeletal system. The intervertebral disc (IVD) in the spine separates the bony vertebrae and permits movement of the spinal column. IVD degeneration is highly prevalent among the ageing population and is a leading cause of lower back pain. The IVD is known to experience diurnal changes in loading patterns driven by the circadian rhythm in rest/activity cycles. In recent years, emerging evidence indicates the existence of molecular circadian clocks within the IVD, disruption to which accelerates tissue ageing and predispose animals to IVD degeneration. The cell-intrinsic circadian clocks in the IVD control key aspects of physiology and pathophysiology by rhythmically regulating the expression of ~3.5% of the IVD transcriptome, allowing cells to cope with the drastic biomechanical and chemical changes that occur throughout the day. Indeed, epidemiological studies on long-term shift workers have shown an increased incidence of lower back pain. In this review, we summarise recent findings of circadian rhythms in health and disease, with the IVD as an exemplar tissue system. We focus on rhythmic IVD functions and discuss implications of utilising biological timing mechanisms to improve tissue health and mitigate degeneration. These findings may have broader implications in chronic rheumatic conditions, given the recent findings of musculoskeletal circadian clocks.

## INTRODUCTION

Evolutionarily conserved circadian (~24 hourly) rhythms persist throughout biology, with almost every aspect of our physiology and behaviour having evolved around the rotation of the earth. Endogenous circadian rhythms define when we sleep, eat and exercise, moderate the scale of an immune response we mount, determine how our body responds to medications and gate daily patterns of metabolism.[1] Evolved as a homeostatic mechanism, this temporal alignment of behaviour and physiology to the external environment ensures optimisation of metabolism and energy allocation to anticipate daily variations in physiological demands.[2] In a tissue context-dependent manner, circadian clocks drive 24 hours rhythmicity in approximately 5%–10% of the genome,[3] 5%–20% of the proteome[4–6] and ~25% of the phosphoproteome.[7]

Like many other regulatory processes, the circadian clock changes during ageing, losing its precise temporal control. The robustness of circadian rhythms, in terms of oscillatory amplitude and circadian phase, declines with age in both animal models and humans.[8] As such, the functional decline of circadian rhythms has been proposed as a potential mechanism driving an increased risk of various diseases, including metabolic syndromes, cancer and musculoskeletal conditions.[8–10] Indeed, genetic models of circadian disruption are associated with age-related rheumatic conditions including osteoporosis, osteoarthritis and tendinopathy.[11–14] In concordance, clocks no longer exhibit robust circadian oscillations in human osteoarthritis and rheumatoid arthritis (RA) synovial fibroblasts.[15] Interestingly, a dosing scheme of anti-inflammatory or analgesic drugs that takes into account circadian rhythms (chronotherapy) has shown increased tolerance and effectiveness in osteoarthritis and patients with RA.[16–19] Prolonged misalignment of internal circadian rhythms with environmental rhythms in humans, such as those seen in chronic shift workers or frequent long-haul travellers, is associated with profound consequences for health and wellbeing.[20 21] Given that both population ageing and chronic circadian misalignment are increasingly prevalent, the importance of understanding biological timing mechanisms behind age-related diseases is becoming more and more relevant. In fact, over the past two decades, circadian clock research has been increasingly recognised as a fundamental branch of biology that could have profound biomedical implications. This notion culminated in the 2017 Nobel Prize in Physiology and Medicine, awarded to the discovery of the molecular mechanisms underpinning the circadian clock.

One highly prevalent age-related disease is the degeneration of the intervertebral disc (IVD). The IVD is a specialised fibrocartilaginous tissue of the spine that permits the shock absorption and mechanical movement of the spinal column. IVD degeneration is a common affliction of ageing and is strongly associated with the incidence of lower back pain (LBP),[22] which has been cited as the highest-ranking condition causing life years lost due to disability.[23] Current treatment strategies largely focus on pain management or invasive procedures such as spinal fusion, the success of which depends on degenerative subtype and can be associated with

later complications.[24 25] The high prevalence and its burden on healthcare services highlight a crucial need for in-depth understanding and additional treatment strategies for IVD degeneration, particularly as ageing populations grow.

The notion of daily ('diurnal') variations in the spine physiology was recorded as early as 1724 by Reverend Mr Wasse, published in the Philosophical Transaction of the Royal Society, noting a variation in body height between morning and night. He hypothesised that 'The alteration in the human stature, I imagine, proceeds from the yielding of the cartilages between the vertebrae, to the weight of the body in an erect posture'.[26] Subsequent observations and measurements by modern imaging techniques confirmed a daily change of the cumulative height of the IVDs by about 15–20 mm.[27–29] These height changes bring about daily fluctuations in extracellular osmolarity of the IVD, drawing in nutrients then flushing away metabolites and waste products on a daily basis. This necessitates IVD cells to be adapted to cope with such drastic daily fluctuations in their microenvironment. However, it remains largely unknown whether IVD cells are passively responding to such changes, or whether they have evolved an intrinsic mechanism to anticipate these changes and respond accordingly. Recently, endogenous circadian rhythms have been discovered within the IVD tissue, which temporally control key aspects of IVD physiology in synchrony with the 24-hour day. Disruptions to these molecular time-keeping mechanisms in mice causes premature ageing and degeneration of the IVD, implicating the circadian clocks as a critical regulator of IVD tissue function.[30]

In this review, we summarise the current understanding of circadian rhythms, their molecular mechanisms as well as biological functions. We use the IVD as one exemplar tissue. However, the mechanistic links between diurnal loading, circadian clocks and rhythmic IVD functions, and the potential avenues for exploiting biological timing mechanisms to mitigate tissue degeneration and promote repair could have much broader implications in musculoskeletal system and rheumatic diseases.

## CIRCADIAN RHYTHMS IN HOMEOSTASIS AND AGEING

### Central and peripheral circadian clocks

The circadian system is organised in a hierarchical structure, whereby the suprachiasmatic nucleus (SCN) of the hypothalamus acts as the central pacemaker of the body. Projections from the SCN to other brain centres that can generate humoral and neuronal signals impart circadian timekeeping on peripheral tissues, thereby ensuring their synchronisation with the external environment. This alignment is derived primarily from external daylight cycles, transmitted directly from the retina to the SCN. The SCN and its role as the core circadian pacemaker has been widely reviewed.[31–33]

It is well known that most peripheral tissues possess their own molecular clocks that are capable of generating self-sustaining oscillations to drive tissue-specific rhythms in gene transcription and translation. These rhythms have been identified in heart, kidney, liver, pancreas, bone, skeletal muscle, tendon and joint (articular cartilage and synovium), among others[3 13 14 34–41] (figure 1). Despite the numerous peripheral clocks, circadian rhythms show a high degree of tissue specificity. This permits individual tissues to anticipate their relevant daily demands and stressors and respond accordingly. For example, the liver clock is highly tuned to anticipate diurnal feeding–fasting cycles by generating circadian rhythmicity in energy metabolism, among other processes. The skin clock regulates ultraviolet (UV) damage-induced DNA repair response, giving the skin a time-of-day-dependent sensitivity to UV exposure.[42] Within the musculoskeletal system, the skeletal muscle clock gates insulin sensitivity and metabolism, which is thought to prime the muscle for optimal response to daily rest–activity patterns.[41] Circadian clocks in tendon temporally regulate the protein secretory pathway, allowing for efficient daily synthesis of a pool of collagen that may enable the repair of collagen fibrils in highly loaded tissue.[43] These studies highlight the notion that peripheral circadian rhythms are highly tuned to their specific physiological niches. Not only do homeostatic processes show daily rhythms, the symptoms of rheumatic diseases such as RA are known to show diurnal variations.[44] Chronic environmental circadian disruption has been linked to a predisposition towards development of osteoarthritis.[45] Taken together, these findings represent a potential opportunity for therapeutic interventions to rheumatic diseases based on the anticipation of intrinsic rhythms in symptom severity and tissue pathophysiology. For a further in-depth review of circadian clocks in non-IVD musculoskeletal tissues, please see other reviews.[40 46–48]

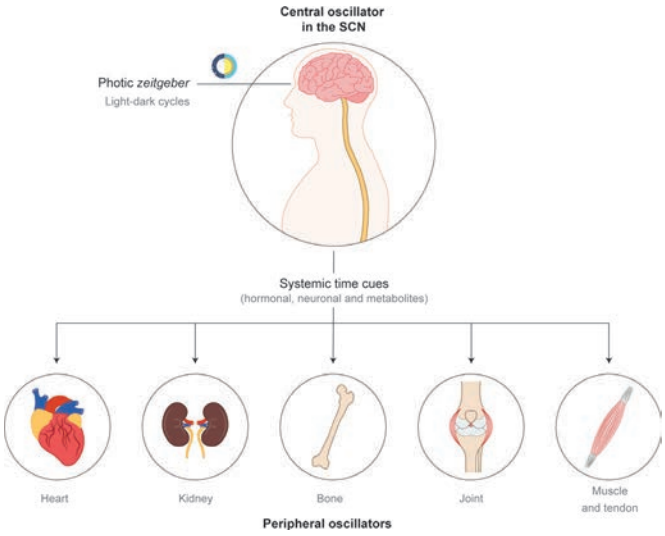


**Figure 1** The hierarchical organisation of the mammalian circadian system. The central pacemaker of the mammalian circadian clock is located in the SCN of the anterior hypothalamus. Photic zeitgebers (time cues) entrain the central clock through the retinohypothalamic tract. The central clock drives the rhythmicity of numerous physiological processes, including sleep–wake, rest–activity and feeding–fasting cycles, as well as neuronal and hormonal signals. These processes in turn function as time cues to synchronise autonomous circadian oscillators present in peripheral tissues, with varying strength depending on the tissue context. These peripheral clocks are responsible for generating local tissue-specific 24 hours rhythms that control tissue physiology and metabolism to their daily demands. SCN, suprachiasmatic nucleus.

### Circadian entrainment factors

Circadian entrainment is the synchronisation of circadian output rhythms to physiological cues to ensure rhythmicity in a given tissue is aligned with the external environment and the rest of the body. Daylight is the primary entrainment cue (referred to as a photic 'zeitgeber') for the SCN. The SCN in turn transmits rhythmic outputs through non-photic time cues that entrain peripheral tissues, such as neural activity, daily hormonal surges (such as corticosteroids and melatonin) and feeding/fasting cycles (metabolites, figure 1). Chronic misalignment of internal circadian rhythms with daily metabolic demands likely exerts an accumulative stress and homeostatic imbalance, which may play

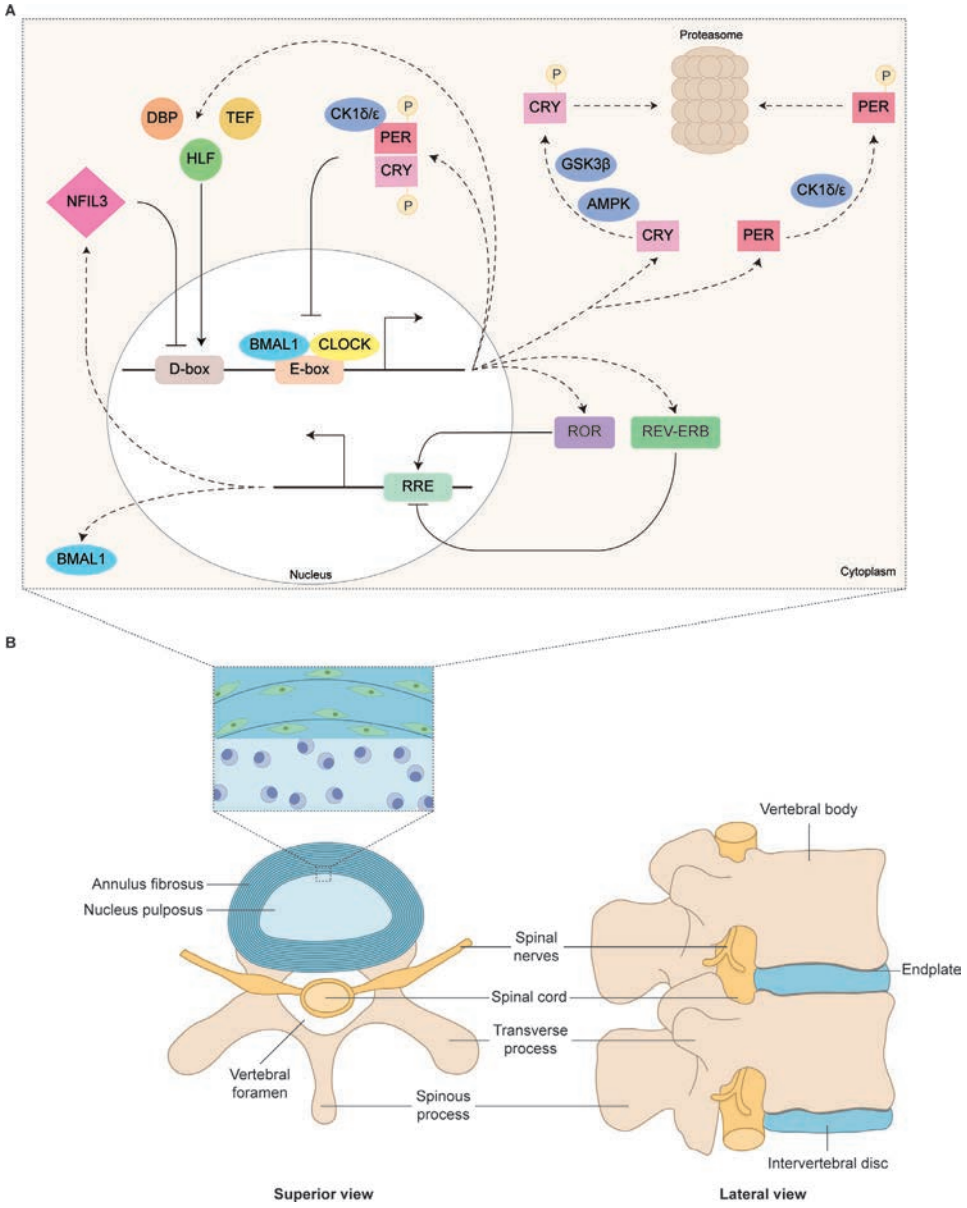


**Figure 2** Molecular clocks in the IVD. (A) The mammalian circadian clock is a network of transcriptional–translational feedback loops. The main feedback loop is initiated when heterodimers of BMAL1/CLOCK bind to E-box elements on the promoter region of target genes, including Per1/2 and Cry1/2. In the cytoplasm, PER1/2 and CRY1/2 form a multimeric complex with each other and with CK1ε/δ; after which they translocate into the nucleus and repress the transcriptional activity of BMAL1/CLOCK. The stability of PER and CRY is regulated by several proteins, including CK1ε/δ, AMPK and GSK3β. The nuclear hormone receptors REV-ERB and ROR constitute an auxiliary feedback loop involved in the regulation of Bmal1 transcription. A third feedback loop comprises the transcriptional activators DBP, TEF and HLF and the repressor NFIL3 rhythmically bind to D-box elements, modulating the expression of genes like Per1/2 and Cry1/2. (B) In the IVD, these molecular clocks can be found in NP and AF cells. This fibrocartilaginous structure is located between adjacent vertebral bodies comprising bony transverse and spinous processes. At the interface between the vertebral bone and the disc lies the cartilage endplate, providing strength and stability, and allowing nutrients to reach the avascular NP. The spinal nerves branch off from the spinal cord, an extension of the central nervous system that passes through the passageway created by adjacent vertebrae—the vertebral foramen. AF, annulus fibrosus; IVD, intervertebral disc; NP, nucleus pulposus.

a role in the premature development of age-related diseases. For example, simulation of chronic shift work in rodents imparts a predisposition towards metabolic dysfunction, immune defects, osteoarthritis-like changes, tumour progression and premature mortality.[45 49–54]

## Core molecular circadian clock

Rhythmicity in the expression and activity of the circadian clock and downstream target genes is generated by the core molecular circadian clock, a transcriptional–translational feedback loop that cycles with a ~24-hour periodicity[55 56] (figure 2A). With the onset of waking hours, transcription of clock activators *Bmal1* and *Clock* genes is induced. The resultant proteins form a BMAL1/CLOCK heterodimer that binds E-box response elements in target gene promoters. Key targets of this heterodimer include PER and CRY proteins, the levels of which accumulate throughout waking hours, heterodimerise and translocate to the nucleus in the evening to induce negative feedback on BMAL1 and CLOCK transactivation, thereby inhibiting their own transcription. Subsequent degradation of PER and CRY

proteins during resting hours alleviates this negative feedback and permits a new cycle of the circadian clock to commence. Degradation of PER proteins is mediated by casein kinases CK1δ and CK1ε which phosphorylate PER proteins, targeting them for proteasomal degradation.[57 58] CRY proteins are degraded via GSK3β-mediated and AMPK-mediated phosphorylation and subsequent proteasomal degradation.[59 60] This regulatory circuit forms the core feedback loop of the circadian clock. Auxiliary feedback loops also feed into this network, stabilising the core circadian circuit. Nuclear receptors REV-ERBα/β and RORα/γ bind RRE elements within the *Bmal1* promoter to inhibit or activate its transcription, respectively. Additionally, the expression of PAR bZIP family transcription factors *Dbp*, *Hlf* and *Tef* is driven by the BMAL1/CLOCK heterodimer. These proteins subsequently drive expression of PER/CRY proteins and other genes through D-box elements, while NFIL3 competes with DBP to suppress D-box genes.

Rhythmic expression of downstream target genes is driven primarily through binding of the aforementioned clock transcription factors to E-boxes, D-boxes and RREs in their promoters. Mechanisms underlying tissue-specificity in circadian transcription are as of yet unclear. It is however known that the colocalisation of tissue-specific transcription factors with clock factors at promoters and enhancers accounts for much of this phenomenon.[61–63] For example, muscle-specific transcription factor MyoD colocalises with the BMAL1/CLOCK complex at E-boxes to drive high-amplitude rhythmicity in the muscle transcriptome.[64] In addition to transcriptional activity, the circadian clock drives rhythmicity in alternative splicing,[65] microRNAs[66] and protein phosphorylation[6] in a tissue-specific manner. Multiple levels of post-transcriptional and post-translational control can thus generate circadian rhythms at both the messenger ribonucleic acid (mRNA) and protein level.

## SPINAL IVD IN HEALTH AND DISEASE

### IVD physiology

The IVD is composed of three distinct areas: the nucleus pulposus (NP), annulus fibrosus (AF) and cartilage endplate (CEP, figure 2B). Each has a key role to play in the homeostasis of the IVD and display unique pathological changes in ageing and degeneration. The NP constitutes the central portion of the IVD and permits compression of the spine, due to a high aggrecan content that confers a high hydration level and gel-like consistency to the tissue. As such, NP cells must withstand an extracellular environment that exhibits extremes of osmotic pressure not typically seen in other tissues. The NP serves not only as a conduit to distribute mechanical pressure but also acts as a signalling hub for the whole IVD.[67] Encircling the NP, the AF is a fibrous and collagen-rich ring-like structure that prevents extrusion of the gelatinous NP. The AF itself can be separated into the inner AF, adjacent to the NP, and an outer segment to the peripheral boundary of the IVD. Collagen II is enriched in the inner portion of the AF but becomes less abundant towards the outer AF, where type I collagen is highly expressed. The outer AF shares similarities to tendon tissue, including abundant type I collagen expression, and expression of tendon markers such as *Mkx*, *Tnmd* and *Scx*.[68–70] Sandwiching the NP and AF, the CEPs fuse the IVD to the adjacent vertebral bodies. This layer of hyaline cartilage acts as an essential conduit for the movement of oxygen, metabolites and waste products into and out of the IVD. Movement of molecules is generated by compressive loading that pushes out fluid of the hydrated NP and reabsorbs fluid back into the tissue when loading pressure is alleviated. Under physiological conditions, the non-degenerate IVD possesses little to no vasculature and instead relies on a relatively limited supply of essential molecules moving through osmotic pressure and diffusion. Therefore, the IVD constitutes a unique and demanding physiological niche. Not only are cells exposed to daily mechanical strain, they must also withstand low levels of oxygen, glucose and extracellular pH. This is particularly true of the NP. Despite these pressures, cells are responsible for the maintenance of a large volume of extracellular matrix (ECM) due to the low cell to ECM ratio within the IVD. This limits the endogenous capacity for tissue repair and as such, the IVD is particularly vulnerable to injury and degeneration.

### IVD degeneration

IVD degeneration is a progressive age-related condition reported to affect approximately 90% of individuals over the age of 50 years.[71] IVD degeneration can present asymptomatically, however, it is frequently associated with the incidence of LBP. Currently, LBP is the leading cause of years-lived-with-a-disability worldwide[23] and exerts significant societal burden due to lost time from work, strain on healthcare services and reduction in quality of life of afflicted individuals.[72 73]

Degeneration of the IVD is characterised by a host of changes including reduced tissue hydration, fibrosis, calcification and altered cell population.[74–78] These pathological changes form a cascade whereby alterations in cell behaviour and matrix composition impair tissue mechanics, thus further exacerbating the process of degeneration. For example, calcification of the CEPs impairs the movement of metabolites and waste products which creates an increasingly hostile environment within the NP. In more extreme cases of degeneration, NP herniation and the formation of fissures within the AF can seriously compromise tissue function. Ultimately this degenerative cascade reduces the IVD's capability to support normal mechanics and can subsequently potentiate the degeneration of adjacent discs by alteration to movement mechanics of the motion spine segment.

Ageing is currently the only conclusive risk factor known for the incidence and progression of IVD degeneration.[79–81] Research on IVD ageing has focused heavily on senescence and changes in the native cell population (including the loss of notochordal and progenitor-like cells, and alteration of phenotype from an anabolic to catabolic profile).[82–84] These factors limit the ability of the cell population to turn over its ECM in response to daily mechanical stress, ultimately leading to an accumulation of tissue and cellular stresses. Other described risk factors include genetic predisposition, obesity, smoking and occupation (including chronic shift work).[85] Obesity and smoking are thought to drive pathology by indirectly increasing levels of proinflammatory cytokines such as Interleukin-1β (IL-1β) and tumour necrosis factor alpha (TNF-α) within the IVD, which in turn promotes cell senescence and tips the homeostatic balance towards a catabolic state.[86–88] This triggers cell death and matrix degradation which promotes further tissue breakdown. Occupations associated with frequent need for postures such as lifting and extreme bending likely increase the risk of degeneration through cumulative overloading of the tissue that triggers pathological responses, such as cell death,[89 90] vascular invasion[91] and induction of inflammatory cytokines.[92]

More recently, the notion that the circadian clock may play a role in IVD degeneration has come to attention. Circadian rhythms and the ageing process are closely intertwined, as indicated by the prevalence of age-related diseases, including IVD degeneration, in individuals who undergo chronic shift working.

Misalignment of daily rhythms in IVD physiology with the external environment is thought to contribute towards ageing and degeneration of the IVD.

## CIRCADIAN RHYTHMS IN THE IVD

### Evidence and molecular targets

Despite the well-documented observations of diurnal changes of stature and IVD height, there was previously little evidence nor efforts to explore the possibility of an intrinsic circadian rhythm within the cells of the IVD. Expression of core circadian clock genes in the IVD was first described by Numaguchi *et al* in their investigation into the effects of passive cigarette smoking on clock gene expression within the IVD. Rat NP and AF/CEP tissues exhibited time-dependent expression of core clock genes over a 24 hours period as shown by quantitative polymerase chain reaction (qPCR). The expression patterns of these clock genes showed phase-shifts and amplitude dampening in rats exposed to passive cigarette smoking, which was postulated to be a result of modulation of feeding and waking behaviour through phase-shifting of the central clock.[93] Soon after, a study from Suyama *et al* identified *Bmal1* and *Rora* mRNA expression in rat AF and NP tissue collected at a single time point, and protein products of these genes within the NP. These clock factors were found to promote the transcriptional activity of HIF-1α, a key regulator of homeostasis within the hypoxic IVD.[94] An autonomous functional molecular clock in the IVD was first demonstrated by Dudek *et al* utilising the PER2::Luc reporter mouse (which expresses a fusion protein consisting of PER2 tagged with firefly luciferase).[95] Mouse IVD explants were shown to exhibit self-sustained circadian oscillations in PER2::Luc expression with an approximately 24 hours rhythmicity that was maintained for several days *ex vivo*. Circadian oscillations are visible in both the NP and AF compartments, suggesting both cell populations possess core clocks that may direct tissue-specific circadian rhythms (figure 3A). Furthermore, circadian time series RNA-seq analysis of mouse IVDs collected over a 48 hours period identified 24-hourly oscillations in the expression of 607 genes,[30] accounting for 3.5% of the identified IVD transcriptome (figure 3B). The majority of identified circadian genes cycled with a peak expression during waking hours. This 'active phase' group consisted of genes relevant to ECM turnover (eg, *Adamts1*, *Timp4*, *Serpinh1*, *Itgb1*) and endoplasmic reticulum (ER) stress (eg, *Pak1*, *Atf6*, *Chac1*), among others. Genes expressed with a peak during resting hours included those involved in processes such as apoptosis (eg, *Bak1*, *Aifm1*, *Lrdd*).[30] Highlighting the high tissue-specificity of peripheral circadian rhythms, the IVD circadian transcriptome showed very little overlap with those of cartilage[13] and tendon,[14] despite these being closely related skeletal tissues.

### Ageing, environmental disruption and inflammation

Though the IVD tissues of young mice exhibit strong circadian rhythmicity, a profound amplitude dampening and lengthening of circadian period is observed in the aged mouse IVD.[30] This is unsurprising, given the reported age-related dampening of circadian oscillations in other peripheral oscillators.[14 96 97] The age-related loss of circadian robustness could be intrinsic to the tissue itself and/or is a result of altered diurnal behavioural patterns, entrainment signals or other systemic factors with ageing. The impact of environmental circadian disruption on skeletal tissue health was demonstrated by Kc *et al* who subjected mice on high-fat diet to a chronic circadian disruption protocol. In these mice, the typical 12 hours light and 12 hours dark environment was reversed every week, mimicking severe 'jet-lag'. After 22 weeks of circadian disruption mice exhibited pathological changes in IVDs with a significant decrease in proteoglycan content in the AF, whereas IVDs from the control mice, also on high-fat diet but no circadian disruption, were unaffected.[45] This relates to the observation that shift work is associated with a higher incidence of LBP,[98 99] suggesting that chronic disruption to circadian rhythms may synergise with other risk factors to drive degeneration of the IVD. However, it is not possible to infer in this model whether this may be imparted directly through disruption of peripheral clocks or whether it is a result of systemic or behavioural factors, such as SCN clock misalignment, altered hormonal signalling or altered sleep patterns.

Reciprocal regulation between circadian clocks and inflammation is well established (see reviews by Cermakian *et al*, Curtis *et al* and Haspel *et al*).[100–102] There are many examples where aspects of the immune system are gated in a time-of-day dependent manner by circadian rhythms. On the other hand, immune responses and inflammatory cytokines can disrupt circadian rhythms in a cell type-dependent context. With ageing, levels of proinflammatory cytokines (eg, IL-1β and TNF-α) in the synovial joints and IVD are elevated.[103 104] Progressive degeneration of the IVD tissue, partly caused by increased catabolism driven

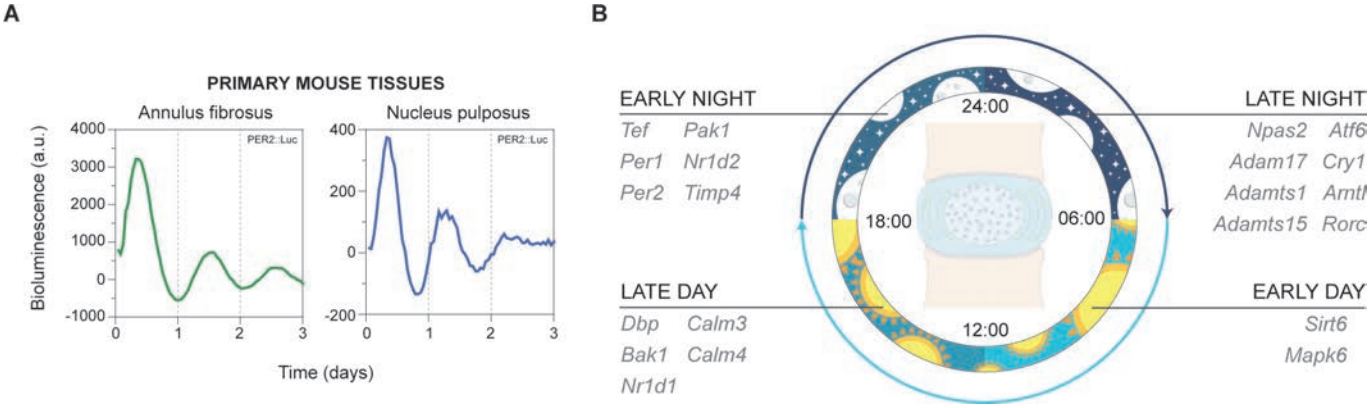


**Figure 3** Circadian rhythms in gene and protein expression and physiology in the IVD. (A) Bioluminescence profiles of murine IVD tissue explants isolated from the dissected AF (green) and NP (blue) regions of a PER2::Luc protein fusion reporter mouse. Note the clear ~24 hour rhythmicity of PER2::Luc expression. The dampening of rhythm in culture is due to desynchronisation among individual cells over time. (B) In mice, 3.5% of the IVD transcriptome oscillates with a 24-hour rhythm. Phase clustering of rhythmic genes revealed four main clusters, with more than 70% of the oscillatory genes peaking at night when the mice are active. Data retrieved from Dudek *et al*.[30] IVD, intervertebral disc.

by inflammatory/catabolic cytokines, is a major contributing factor in LBP.[105] Interestingly, PER2::Luc rhythms of the mouse IVD were shown to dampen on exposure to inflammatory cytokine IL-1β, through an NF-κB-dependent mechanism.[30] Similar findings have been reported in articular cartilage.[106] Inflammatory signalling, especially via IL-1β, is known to be increased during degeneration of the IVD[103] and may therefore initiate or exacerbate age-related circadian disruption to the IVD clock. Therapeutic interventions based on suppressing proinflammatory signalling within the IVD may promote circadian homeostasis, among other benefits.

### Genetic clock disruption

Genetic clock disruption models provide more direct evidence for a role of clock genes in IVD homeostasis. Mice carrying a global deletion in *Bmal1* show age-related development of calcification of the IVD and osteophyte formation at sites on the spine and load-bearing joints, highlighting a role for circadian rhythms in the maintenance of tissue homeostasis within the musculoskeletal system.[107] Suyama *et al* further examined the IVD phenotype in the global *Bmal1* knockout mouse, describing reduced IVD height and hyperplasia within the AF region in 10-week-old knockout mice.[94] Though global circadian disruption models provide interesting insights into peripheral tissue physiology, the use of such models produces difficulties in separating the influences of central versus peripheral clock inputs on the pathology of individual tissues. As such, the role of local clocks in the homeostasis and degeneration of peripheral tissues has been explored in recent years. Knockout of *Bmal1* targeted to *Col2a1*-expressing cells ablates circadian rhythms in the IVD and results in an accelerated ageing-like phenotype in the disc, characterised by hyperplasia of the AF and tissue calcification at IVD–vertebral body junctions.[30] These findings clearly suggest a critical role for locally expressed core clock factor *Bmal1* (clock-independent functions of BMAL1 have also been described)[108 109] and/or IVD circadian rhythms in ageing and degeneration of the IVD. Supporting this, Qiu *et al* show a significant downregulation of the circadian rhythm Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway in human geriatric NP tissue relative to foetal NP, through mass spec analysis.[110] This supports the notion that dampened clock in the aged human IVD could be a contributing factor towards tissue degeneration. More human studies are clearly warranted to address a possible causal link, although difficulty in obtaining young healthy IVD tissues often presents a limiting factor.

As an integral part of the spinal motion segment that contains ligaments, hyaline cartilage and bony vertebral bodies, the IVD is not unique in possessing an intrinsic circadian clock. Cartilage, tendon, bone and muscle have all been reported to possess functional clocks that generate tissue-specific circadian rhythms.[14 39 96 111–113] Much like the IVD, these musculoskeletal clocks dampen with ageing[14 96] and are sensitive to inflammatory signalling,[106 114] implicating a role for clock disruption in the pathogenesis of musculoskeletal degeneration. For example, *Bmal1* knockout mouse studies show phenotypes resembling accelerated ageing in tissues including articular cartilage, tendon, muscle and bone.[11 13 43 115] Common traits such as accelerated ageing in response to prolonged circadian disruption highlight the importance of circadian rhythms in the long-term maintenance of skeletal tissue homeostasis.

## HOW DO IVD CELLS COMMUNICATE TIMING INFORMATION WITH THE BRAIN?

Maintaining circadian entrainment of a given tissue to physiological time cues is paramount to ensure rhythmicity is aligned with the external environment. The largely avascular and aneural nature of the non-degenerate IVD makes it a unique niche in terms of circadian entrainment. In this tissue context, conventional zeitgebers such as neural innervation is absent, therefore may be reliant on the diffusion of molecules into the IVD space. Alternatively, factors such as core body temperature cycles and daily activity patterns may act as endogenous time cues for the IVD clock (figure 4A). Of note, these factors can be negatively affected by ageing due to structural changes to the IVD, lowered circadian amplitude of the central clock and impaired mobility in later life. As such, the age-related dampening of circadian rhythms in the IVD could be partially due to weakened systemic cues.

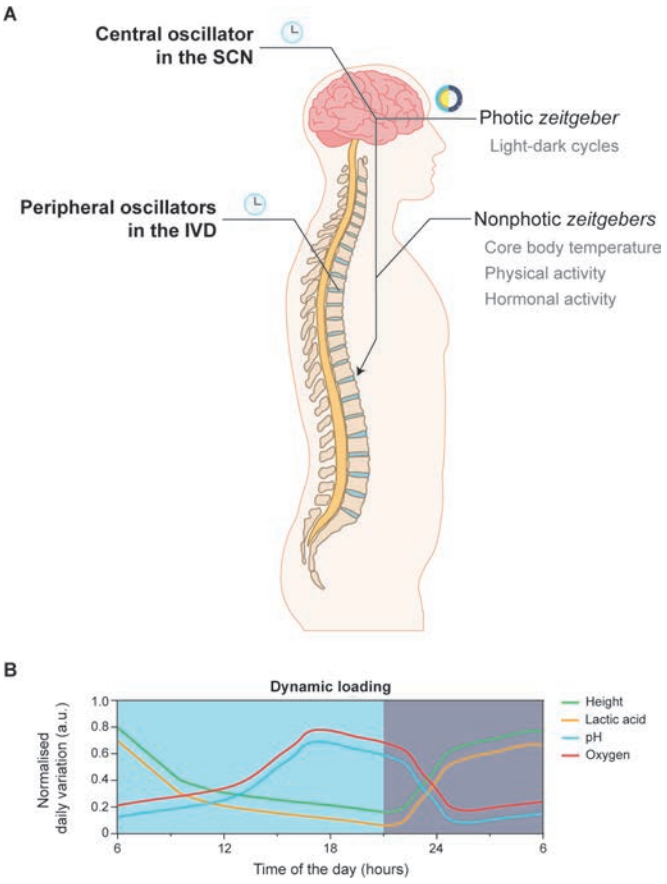


**Figure 4** The circadian control of homeostasis in the IVD. (A) Photic *zeitgeber* (time cue) entrains the central clock in the SCN, which in turn synchronises the circadian rhythms of the spinal IVD via fluctuations of core body temperature, diurnal cycles of physical activity, as well as daily surges of hormonal signals. (B) Variation in IVD height occurs in response to changes in compressive loads acting along the spine, which in humans results in a height loss throughout the day (maximum at ~06:00 and minimum at ~21:00 hours) and recovery during the night time. The majority of IVD height change occurs within the first few hours after resuming an upright position (for height loss) and lying down (for regaining of height), respectively, followed by a slower phase. Dynamic changes in loading also affect the transport and metabolism of oxygen and lactic acid in the IVD, promoting pH changes. This graph provides a simplistic overview of these variations and the metabolism of oxygen and lactic acid depends on strain level and loading frequency. Information adapted from Huang and Gu[153] and Krag *et al*.[154] IVD, intervertebral disc.

### Diurnal hormonal signals

Among the strongest known entrainment cues for peripheral oscillators is glucocorticoid signalling. Robust diurnal variation

in blood glucocorticoid levels, driven by oscillators in the SCN, hypothalamus and adrenal gland, acts as a potent zeitgeber for numerous peripheral clocks.[116] These glucocorticoids directly regulate the molecular clock through binding of the glucocorticoid receptor to glucocorticoid response elements in target clock genes, regulating their transcription.[117] Circadian clocks in cartilage, tendon, bone and IVD are responsive to the synthetic glucocorticoid dexamethasone in *ex vivo* cultures.[14 30 39 96 113] It is likely that endogenous glucocorticoids are capable of synchronising these tissue clocks *in vivo*. In the context of the IVD, the limited capacity for transport via diffusion may present a barrier to this conventional entrainment mechanism. Although, hormones such as parathyroid hormone (PTH) and oestrogen have been postulated to be able to enter the IVD.[118] Interestingly, PTH itself shows a diurnal variation in serum concentration[119 120] and can reset the mouse cartilage circadian clock *ex vivo*,[121] possibly through induction of *Per1/2* transcription through PKA–CREB pathway.[122] NP cells express the type 1 PTH receptor and are responsive to PTH stimulation,[123] suggesting that these cells may be sensitive to circadian entrainment by PTH. In ageing and degeneration, sclerosis of the CEPs may reduce the supply of nutrients, and possibly other humoral factors, into the IVD,[124 125] which could contribute to compromised entrainment of the IVD clock.

### Endogenous temperature rhythms

Observations that the temperature of the human body is not constant throughout the day were first reported in the 18th century.[126 127] The daily rhythm was later shown to persist in subjects who were sleep deprived[128] or who lived under constant conditions without external time cues.[129] In humans, with wakeup time at ~07:00 and bedtime at ~23:00 hours, rectal temperature starts rising 3 hours before awakening from a night-time low of approximately 36.5°C, reaching 37.2°C by 09:00 hours, and continuing to rise slowly to a peak of 37.4°C at about 20:00 hours before falling to 36.5°C at 04:00 hours.[130] Studies of human knee temperature using sensors have shown that the mean intra-articular temperature of the knee in healthy subjects is 32.6±0.9°C.[131] To the best of our knowledge, no studies have assessed temperature in the human IVD in a physiological context. Like cartilage, the IVD is also largely avascular and poorly innervated, suggesting that in this tissue the average temperature will also be lower than the core body temperature.

Recently, it has been shown that 12 hours/12 hours temperature cycles that approximate daily body temperature fluctuations are sufficient to entrain peripheral clocks in various murine tissues, such as the liver, lung and cartilage.[96 132 133] Importantly, Dudek *et al* showed that the circadian phase of oscillations in IVDs and articular cartilage can be driven to 180° out of phase to each other by oppositely phased cyclic temperature changes (38.5°C for 12 hours/35.5°C for 12 hours) and their amplitude enhanced.[13 30] The amplitude effect on these skeletal clocks is particularly intriguing as the IVD and cartilage tissues lose circadian amplitude during ageing. This has raised the interesting possibility that temperature-based interventions could be utilised to ameliorate clock-controlled homeostatic functions in the skeletal system.

In another tissue context, it has been suggested that the temperature-mediated entrainment of the clock is regulated by the heat shock pathway. The activity of heat shock factor 1 (HSF1) in the liver is circadian and can be driven by physiologic temperature fluctuations.[134] Remarkably, heat shock elements can be found in the promoter of *Per2* and several *Hsp* genes oscillate in a similar phase to *Per2*.[37]

Heat shock pathway molecules have been found in human IVDs and shown to be key players in tissue homeostasis. For instance, HSF1, HSP27 and HSP72 immunostaining was observed in human AF and NP cells and more frequently seen in organised clusters of cells, a hallmark of tissue degeneration.[135 136] *In vitro* heat shock of porcine NP induced the expression of HSP70.[137] In future, it would be interesting to examine whether the response of the IVD clock also depends on the heat shock pathway or whether additional, tissue-specific mechanisms are involved.

Aberration of circadian rhythms of body temperature in aged individuals is well documented (see reviews by Kenney and Munce and Hood and Amir).[138 139] Since peripheral circadian clocks are closely intertwined with the heat shock response pathway to regulate tissue physiology, this provides a potential avenue to address homeostatic imbalances and counteract degenerative processes. Interventions based on imposed daily patterns of temperature stimuli may present a promising avenue to preserve and sustain circadian rhythmicity in the ageing tissues.

### Daily loading patterns and related osmolarity changes

The IVD is one of the most highly loaded tissues in the body and routinely experiences high (0.5 MPa) compressive pressures while standing and low pressure during sleep.[140] These pressures result in a significant volume of fluid being pressed from the disc during daily loading, which is then reabsorbed overnight during rest, driven by osmotic pressure.[141–143] Various studies estimate the proportion of lost water to be between 10% and 25% of total volume,[27–29] which is in line with earlier observations of loss in height. As a result, the osmolarity of the ECM of IVDs varies widely within a few hours of resting or activity onset (figure 4B).

The changes in the osmolarity of the IVD have several implications. First, hydration is essential for maintaining IVD tissue stiffness and energy absorption capacity. The reduction of water content in the evening affects the IVD's response to mechanical loading, leading to more load being transferred to the AF and reduced stability.[144–147] Second, the expression of key IVD genes is different depending on different osmolarities and mechanical loading. For example, the expression of aggrecan and collagen type II is higher, and collagen type I lower in high osmolarity static culture.[148–152] Last but not least, the changes in water content of human IVDs occur rapidly after assuming upright position in the morning and after recumbence in the evening[153 154] (figure 4B). As a consequence, the IVD cells experience hyperosmotic stress in the morning and hypo-osmotic stress in the evening, which differentially activate downstream signalling pathways to adapt to such drastic daily changes in their osmotic environment.

Fluctuations in extracellular osmolarity regulate IVD matrix homeostasis. When hyperosmotic shock was applied for 16 hours followed by 8 hour iso-osmotic recovery for 7 days, it promoted NP matrix synthesis in a porcine IVD organ culture model.[155] Similarly, when hyperosmotic stress was applied daily for only 1.5 hours a day over a period of 11 days, it increased matrix synthesis and stabilised the notochordal cell phenotype in a mouse IVD organ culture model.[156] Mechanistically, hypo-osmotic challenge was found to activate the mechano-sensitive and osmo-sensitive calcium channel TRPV4 in IVD cells and chondrocytes.[157–159] In contrast, hyperosmotic challenge was mediated largely through the transcription factor *TonEBP* (*Nfat5*).[160] *TonEBP* plays a critical role in osmo-adaptation and ECM homeostasis of NP cells.[161] Particularly, *TonEBP* is

responsible for upregulation of aggrecan as the *Acan* promoter was found to contain two conserved TonE motifs.[160]

Interestingly, one of the top upregulated genes in human IVD cells following hyperosmotic stress is the core circadian clock component *Bmal1* (*Arntl*).[162] *Arntl* was also identified as one of the key hubs in protein interaction network analysis of differentially regulated genes in response to hyperosmotic challenge in IVD cells.[163] Given the diverse pathways controlled by the circadian rhythm in the IVD, it is plausible to suggest that the circadian clock may act as a mediator between daily osmotic challenge and changes in the expression of downstream genes and proteins. Reduced mobility in aged individuals may confer further reduction in circadian entrainment of the IVD functions through reduced mechanical loading. Reduction in tissue water content and associated extracellular osmolarity is a hallmark of IVD ageing and degeneration.[164] Diurnal changes in osmotic pressure are also attenuated in the degenerate disc,[29] which could further exacerbate age-related clock disruption.

Mechanical loading could potentially affect the circadian clock independently of osmolarity, through activation of mechanosensitive pathways. For example, the TRPV4 calcium channel, aside from sensing hypo-osmolarity, was shown to mediate the effect of low magnitude compression on IVD cells and induce accumulation of GAGs and expression of aggrecan and collagen type II.[165] Loading of cells can also act directly on the cytoskeleton by promoting F-actin reorganisation, which can in turn act as a mechanism for transducing mechanical stimuli to the nucleus and the circadian time-keeping mechanisms.[166 167] Interestingly, a microarray study in an *in vitro* 3D sponge culture model of chondrocytes identified the *Clock* gene as being downregulated by mechanical stress.[168] Mechanisms such as cell-matrix and cell–cell interactions are also implicated in response of IVD cells to mechanical loading (for an extensive review please see Fearing *et al*[169]). However, further studies are needed to determine if any of the above pathways can transmit mechanical stimuli to the circadian clock.

### Chronotherapy: time for a change

Disruption to circadian entrainment signals such as humoral signalling, mechanical loading, osmotic pressure and body temperature cycles may contribute towards the disruption of the IVD clock in ageing, leading to a vicious cycle which exacerbates tissue degeneration. In this regard, future work should explore whether harnessing the power of entrainment signals may present an opportunity to sustain or restore circadian rhythmicity in the IVD and other musculoskeletal tissues. Timed exercise has been shown to be a potent entrainment factor for clocks in mouse skeletal muscle[170] and human skeletal muscle.[171] Regular low-intensity to medium-intensity exercise is thought to be beneficial for the maintenance of musculoskeletal tissues, including the IVD.[172–174] Although not tested yet, it is reasonable to hypothesise that scheduled exercise could impart a beneficial effect on skeletal tissue homeostasis through mitigation of age-related circadian dampening and misalignment.

In addition, there is also scope for taking advantage of circadian clock principles to guide the timing of interventions and medications to improve efficacy of current treatments (eg, anti-inflammatory drugs for RA, disease-modifying osteoarthritis drugs for osteoarthritis), while mitigating side effects. Such chronotherapeutic principles have been successfully applied in cancer treatment, particularly chemotherapy, whereby the appropriate timing of treatment can simultaneously enhance efficacy and reduce toxicity.[175 176] Interestingly, systemic analysis of clinical trials revealed that of ~100 human trials that evaluated time-of-administration of drugs, 75% of clinical trials (78/105) showed dosing-time-dependent efficacy or toxicity in treating hypertension, cancer, asthma and arthritis.[177] Indeed, analysis has shown that 56 of the top 100 best-selling drugs in the USA target the product of a circadian gene, highlighting circadian timing as a critical yet often underappreciated factor when considering drug efficacy in clinical trials and medical practices.[3] One of the best examples of a real-life application of chronotherapy in rheumatic diseases is in the treatment of RA. In RA, pain severity is known to show a diurnal pattern at least partly due to a diurnal rhythm in inflammatory cytokine levels.[17 18] A modified-release formula of prednisone in patients with RA enables high serum glucocorticoid concentration in anticipation of peak cytokine levels and symptom severity in the early morning, resulting in improved morning stiffness when compared with standard prednisone treatment.[19] In the musculoskeletal system, it has been reported that timed dosing increases the tolerance and analgesic effectiveness of anti-inflammatory drug indomethacin in patients with osteoarthritis.[16] A diurnal variation in LBP symptoms has been alluded to by several studies.[178 179] However, to the best our knowledge, a ‘chrono-pain management’ approach has not been trialled for patients with pronounced diurnal pain presentation.

Chronotherapy approaches such as these are of little cost to healthcare services and patients but have the potential to impart significant benefits. Given the diverse pathways that are regulated by circadian rhythms in various tissue systems, future therapeutic interventions for chronic rheumatic and skeletal conditions may also benefit from such principles.

## CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The genetic decoding of the molecular circadian clockwork is a standout success in revealing the intricate feedback loops that generate 24 hours rhythms. Since the discovery of cell intrinsic peripheral circadian clocks,[95 180] there has been an acceleration of research into the functions of these local oscillators in coordinating tissue physiology. It is becoming increasingly clear that most aspects of our physiology and pathophysiology are under daily rhythmic control, including musculoskeletal tissues and chronic rheumatic diseases, for example, RA (see review by Buttgereit *et al*),[181] osteoarthritis (see reviews by Berenbaum and Meng, Morris *et al* and Dernie and Adeyoju),[182–184] osteoporosis (see reviews Gonçalves and Meng and Swanson *et al*),[47 185] IVD degeneration and associated back pain. Despite the highly conserved expression of core clock genes, cross tissue comparisons of circadian functions have revealed an amazing degree of tissue specificity, both in terms of the input pathways to the clock (entrainment factors), and output clock-controlled targets.[3 186] Conversely, demographic ageing and an increasingly 24/7 society (involving too much artificial light at night and less exposure to natural light during the day) frequently disrupt the circadian timing mechanisms. Therefore, further investigation of how the central and peripheral clocks regulate diverse tissue functions and how their dysregulations contribute to disease are clearly needed.

In this regard, we used the highly rhythmic IVD as an exemplar peripheral clock tissue which experiences profound diurnal changes associated with daily loading and temperature cycles. Degeneration of the IVD is a complex and multifactorial process associated with ageing. Due to the intertwined links between ageing and circadian rhythms, and the diverse molecular targets within the IVD that are under rhythmic control, it is likely that the recently described IVD circadian clock plays a key

modulatory role in this degenerative process. Significantly, these IVD clocks are disrupted by ageing and inflammation, known risk factors for IVD degeneration, suggesting the involvement of clock mechanisms in driving a predisposition to the development of advanced ageing and degeneration in the IVD.

However, despite recent advances linking the circadian clock to homeostasis and degeneration of the IVD, our current understanding of circadian biology in the IVD represents only the tip of the iceberg. As the IVD is not a homogenous tissue but consists of different cell types and distinct anatomical structures, further investigation of cell type-specific clock functions will enable a better understanding of the role that circadian rhythms play in the daily tissue physiology. Enhanced understanding of how the clock changes during the process of human IVD degeneration and what role this plays in the aetiology and progression of degeneration is also needed. This will help identify molecular targets and design therapeutic interventions accordingly. Finally, it remains to be understood how the circadian rhythm of the IVD is weakened during ageing. Plausible hypotheses include proinflammatory signalling, altered daily loading patterns, reduced IVD tissue osmolarity and disrupted core body temperature rhythms, as well as cell intrinsic changes such as senescence and ER stress. Further research will shed light on the role of molecular circadian clocks in this unique tissue niche and enable new therapeutic approaches for better management of IVD degeneration and LBP.

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CLINICAL SCIENCE

# EULAR recommendations for the reporting of ultrasound studies in rheumatic and musculoskeletal diseases (RMDs)

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## ABSTRACT

**Objective** To produce European League Against Rheumatism (EULAR) recommendations for the reporting of ultrasound studies in rheumatic and musculoskeletal diseases (RMDs).

**Methods** Based on the literature reviews and expert opinion (through Delphi surveys), a taskforce of 23 members (12 experts in ultrasound in RMDs, 9 in methodology and biostatistics together with a patient research partner and a health professional in rheumatology) developed a checklist of items to be reported in every RMD study using ultrasound. This checklist was further refined by involving a panel of 79 external experts (musculoskeletal imaging experts, methodologists, journal editors), who evaluated its comprehensibility, feasibility and comprehensiveness. Agreement on each proposed item was assessed with an 11-point Likert scale, grading from 0 (total disagreement) to 10 (full agreement).

**Results** Two face-to-face meetings, as well as two Delphi rounds of voting, resulted in a final checklist of 23 items, including a glossary of terminology. Twenty-one of these were considered 'mandatory' items to be reported in every study (such as blinding, development of scoring systems, definition of target pathologies) and 2 'optional' to be reported only if applicable, such as possible confounding factors (ie, ambient conditions) or experience of the sonographers.

**Conclusion** An EULAR taskforce developed a checklist to ensure transparent and comprehensive reporting of aspects concerning research and procedures that need to be presented in studies using ultrasound in RMDs. This checklist, if widely adopted by authors and editors, will greatly improve the interpretability of study development and results, including the assessment of validity, generalisability and applicability.

## Key messages

**What is already known about this subject?**

- Nomenclature, definitions of ultrasound-detected pathologies, scoring systems and technical issues may affect the validity and generalisability of results of ultrasound studies in rheumatic and musculoskeletal diseases.
- These aspects, along with critical design characteristics, are often suboptimally reported in current ultrasound studies.

**What does this study add?**

- A 23-item recommendation checklist was developed by a European League Against Rheumatism taskforce to ensure transparent and comprehensive reporting of ultrasound research.
- This is the first reporting checklist focused on how to report characteristics of imaging measurement tools.

**How might this impact on clinical practice or future developments?**

- The use of this checklist may improve the interpretability, reproducibility and generalisability of study results.

Ultrasound is an imaging technique widely used in patients with rheumatic and musculoskeletal diseases (RMDs) to detect signs of inflammation and destructive changes.[1] Despite an increased use in clinical practice facilitated by major technical advances in the resolution of soft tissue contrast (B-mode or grey scale (GS)) and of vascular perfusion (Doppler techniques), a relatively long learning curve[2] and, until recently, the absence of agreed scoring systems have hampered its utilisation for research.[3 4]

The European League Against Rheumatism (EULAR) and the Outcome Measures in Rheumatology (OMERACT) Ultrasound Working Group have actively worked towards the standardisation of the technique by developing educational programmes and by performing several studies evaluating its reliability, validity and feasibility.[5–8] These initiatives have underlined that factors such as nomenclature, definitions of ultrasound-detected pathologies, scoring systems and technical issues

with the ultrasound equipment may affect the validity and generalisability of these results. These aspects, along with critical design characteristics, such as reproducibility, blinding, patient selection and clearly defined purposes of the ultrasound evaluation, are often suboptimally reported in the current ultrasound studies.[5 6 9 10]

Complete and accurate reporting is necessary to detect potential biases in the study (internal validity) and to assess the generalisability and applicability of the results (external validity). Over the last 20 years, many guidelines have been developed to improve the quality of reporting of research articles, including those for randomised controlled trials (RCT) (Consolidated Standards of Reporting Trials)[11] and diagnostic accuracy studies (Standards for Reporting Diagnostic accuracy studies).[12 13] EULAR has also contributed by developing recommendations for reporting registers and clinical trial extension studies.[14 15] We are not aware of recommendations focused on how to report characteristics of imaging measurement tools such as the equipment characteristics, procedures or scoring, which can influence the validity and generalisability of study results. Therefore, an EULAR taskforce was convened to propose recommendations for the reporting of such aspects in ultrasound studies in RMDs.

## METHODS

The convenor (MADA), EULAR methodologist (LC) and project fellow (FC) led a multidisciplinary taskforce in accordance with the EULAR Standardised Operating Procedures (SOPs).[16] The taskforce included 23 members from 11 European countries and from the USA and was composed as follows: 11 experts in ultrasound in RMDs, 7 in methodology, 1 in both ultrasound and methodology, 2 in biostatistics, 1 patient research partner and 1 health professional in rheumatology. Three of the 23 members were members of EMEUNET and 13 of them were also part of an editorial board.

The taskforce employed a stepwise process summarised in figure 1, including two face-to-face meetings and several Delphi rounds. First the EULAR methodologist, convenor and fellow searched for evidence of quality of reporting of ultrasound studies in RMDs. The choice was made to focus on an extensively studied topic, that is, ultrasound assessment of synovitis in rheumatoid arthritis. In PubMed Clinical Queries, a broad search was performed; 80 studies were randomly selected and divided in four categories: diagnosis, aetiology, prognosis and therapy. The articles were summarised in table format to highlight objective, design, technical data, measures and outcomes (online supplemental file 1). These tables were sent to each member of the taskforce prior to the first face-to-face meeting, with the request to identify possible sources of bias and error and the absence of information considered important for the generalisability of the results. During the first face-to-face meeting, the members of the taskforce discussed the results and the unmet requirements in the selected literature, agreed on the format of presentation of the project (checklist or statement document) and elaborated a first list of items to be included. Other objectives of this meeting were the definition of a target audience and the need for systematic reviews. After the meeting, a number of focused literature reviews addressed specific issues; a summary of their results, along with the total list of items, were subsequently sent to the taskforce members. Relevance and comprehensibility of each proposed item were tested in a Delphi exercise, first by the taskforce members (excluding the convenor, EULAR methodologist and fellow), then by a panel of external experts chosen from the fields of musculoskeletal imaging, epidemiology and methodology, including journal editors. External experts were also asked if no key aspects were missed (comprehensiveness). During the second face-to-face meeting, the optimal format of the checklist document was established. Inclusion of each item was either supported by empirical evidence, when available, or by consensus within the task force, that the information requested by the item was methodologically important to assess in a study, as recommended by the Enhancing the QUAlity and Transparency Of health Research (EQUATOR) 'guidance for developers of health research reporting guidelines'.[17] In the same way, it was agreed not to include a level of evidence for each proposed item. The external experts were then invited to apply the checklist to a selection of ultrasound articles and to comment on its feasibility and comprehensibility; this resulted in minor modifications to the items. Finally, an online Delphi survey was performed among the taskforce experts to obtain their level of agreement with each final item, including each term of an accompanying glossary, included to define the checklist terminology. Agreement was assessed with a Likert scale, grading from 0 (total disagreement) to 10 (full agreement). Consensus was defined as a mean agreement ≥7 and with at least eight responders (2/3 of participants) having an agreement ≥7.

## RESULTS

Figure 1 shows the flowchart of the project. During the first face-to-face meeting, a preliminary checklist of 43 items was established, and three scoping reviews were requested on factors potentially influencing the ultrasound evaluation and therefore the generalisability of the results: (a) contextual factors (eg,

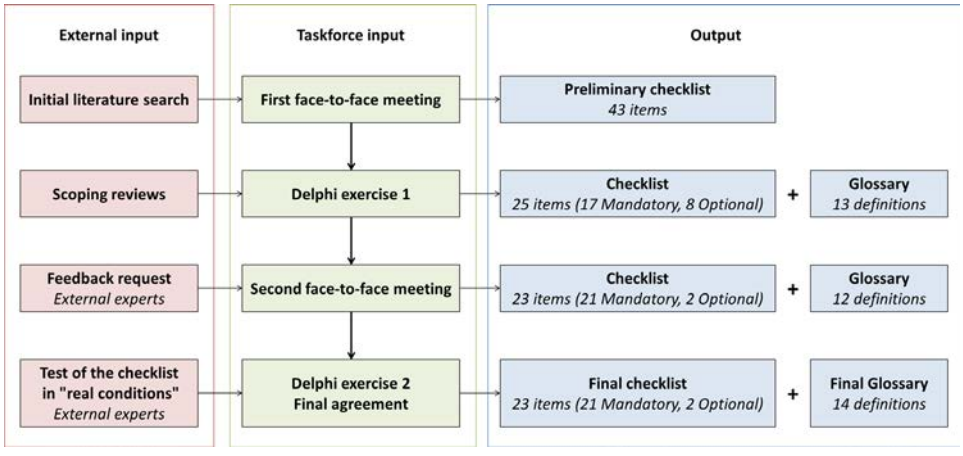


**Figure 1** Process flowchart of the project.

smoking, temperature), (b) machine quality (eg, device, settings) and acquisition methods (eg, joint or transducer position) (online supplemental file 2).

A first Delphi exercise helped decide which of the 43 items should be considered as 'mandatory' (always reported in every ultrasound study) or 'optional' (reported only according to specific study designs). After two voting rounds, several items were rephrased, deleted or combined, resulting in a checklist of 17 'mandatory' and 8 'optional' items (figure 1).

This new checklist was distributed among 218 external panellists (external Delphi exercise): 123 experts in muscoloskeletal imaging, 67 in epidemiology, 7 in methodology as well as 21 journal editors. Seventy-nine of them (36%) were participated. The external experts rated the initiative as very important (96%), the checklist comprehensive (95%), and all items were considered clear by the majority of them (median: 96%, range: 86%–96%). Additional suggestions were made to clarify some terminology. The results were discussed during the second face-to-face meeting, where the format of the checklist was agreed. Each item was verified to ensure comprehensibility, and a preliminary glossary including 12 terms was prepared. After this process, the checklist included 23 items (21 'mandatory' and 2 'optional') organised into 13 categories. This version and the glossary were distributed to the 79 external experts who had participated in the previous evaluation. Twenty-nine (37%) of them agreed to test the new checklist on additional selected articles and to comment on the comprehensibility and comprehensiveness of the items and the glossary as well as on the feasibility of applying the checklist. The median time needed to assess the articles for reporting the checklist items was 30 min (range 10–240). Comprehensibility was assessed as good with a median of 8 (range 1–10); and additional suggestions on the glossary terminology were made. The convenor and fellow incorporated all suggestions, and the final product (checklist with accompanying recommendation guidelines and glossary) was submitted to the taskforce members. Two rounds of an additional Delphi exercise (the second) were needed to obtain a final agreement on each item of the checklist and each term of the glossary.

The final checklist, composed of 23 items (21 'mandatory' and 2 'optional'), organised into 13 categories, along with the level of agreement for each item, is reported in table 1; the accompanying glossary and the recommendation guidelines on how to use and interpret each item in the following paragraphs are presented in table 2.

### Target audience and when to apply the recommendations

The target audience comprises health or scientific researchers reporting or assessing observational and interventional studies using ultrasound in RMDs: that is, rheumatologists, radiologists and healthcare professionals using ultrasound, manuscript reviewers, grant applicants and reviewers, journal editors. Each mandatory item of the checklist should be considered as essential to be reported in every ultrasound study regardless of the purpose of the study. Such a report will allow proper appraisal of the validity and applicability of the results. The checklist is meant to be applied whenever ultrasound is used (investigation of measurement properties, diagnostic or prognostic accuracy and therapeutic studies). It is focused specifically on ultrasound issues and is neither intended to be totally comprehensive for all study design issues, nor intended to replace other existing reporting guidelines (eg, RCT, observational diagnostic studies, etc).

### General items

The first six items on 'objective', 'design', 'participants' and 'blinding' as well as items 20 ('statistical analysis') and 21 ('disclosures') are not specifically related to ultrasound and some of them have already been included in other reporting checklists. However, the taskforce members felt it was essential to include them in this checklist to emphasise their importance. For example, the objective of the ultrasound measurement in a study might be different from the main objective of the study, and then such difference should be clearly described. Another example is the blinding of the ultrasound evaluation. Blinding is of utmost importance, especially in diagnostic or therapeutic studies, since the lack of patient or operator blinding can influence the results.[18 19] Item 20 ('statistical analysis') refers mainly to the way in which the analysis of ultrasound variables should be performed, for example, the importance to clearly state whether analyses are performed at patient or at joint/site level.

### Ultrasound features

Item 7 refers to the ultrasound definition of the pathological lesions under study. It is crucial to be able to check for consistency between what authors say they want to measure (eg, erosions (target domain) as a measure of structural damage (broad domain of interest)) and what was really measured with ultrasound. The concepts of broad and target domains are explained in table 2. In such cases, reporting the domain components (ie, elementary lesions) really measured by ultrasound will help evaluate consistency. A precise definition of ultrasound elementary lesions used in the study helps to check whether ultrasound is able to measure what it is supposed to measure (domain match). Here we used the terminology proposed by OMERACT for the development of imaging outcome measurement instruments,[20] and in particular of ultrasound.[5] In recent years, the OMERACT ultrasound working group, frequently in collaboration with EULAR, has undertaken considerable efforts to develop and improve definitions of ultrasound elementary lesions for a defined pathology (eg, synovitis, enthesitis, bone erosion).[5 8]

### Scanning/acquisition procedures

Several sources of variability may affect the reliability of ultrasound measurements and generalisability of the study results. These include the quality of the equipment, positioning of patient and transducer and training of the examiner. One of the scoping reviews (online supplemental file 2) studied whether acquisition methods (ie, joint or transducer position and dynamic acquisitions) affected the reliability and accuracy of ultrasound. All retrieved studies confirmed the importance of a standardised joint position for the reliability and generalisability of the results; this applies to all anatomical sites (eg, knee, wrist, Achilles tendon, etc) and all target pathologies under study (eg, synovitis, joint effusion, etc),.[21–26] In addition, appropriate transducer manipulation is needed to avoid artefacts.[23–25] For example, transducer pressure may cause the synovial hypertrophy or Doppler signal to disappear or be reduced.[27] The 2001 EULAR guidelines for performing ultrasound in rheumatology (updated in 2017) addresses both joint (and patient) positioning as well as transducer use.[28 29] Authors are invited to refer to these latest guidelines in their studies. Items 8 and 9 describe what details should be provided about the scanning and acquisition procedures to assess compliance with these guidelines.

**Table 1** Recommendations checklist for reporting studies using ultrasound in rheumatic and musculoskeletal diseases

| Topic | Number | Item to report | Agreement† (mean±SD) |
|---|---|---|---|
| Objective | 1 | Objective of the ultrasound measurement in the study (*eg, description, prediction, diagnosis, validation…*) | 9.9±0.3 |
| Design | 2 | Study design (*eg, cross-sectional, case-control, cohort, randomised clinical trial, …*) | 9.9±0.3 |
| | 3 | Prospective or retrospective data collection* | 9.7±0.7 |
| Participants | 4 | Informed consent procedure (written, oral) | 9.2±1.0 |
| | 5 | Source, selection criteria and sampling of the participants (including controls where appropriate) | 9.9±0.3 |
| Blinding | 6 | Procedures for blinding of sonographers and participants | 9.3±1.0 |
| Ultrasound features | 7 | a. Broad domain* of interest (*eg, inflammation or structural damage*) | 8.9±1.4 |
| | | b. Target domain* with corresponding theoretical ultrasound definition(s)* (eg, synovitis: synovial hypertrophy plus increased synovial blood flow) | |
| | | c. Domain components (ie, elementary lesions)* with corresponding operational definitions* (*eg, synovial hypertrophy: increased thickness of synovium with hypoechoic appearance*) | |
| Scanning/acquisition procedures | 8 | a. Anatomical region(s)* or structure(s)* that were studied | 9.2±1.2 |
| | | b. Rationale for choosing these anatomical region(s)/structure(s) | |
| | 9 | a. Patient position (eg, prone, supine…) | 9.6±0.8 |
| | | b. Anatomical region position (eg, neutral…) | |
| | | c. Surfaces scanned (eg, volar, dorsal) | |
| | | d. Transducer position (eg, transverse, longitudinal) | |
| | | e. Whether the examination was dynamic* | |
| Ultrasound scoring system | 10 | Scoring system used:<br>a. Type (*eg, quantitative, semiquantitative, binary*) | 9.6±0.5 |
| | | b. Level: (*eg, patient level, joint/anatomical region level*) | |
| | 11 | For existing scoring systems:<br>a. References or results of previous validity and reliability studies | 9.3±1.2 |
| | | b. Score range (minimum-maximum), and meaning of the score (*eg, higher is ……*) | |
| | | c. Rationale for any thresholds or cut-offs | |
| | | d. Training session details if performed | |
| | | e. The reliability* of the scoring system in the hands of the study sonographers/readers | |
| | 12 | For new scoring systems:<br>a. Rationale for developing a new scoring system | 9.4±0.8 |
| | | b. Detailed description of the scoring system | |
| | | c. Reliability assessment: | |
| | | I. Type of reliability: inter-reader, other | |
| | | ii. Training session if performed | |
| | | Iii. The reliability of the scoring system as applied by the study sonographers/readers | |
| | | iv. Whether reliability was assessed on static images, video-clips or real-time examination of patients | |
| | | v. Sample size of the reliability study | |
| | | vi. Reliability results (eg, kappa or ICC with 95% CI and type of kappa or ICC, prevalence of observed lesions, smallest detectable change, SE of measurement) | |
| Sonographer(s)*/reader(s)* | 13 | a. Whether acquisition and reading were performed at the same time | 9.6±0.6 |
| | | b. Whether acquisition and reading were performed by the same person | |
| | | c. Number of sonographers or readers | |
| | | d. In longitudinal studies, whether the same sonographer scanned the same patient at each assessment | |
| | *14* | *Optional: Information about the experience of sonographer(s) and reader(s) (eg, numbers of scanned performed, certification, qualification…)* | 8.7±2.3 |
| Equipment | 15 | a. Brand and model of the ultrasound device | 9.1±1.3 |
| | | b. Type and model of the transducer | |
| | | c. Whether the ultrasound device (or software) was changed during the study | |
| | 16 | Ultrasound modalities* and settings<br>a. Grey scale | 9.7±0.9 |
| | | b. Doppler | |
| | | c. Other | |
| Images (pictures and drawings) | 17 | For images included into the manuscript, verify that:<br>a. Information identifying patient is deleted | 9.3±1.6 |
| | | b. Essential targets in the image(s) are clearly labelled | |
| | | c. Images match the content of the manuscript | |
| | | d. Quality of the images is adequate | |
| Contextual factors | 18 | Duration of ultrasound examination when relevant for the study question | 8.8±1.6 |
| | *19* | *Optional:*<br>*a. Whether ambient conditions (eg, temperature, time of day) were kept stable during the study*<br>*b. Potential confounding factors (eg, exercise, alcohol, caffeine, smoking)* | 7.3±2.8 |

Continued

**Table 1** Continued

| Topic | Number | Item to report | Agreement† (mean±SD) |
|---|---|---|---|
| Statistical analysis | 20 | a. Existence of a pre-specified statistical analysis plan and specification of post-hoc analyses<br>b. Analyses performed<br>c. Whether the analyses were performed at patient or at joint/region level<br>d. Extent of missing data<br>e. Handling of missing data | 9.3±1.2 |
| Disclosures | 21 | Potential conflicts of interest including those related to ultrasound | 9.2±1.4 |

*Items are explained in detail in the glossary (table 2).
ICC, intra class correlation; SE, standard error.

## Ultrasound scoring systems

Items 10, 11 and 12 focus on a clear description of ultrasound scoring systems, especially if a newly developed scoring system is used. Special attention should be paid to the documentation of the development of the scoring system. As ultrasound is frequently considered the most operator-dependent imaging technique, intra-rater and inter-rater reliability is an important concern and a strong argument for standardisation. For new scoring systems, results of intra-sonographer and inter-sonographer/reader reliability studies should be reported. For existing scoring systems, reference to previous reliability studies should be given as well as the results of reliability assessments among the sonographers/readers in the context of the study.

## Sonographers/readers

Depending on the setting, the person who performs the ultrasound acquisition of the images (sonographer) can be a healthcare professional or a medical doctor (radiologist or rheumatologist). The images can be interpreted at the time of acquisition or later, by the same or another person. Choices made here affect ultrasound scores and generalisability, so details on who performs

**Table 2** Glossary

| Item | Terminology | Definition | Agreement† (mean±SD) |
|---|---|---|---|
| 3 | Prospective data collection | Data collection that starts before the outcome has occurred. | 9.2±1.7 |
| | Retrospective data collection | Data collection that starts after outcome status has been determined and refers to information up to that moment. | 9.4±1.2 |
| 7 | Broad domain | A pathological (or pathophysiological) manifestation we are interested in assessing/measuring. For example, current broad domains in rheumatic and musculoskeletal diseases measured by ultrasound are 'inflammation' and 'structural damage'. | 9.7±0.8 |
| | Target domain | Further specification of the broad domain we are interested in assessing/measuring with ultrasound. For example, synovitis, enthesitis, erosion. | 9.7±0.6 |
| | Theoretical definition of target domain | The ultrasound definition of the target domain we want to measure, made up of domain components. | 8.9±1.7 |
| | Target domain component | An individual characteristic of the target domain that can be measured.<br>All domain components together constitute the theoretical definition.<br>For example, synovial hypertrophy and synovial hyperaemia are the domain components that can be measured, and together define<br>the target domain of synovitis as assessed by ultrasound. | 9.1±1.1 |
| | Operational definition of target domain component | The ultrasound definition of a target domain component<br>(ie, the 'signal' that can be detected by ultrasound).<br>For example, synovial hypertrophy is defined as hypoechoic thickening of the synovium; and synovial hyperaemia as increased Doppler signal within the synovial hypertrophy. | 8.9±2.2 |
| 8 | Anatomical region | The region of the body which is the focus of the ultrasound examination; it may include more than one related structure.<br>For example, muscles, nerves and arteries, or joint cavity and tendons. | 9.9±0.2 |
| | Anatomical structure | Isolated tissue(s) or organ(s) which is/are examined by ultrasound.<br>For example, synovium, bone, tendon, muscle. | 9.6±1.3 |
| 9 | Dynamic examination | Procedure in which the transducer is moved along the anatomical region under study; or the anatomical region is moved during the ultrasound examination, for example through muscle contraction or tendon movement. | 9.4±1.3 |
| 11,12 | Reliability | The degree to which the measurement is free from measurement error. | 9.1±1.4 |
| 11,12,13 | Reader | The person who is reading (ie, interpreting) the ultrasound images or the video-clips of the examination. This interpretation may take place at the same time as the acquisition of the ultrasound images/video-clips, or later.<br>In the latter case, the reader may be the same person who performed the ultrasound examination and the acquisition of the images/video-clips or a different person. | 9.9±0.3 |
| | Sonographer | The person performing the ultrasound evaluation.<br>Usually the sonographer is a health professional with the appropriate skills to perform an ultrasound examination in RMDs. | 9.7±0.9 |
| 16 | Ultrasound modalities | The ultrasound technique(s) used, that is, grey scale mode (or B mode),<br>Doppler (colour, power, pulse), elastography, contrast-enhanced ultrasound, etc. | 9.9±0.2 |

RMD, rheumatic and musculoskeletal disease.

the acquisition and the interpretation are a mandatory reporting requirement. Item 14 on the experience of sonographer(s) and/or reader(s) is optional, mainly because no consensus exists on how to report such experience. EULAR and American College of Rheumatology suggest a competency assessment in ultrasound to improve the quality of the examination.[30 31]

### Equipment

Technical characteristics of the imaging device (item 15), ultrasound modalities and settings used (item 16) may affect the intrasonographer and intersonographer/reader reliability and generalisability of the results. A second scoping review (online supplemental file 2) addressed this question, that is, whether the ultrasound device (model, age, acquisition software, transducers and settings) affect the reliability or accuracy of the ultrasound examination. We found no study investigating the influence of device age or software on ultrasound results. However, seven studies assessed the influence of the machine (eg, ultrasound device, transducer frequency, settings) on ultrasound results whatever the anatomical site studied, the ultrasound modality used (ie, Doppler, GS) or the target pathology under study (eg, joint effusion, synovitis, erosion).[32–38] Three studies used a phantom to compare the ultrasound devices.[34 36 37] Five of the seven studies showed differences in the performance between machines and therefore an influence on the study results.[35 36] However, in these studies, the relevance and the magnitude of such differences were reported, but no sensitivity analysis was conducted.

### Images, pictures and figures

Since ultrasound is a tomographic imaging modality, the appearance of the structures may change following the orientation and position of the transducer. Standardised images should always be presented (item 17) so that the reader can easily recognise the anatomical structures as well as the target pathology and elementary lesions described in the study. The use of drawings can facilitate the interpretation of the images for readers not experienced in ultrasound. Images should never contain patient information and should be accompanied by clear legends and points of reference.

### Contextual factors

Item 18 deals with the feasibility of ultrasound, in particular, the time necessary for the evaluation, which depends on the number of sites (or joints) examined and the number of ultrasound examinations performed over the duration of the study. Although these aspects are highly important for the acceptability of the technique, the taskforce members felt that time spent should be reported only if relevant to the study question.

Item 19 refers to additional potential sources of variability: ambient and patient conditions. This item was made optional because the third scoping review (online supplemental file 2) failed to find strong evidence of the influence of these factors on the ultrasound results. It reviewed the effect of three ambient conditions (room temperature, atmospheric pressure and time of the day) and five patient conditions (exercise, skin temperature, smoking, alcohol consumption and caffeine) on ultrasound measurements. There was a potential influence of time of the day on Doppler signal evaluation (with contradictory effects)[39 40] and on GS results[41]; and potential influences on Doppler signal following the application of cold (ice and cold water)[42–44] and after physical exercise.[45–47]

## DISCUSSION

This EULAR taskforce developed a recommendation checklist to ensure transparent and comprehensive reporting of ultrasound research and procedures aspects, which may affect the interpretation and generalisability of the results. The checklist consists of 23 items (21 'mandatory' and 2 'optional'), organised into 13 categories. Its organisation allows authors to choose the order and format for presenting information, depending on their preferences and on journal style. Content validity of the recommendations checklist was confirmed by a panel of external experts, who considered each item of the checklist an essential reporting point, crucial to make an informed judgement on the quality of the scientific report. Moreover, all items were considered comprehensible and the checklist as a whole was considered to comprehensively cover all relevant reporting issues.

Along with sufficient content validity of this checklist, additional strengths include the development process that followed EULAR SOPs for a stepwise consensual approach[16] and the guidance from the EQUATOR network,[17] also, the panel members reflected a wide range of expertise and stakeholders. In addition, agreement on comprehensiveness and comprehensibility of the checklist was obtained in the first round of voting for all items of the checklist and all definitions of the glossary.

A possible limitation of this project is the fact that the face-to-face meetings comprised mostly Europeans, with only one colleague from USA, and only one patient. We partially overcame this in the external Delphi panels, including more international experts, including several radiologists.

The checklist was purposefully focused and is complementary to other existing guidelines, depending on the study design. It has not been developed as a tool to assess the quality of published research; however, it can certainly serve as a basis to develop such a tool, and its use may improve the quality of studies, as seen with other reporting recommendations.[48 49] We hope that this reporting checklist will be widely adopted by authors and editors, which, in turn, will greatly improve the interpretability, reproducibility and generalisability of the study results.

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CLINICAL SCIENCE

# Filgotinib versus placebo or adalimumab in patients with rheumatoid arthritis and inadequate response to methotrexate: a phase III randomised clinical trial

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## ABSTRACT

**Objective** To evaluate the efficacy and safety of the Janus kinase-1-preferential inhibitor filgotinib versus placebo or tumour necrosis factor-α inhibitor therapy in patients with active rheumatoid arthritis (RA) despite ongoing treatment with methotrexate (MTX).

**Methods** This 52-week, multicentre, double-blind, placebo-controlled and active-controlled phase III trial evaluated once-daily oral filgotinib in patients with RA randomised 3:3:2:3 to filgotinib 200 mg (FIL200) or filgotinib 100 mg (FIL100), subcutaneous adalimumab 40 mg biweekly, or placebo (through week 24), all with stable weekly background MTX. The primary endpoint was the proportion of patients achieving 20% improvement in American College of Rheumatology criteria (ACR20) at week 12. Additional efficacy outcomes were assessed sequentially. Safety was assessed from adverse events and laboratory abnormalities.

**Results** The proportion of patients (n=1755 randomised and treated) achieving ACR20 at week 12 was significantly higher for FIL200 (76.6%) and FIL100 (69.8%) versus placebo (49.9%; treatment difference (95% CI), 26.7% (20.6% to 32.8%) and 19.9% (13.6% to 26.2%), respectively; both p<0.001). Filgotinib was superior to placebo in key secondary endpoints assessing RA signs and symptoms, physical function and structural damage. FIL200 was non-inferior to adalimumab in terms of Disease Activity Score in 28 joints with C reactive protein ≤3.2 at week 12 (p<0.001); FIL100 did not achieve non-inferiority. Adverse events and laboratory abnormalities were comparable among active treatment arms.

**Conclusions** Filgotinib improved RA signs and symptoms, improved physical function, inhibited radiographic progression and was well tolerated in patients with RA with inadequate response to MTX. FIL200 was non-inferior to adalimumab.

**Trial registration number** NCT02889796.

## Key messages

**What is already known about this subject?**

- Methotrexate (MTX) is the recommended initial treatment for rheumatoid arthritis, with tumour necrosis factor α inhibitors (TNFαi) as common second-line therapy in patients with inadequate response.
- Oral therapies that match or exceed TNFαi efficacy in this population are still needed.
- Filgotinib—a once-daily, oral, Janus kinase-1-preferential inhibitor—with or without MTX is superior relative to placebo treatment in patients with rheumatoid arthritis with inadequate response to MTX or prior biologic failure.

**What does this study add?**

- This is the first study to evaluate filgotinib compared with TNFαi standard therapy or placebo with stable background MTX in patients with rheumatoid arthritis with inadequate response to MTX but without prior biologic failure, and to include a radiographic endpoint.
- Filgotinib treatment reduced rheumatoid arthritis signs and symptoms, improved physical function, inhibited radiographic progression and appeared well tolerated for up to 52 weeks in this population.

**How might this impact on clinical practice or future developments?**

- Filgotinib with background MTX could be considered a treatment option in patients with rheumatoid arthritis with inadequate response to MTX.

## INTRODUCTION

Scientific innovations have changed the landscape of rheumatoid arthritis (RA) treatment. The cornerstone of RA treatment remains disease-modifying antirheumatic drugs (DMARDs), including conventional synthetic DMARDs (csDMARDs), of which methotrexate (MTX) is the gold standard, and biologic DMARDs (bDMARDs) such as those targeting cytokines (eg, tumour necrosis factor α (TNFα), interleukin 6 or interleukin 1) and B or T

cells. Availability of TNFα inhibitors (TNFαi) in the late 1990s, non-TNFαi biologics in the 2000s and recently the targeted synthetic DMARDs has helped to reduce disease severity in patients with RA. Advances in RA management have further improved patient outcomes by focusing on treat-to-target strategies, pain and inflammation reduction, and administration convenience, in addition to efficacy and safety.[1 2] Despite this focus, many patients do not achieve long-term responses with currently available therapies[3]; in one study, only 10%–21% of patients initiating csDMARDs and 12%–24% initiating TNFαi therapy achieved remission within 12 months.[4] Potential innovations that may further improve patient outcomes in RA include new oral therapies that perform as well as, or better than, existing standard of care (SOC), particularly in patients with intolerance or inadequate response to bDMARDs (bDMARD-IR).

The FINCH phase 3 programme was developed to study filgotinib, a Janus-associated kinase (JAK)-1-preferential inhibitor, for RA treatment. In FINCH 2, filgotinib significantly improved efficacy versus placebo in bDMARD-IR patients with active RA.[5] FINCH 3 examined filgotinib use in patients with MTX-naïve RA. To address the MTX-IR population, the FINCH 1 study examined filgotinib versus placebo or adalimumab, all with background MTX, in MTX-IR patients with active RA.

## METHODS

### Study design and conduct

This randomised, double-blind, 52-week, placebo-controlled and active-controlled phase III trial was conducted at 303 sites in 30 countries from 30 August 2016 to 20 June 2019. The protocol and statistical analysis plan are provided in online supplemental files 1–3. All patients provided written informed consent. An independent data monitoring committee reviewed safety data periodically. An independent adjudication committee periodically reviewed all potential major cardiovascular adverse events (MACE) and thromboembolic events.

### Study participants

Eligible patients were ≥18 years old at the time of consent and met the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism criteria for RA diagnosis.[6] Patients had active moderate-to-severe RA, defined as ≥6 swollen joints and ≥6 tender joints (both at screening and on day 1 despite ongoing MTX treatment for ≥12 weeks and stable at 7.5–25 mg/week for ≥4 weeks). Additional inclusion criteria were seropositivity for anticyclic citrullinated peptide (anti-CCP) antibodies or rheumatoid factor (RF); ≥1 joint erosion on hand/wrist and foot radiographs, or ≥3 erosions if negative for RF and anti-CCP; or serum C reactive protein (CRP) ≥6 mg/L. Key exclusion criteria included previous use of JAK inhibitors (JAKi) or adalimumab, prior non-response or intolerance to any bDMARD, and recent use of csDMARDs other than MTX or stably dosed hydroxychloroquine or chloroquine; concomitant, stably dosed non-steroidal anti-inflammatory drugs or glucocorticoids (≤10 mg/day prednisone/equivalent) were permitted.

### Interventions

Eligible patients were randomly assigned (3:3:2:3) to oral filgotinib 200 mg (FIL200) or filgotinib 100 mg (FIL100) once daily, subcutaneous adalimumab 40 mg every 2 weeks, or placebo, all with stable background MTX; other concomitant medications were to be kept stable as much as possible. Study participants were blinded to treatment and received placebo tablets matching FIL200 and/or FIL100; patients not assigned to active adalimumab received matching placebo injections. At week 24, placebo-treated patients were rerandomised (1:1) to FIL200 or FIL100 and continued background MTX. Per protocol, patients without adequate treatment response (<20% improvement from baseline in either swollen joint count 66 or tender joint count 68) at week 14 or two consecutive visits after week 30 discontinued study treatment but continued study visits, using investigator-specified SOC RA therapy.

### Endpoints and assessments

The primary efficacy endpoint was ACR20 response (20% improvement in ACR criteria)[7] at week 12. Key secondary efficacy endpoints tested hierarchically at week 12 (unless otherwise specified) were change from baseline score on the Health Assessment Questionnaire-Disability Index (HAQ-DI),[8 9] proportion of patients with Disease Activity Score in 28 joints with CRP (DAS28(CRP)) <2.6,[10] change from baseline van der Heijde modified total Sharp score (mTSS)[11] at week 24 (radiographic assessment details in online supplemental methods), non-inferiority of filgotinib versus adalimumab for a proportion of patients with DAS28(CRP) ≤3.2, change from baseline Short Form-36 Physical Component Summary[12] and Functional Assessment of Chronic Illness Therapy-Fatigue score,[13] superiority of filgotinib versus adalimumab for a proportion of patients with DAS28(CRP) ≤3.2, non-inferiority of filgotinib versus adalimumab for a proportion of patients with DAS28(CRP) <2.6, and superiority of filgotinib versus adalimumab for a proportion of patients with DAS28(CRP) <2.6. Other secondary endpoints included ACR50/70; low disease activity defined as Clinical Disease Activity Index (CDAI) ≤10 or Simplified Disease Activity Index (SDAI) ≤11[14]; and remission defined as CDAI ≤2.8, SDAI ≤3.3 or Boolean remission.[15] Safety was assessed from laboratory tests and adverse events (AEs). Positively adjudicated MACE and thromboembolic events were reported.

### Statistical analysis

A sample size of 450 patients per filgotinib and placebo group was estimated to provide >90% power at a two-sided α of 0.05 to test the superiority of FIL200 versus placebo for change from baseline mTSS at week 24, based on other RA studies with radiography.[16–18] This sample size also provided >95% power to detect a 20% difference in ACR20 for filgotinib versus placebo. Assuming similar DAS28(CRP) ≤3.2 response rates for filgotinib and adalimumab, approximately 300 adalimumab-treated patients were required to ensure >90% power at a two-sided α of 0.05 to demonstrate non-inferiority of FIL200 versus adalimumab. Consistent with regulatory guidance, non-inferiority assessments were based on the method of Liu *et al*,[19] which does not require a prespecified fixed non-inferiority margin or constancy and assay sensitivity assumptions.[20] Non-inferiority testing assessed whether the effect of each filgotinib dose (response rate difference between filgotinib and placebo) preserves >50% of the effect of adalimumab (difference in response rate between adalimumab and placebo). The 50% non-inferiority margin of DAS28(CRP) ≤3.2 and <2.6 at weeks 12 and 24 based on FINCH 1 data are presented in online supplemental table S1.

Type I error rate was controlled by hierarchical testing of primary and key secondary endpoints at a two-sided α of 0.05 (online supplemental figure S1). The primary analysis tested the superiority of FIL200 versus placebo for ACR20 at week 12 using a logistic regression model, with treatment and stratification factors included as covariates. Hypothesis testing for

key secondary endpoints commenced only after the primary endpoint reached statistical significance and proceeded sequentially until a null hypothesis was not rejected, after which exploratory p values are reported for the remaining hypotheses.

All analyses were based on data from patients who received ≥1 dose of study drug. For binary endpoints, a logistic regression model including treatment and stratification factors (geographical region, prior exposure to bDMARDs, and RF or CCP antibody positivity at screening) was used. Treatment effect on continuous endpoint change from baseline was evaluated using a mixed-effects model for repeated measures, with treatment, visit, treatment by visit interaction, stratification factors and baseline value included as fixed effects and subject as a random effect. Patients who required rescue therapy or had missing values were defined as non-responders, and non-responder imputation (NRI) was employed for primary and key secondary binary endpoint analyses. Multiple imputation was conducted to determine the impact of NRI on the robustness of results (online supplemental methods and table S2).[21 22] Safety analyses of AEs and laboratory data were summarised by treatment group using descriptive statistics.

### Patient and public involvement

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

## RESULTS

### Study participants

A total of 1755 patients received study treatment (enrolment by country; online supplemental figure S2), and 87.4% completed the study visits through the 24-week placebo-controlled period. The reasons for discontinuation are summarised in figure 1. At week 14, 4.8% of FIL200-treated, 6.0% of FIL100-treated, 4.0% of adalimumab-treated and 8.6% of placebo-treated patients had inadequate response to treatment and were mandated to SOC. After week 24, four patients receiving FIL200, three receiving FIL100, three receiving adalimumab and two in each placebo-to-filgotinib arm discontinued study drug due to lack of efficacy. Baseline demographics, concomitant medications and disease characteristics were similar among the treatment arms (table 1).

### Efficacy

ACR20 responses at week 12 were significantly greater in patients receiving filgotinib versus placebo: 76.6% for FIL200 and 69.8% for FIL100 vs 49.9% for placebo (all p<0.001) (table 2, figure 2A). Significant improvements at week 12 with filgotinib versus placebo treatment were also observed in key secondary endpoints, including HAQ-DI and DAS28(CRP) <2.6 (all p<0.001) (table 2). Radiographic progression of structural joint damage was significantly reduced in both filgotinib dose arms versus placebo at week 24 (p<0.001 for FIL200; p=0.001 for FIL100) (figure 3). FIL200 was non-inferior to adalimumab at week 12 for DAS28(CRP) ≤3.2 (p<0.001); FIL100 did not

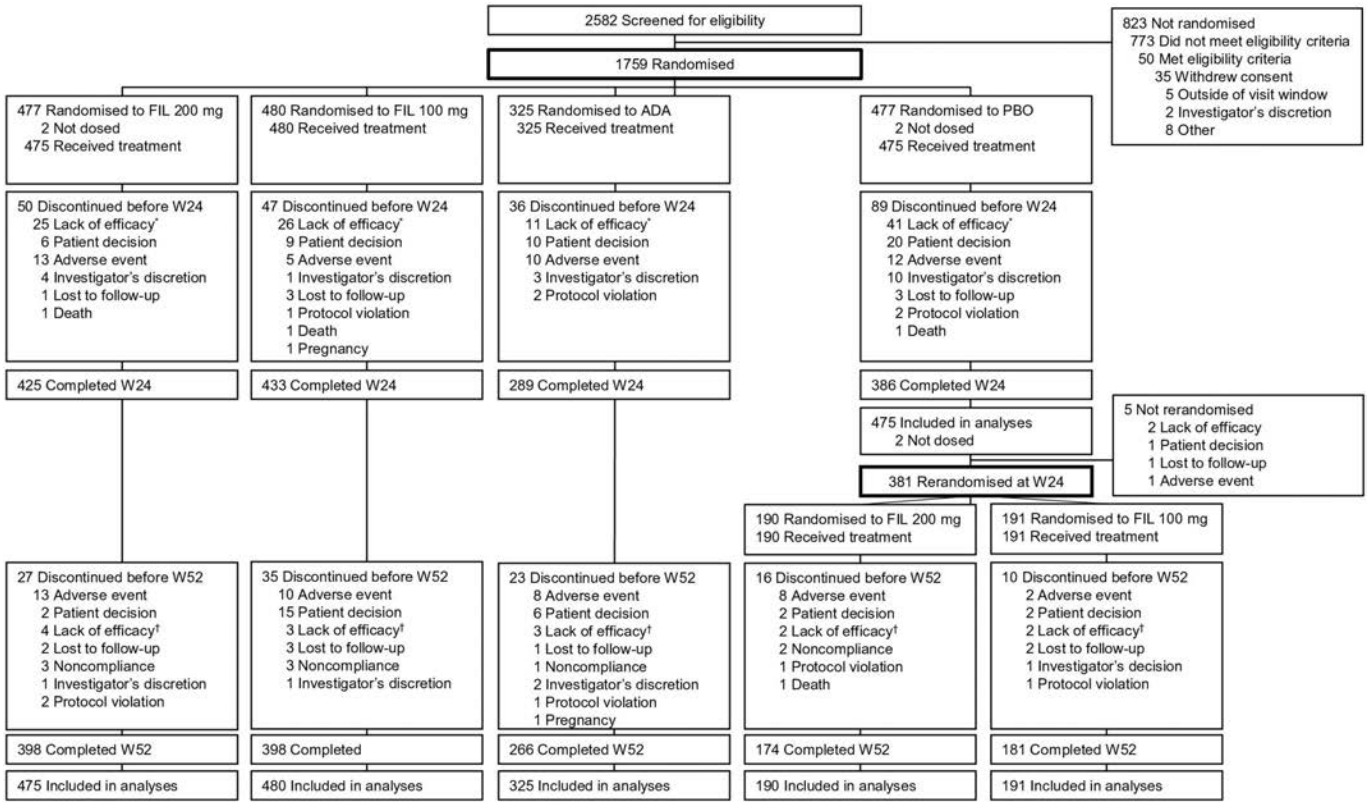


**Figure 1** Patient disposition. *23 (4.8%) patients treated with filgotinib 200 mg, 29 (6.0%) patients treated with filgotinib 100 mg, 13 (4.0%) patients treated with adalimumab, and 41 (8.6%) patients treated with placebo did not have adequate response to treatment per protocol at week 14. †3 (0.7%) patients treated with filgotinib 200 mg, 2 (0.5%) patients treated with filgotinib 100 mg, 3 (1.0%) patients treated with adalimumab, 0 patient treated with placebo and rerandomised to filgotinib 200 mg at week 24, and 4 (2.2%) patients treated with placebo and rerandomised to filgotinib 100 mg at week 24 failed to maintain response to treatment per protocol after week 30. ADA, adalimumab; FIL, filgotinib; PBO, placebo; W, week.

**Table 1** Baseline demographics and disease characteristics

| | FIL200 (n=475) | FIL100 (n=480) | ADA (n=325) | PBO (n=475) | Total (N=1755) |
|---|---|---|---|---|---|
| Sex at birth, n (%), female | 379 (79.8) | 399 (83.1) | 266 (81.8) | 391 (82.3) | 1435 (81.8) |
| Age, years | 52±12.8 | 53±12.6 | 53±12.9 | 53±12.8 | 53±12.7 |
| Weight, kg | 70.6±17.5 | 69.9±16.9 | 71.5±17.4 | 70.6±16.8 | 70.6±17.1 |
| Body mass index, kg/m$^2$ | 26.7±5.7 | 26.4±5.8 | 26.9±6.0 | 27.0±5.9 | 26.7±5.8 |
| Race, n (%) | | | | | |
| White | 312 (65.7) | 324 (67.5) | 229 (70.5) | 319 (67.2) | 1184 (67.5) |
| Asian | 122 (25.7) | 115 (24.0) | 65 (20.0) | 109 (22.9) | 411 (23.4) |
| American Indian/Alaska Native | 27 (5.7) | 27 (5.6) | 20 (6.2) | 29 (6.1) | 103 (5.9) |
| Black/African American | 6 (1.3) | 7 (1.5) | 10 (3.1) | 12 (2.5) | 35 (2.0) |
| Other* | 8 (1.7) | 6 (1.3) | 1 (0.3) | 5 (1.1) | 20 (1.1) |
| Not permitted | 0 | 1 (0.2) | 0 | 1 (0.2) | 2 (0.1) |
| Ethnicity, n (%) | | | | | |
| Not Hispanic or Latino | 404 (85.1) | 399 (83.1) | 268 (82.5) | 400 (84.2) | 1471 (83.8) |
| Duration of RA diagnosis, years | 7.3±7.4 | 8.5±8.2 | 8.0±7.4 | 7.3±7.2 | 7.8±7.6 |
| hsCRP, mg/L | 16.1±21.0 | 16.7±23.0 | 14.6±18.0 | 16.3±24.1 | 16.0±21.9 |
| Median (Q1, Q3) | 8.8 (3.6, 21.2) | 9.0 (3.9, 20.7) | 8.0 (3.4, 17.2) | 7.5 (3.3, 19.8) | 8.2 (3.6, 19.9) |
| ≥6 mg/L, n (%) | 298 (62.7) | 295 (61.5) | 197 (60.6) | 274 (57.7) | 1064 (60.6) |
| RF-positive, n (%) | 352 (74.1) | 362 (75.4) | 241 (74.2) | 365 (76.8)† | 1320 (75.2)† |
| Anti-CCP-positive, n (%) | 380 (80.0) | 381 (79.4) | 253 (77.8)‡ | 378 (79.6) | 1392 (79.3)‡ |
| RF and anti-CCP positive, n (%) | 331 (69.7) | 332 (69.2) | 219 (67.4)‡ | 333 (70.1)† | 1215 (69.2)†‡ |
| mTSS units§ | 32.5±47.9 | 36.7±53.1 | 34.8±55.0 | 31.6±53.2 | 33.8±52.1 |
| Median (Q1, Q3) | 12.0 (2.0, 43.5) | 13.0 (2.5, 52.5) | 12.5 (2.0, 43.5) | 11.5 (2.0, 37.0) | 12.0 (2.0, 43.5) |
| Erosion score >0, n (%)¶ | 399 (84.0) | 411 (85.6) | 277 (85.2) | 404 (85.1) | 1491 (85.0) |
| JSN score | 18.5±25.6 | 19.9±27.3 | 19.6±28.2 | 17.6±26.9 | 18.8±26.9 |
| bDMARD-naïve, n (%) | 458 (96.4) | 464 (96.7) | 317 (97.5) | 469 (98.7) | 1708 (97.3) |
| MTX dose, mg/week** | 15.3±4.9 | 15.5±4.8 | 15.4±4.8 | 14.9±4.5 | 15.3±4.8 |
| Concurrent oral steroids, n (%) | 229 (48.2) | 229 (47.7) | 140 (43.1) | 217 (45.7) | 815 (46.4) |
| ≤5 mg/day, n (%)†† | 152 (66.4) | 160 (69.9) | 96 (68.6) | 152 (70.0) | 560 (68.7) |
| Steroid dose, mg/day‡‡ | 6.2±3.4 | 6.1±2.5 | 5.9±2.2 | 5.9±2.5 | 6.0±2.8 |
| Concurrent antimalarials, n (%) | 64 (13.5) | 59 (12.3) | 39 (12.0) | 63 (13.3) | 225 (12.8) |
| DAS28(CRP) | 5.8±0.9 | 5.7±1.0 | 5.7±0.9 | 5.7±0.9 | 5.7±0.9 |
| SDAI | 41.2±12.3 | 40.2±12.8 | 40.6±11.9 | 41.2±12.4 | 40.8±12.4 |
| CDAI | 39.5±11.9 | 38.6±12.2 | 39.2±11.5 | 39.6±11.7 | 39.2±11.8 |
| SJC66 | 15±8.5 | 15±8.5 | 16±8.4 | 16±8.5 | 16±8.5 |
| TJC68 | 25±13.5 | 25±13.4 | 24±13.2 | 24±13.5 | 24±13.4 |
| SGA, VAS, mm | 67±19.2 | 65±19.7 | 67±19.1 | 68±18.7 | 67±19.2 |
| PGA, VAS, mm | 66±16.0 | 65±16.5 | 67±15.5 | 66±16.2 | 66±16.1 |
| Pain, VAS, mm | 65±20.4 | 64±20.1 | 64±19.5 | 66±19.0 | 65±19.8 |
| HAQ-DI | 1.6±0.6 | 1.6±0.6 | 1.6±0.6 | 1.6±0.6 | 1.6±0.6 |
| SF-36 PCS§§ | 33.4±7.2 | 33.6±7.8 | 32.8±7.7 | 32.9±7.1 | 33.2±7.4 |
| SF-36 MCS¶¶ | 43.9±10.4 | 44.6±10.4 | 44.1±10.4 | 43.4±11.0 | 44.0±10.6 |
| FACIT-F¶¶ | 27.6±10.7 | 27.8±10.6 | 27.2±10.2 | 26.9±10.3 | 27.4±10.5 |

Values are mean±SD.
*Includes patients recorded as Native Hawaiian/Pacific Islander and 'Other'. Race was not recorded for one patient receiving FIL100 and one patient receiving PBO due to local regulations.
†n=1 missing.
‡n=2 missing.
§Campaign A: FIL200, n=467; FIL100, n=471; ADA, n=319; PBO, n=466.
¶Campaign A: FIL200, n=8 missing; FIL100, n=9 missing; ADA, n=6 missing; PBO, n=9 missing.
**FIL100, n=479; ADA, n=324.
††Percent of patients with concurrent oral corticosteroid use on first dosing date.
‡‡FIL200, n=226; FIL100, n=229; ADA, n=140; PBO, n=217.
§§FIL200, n=473; FIL100, n=479; ADA, n=323; PBO, n=474.
¶¶FIL200, n=472; FIL100, n=477; ADA, n=319; PBO, n=469.
ADA, adalimumab; anti-CCP, anticyclic citrullinated protein antibody; bDMARD, biologic disease-modifying antirheumatic drug; CDAI, Clinical Disease Activity Index; DAS28(CRP), Disease Activity Score in 28 joints with C reactive protein; FACIT-F, Functional Assessment of Chronic Illness Therapy-Fatigue; FIL100, filgotinib 100 mg; FIL200, filgotinib 200 mg; HAQ-DI, Health Assessment Questionnaire-Disability Index; hsCRP, high-sensitivity C reactive protein; JSN, joint space narrowing; MCS, Mental Component Summary; mTSS, van der Heijde modified total Sharp score; MTX, methotrexate; PBO, placebo; PCS, Physical Component Summary; PGA, Physician's Global Assessment; Q1, first quartile; Q3, third quartile; RA, rheumatoid arthritis; RF, rheumatoid factor; SDAI, Simplified Disease Activity Index; SF-36, Short Form-36; SGA, Subject's Global Assessment; SJC66, swollen joint count of 66 joints; TJC68, tender joint count of 68 joints; VAS, visual analogue scale.

**Table 2** Primary and key secondary efficacy outcomes during the placebo-controlled period*

| | FIL200 (n=475) | FIL100 (n=480) | ADA (n=325) | PBO (n=475) |
|---|---|---|---|---|
| Primary outcome | | | | |
| ACR20, week 12 | | | | |
| n/N | 364/475 | 335/480 | 229/325 | 237/475 |
| % (95% CI) | 76.6 (72.7 to 80.5) | 69.8 (65.6 to 74.0) | 70.5 (65.3 to 75.6) | 49.9 (45.3 to 54.5) |
| Difference vs PBO (95% CI)† | 26.7 (20.6 to 32.8) | 19.9 (13.6 to 26.2) | 20.6 (13.6 to 27.5) | |
| P value vs placebo | <0.001 | <0.001 | <0.001‡ | |
| Key secondary outcomes with hierarchical testing | | | | |
| HAQ-DI change from baseline to week 12 | | | | |
| N | 457 | 459 | 311 | 435 |
| Mean±SD | −0.69±0.61 | −0.56±0.56 | −0.61±0.56 | −0.42±0.54 |
| Difference vs PBO (95% CI)† | −0.29 (−0.36 to −0.22) | −0.17 (−0.24 to −0.10) | −0.20 (−0.28 to −0.13) | |
| P value vs PBO | <0.001 | <0.001 | <0.001‡ | |
| DAS28(CRP) <2.6, week 12 | | | | |
| n/N | 162/475 | 114/480 | 77/325 | 44/475 |
| % (95% CI) | 34.1 (29.7 to 38.5) | 23.8 (19.8 to 27.7) | 23.7 (18.9 to 28.5) | 9.3 (6.6 to 12.0) |
| Difference vs PBO (95% CI)† | 24.8 (19.6 to 30.0) | 14.5 (9.7 to 19.3) | 14.4 (8.9 to 20.0) | |
| P value vs PBO | <0.001 | <0.001 | <0.001‡ | |
| mTSS change from baseline to week 24 | | | | |
| N | 405 | 404 | 271 | 351 |
| Mean±SD | 0.13±0.9 | 0.17±0.91 | 0.16±0.95 | 0.37±1.42 |
| Difference vs PBO (95% CI)† | −0.27 (−0.43 to −0.12) | −0.25 (−0.40 to −0.10) | −0.22 (−0.39 to −0.05) | |
| P value vs PBO | <0.001 | 0.001 | 0.012‡ | |
| Non-inferiority DAS28(CRP) ≤3.2, week 12 | | | | |
| n/N | 236/475 | 186/480 | 141/325 | 111/475 |
| % (95% CI) | 49.7 (45.1 to 54.3) | 38.8 (34.3 to 43.2) | 43.4 (37.8 to 48.9) | 23.4 (19.5 to 27.3) |
| P value vs ADA | <0.001 | 0.054 | | |
| Key secondary outcomes without multiplicity adjustment | | | | |
| SF-36 PCS change from baseline to week 12 | | | | |
| N | 459 | 463 | 310 | 440 |
| Mean±SD | 9.2±8.1 | 8.5±7.7 | 8.4±7.9 | 5.8±7.1 |
| Difference vs PBO (95% CI)† | 3.7 (2.8 to 4.6) | 3.1 (2.2 to 4.0) | 2.6 (1.6 to 3.6) | |
| Exploratory p value vs PBO | <0.001 | <0.001 | <0.001 | |
| FACIT-F change from baseline to week 12 | | | | |
| N | 452 | 455 | 304 | 432 |
| Mean±SD | 9.2±9.8 | 9.1±10.2 | 8.8±9.2 | 6.8±9.9 |
| Difference vs PBO (95% CI)† | 2.8 (1.7 to 3.9) | 2.6 (1.5 to 3.7) | 2.1 (0.9 to 3.3) | |
| Exploratory p value vs PBO | <0.001 | <0.001 | <0.001 | |
| Superiority DAS28(CRP) ≤3.2, week 12 | | | | |
| Difference vs ADA (95% CI)† | 6.3 (−1.0 to 13.6) | −4.6 (−11.8 to 2.6) | | |
| Exploratory p value vs ADA | 0.069 | 0.18 | | |
| Non-inferiority DAS28(CRP) <2.6, week 12 | | | | |
| Exploratory p value vs ADA | <0.001 | 0.002 | | |
| Superiority DAS28(CRP) <2.6, week 12 | | | | |
| Difference vs ADA (95% CI)† | 10.4 (3.9 to 17.0) | 0.1 (−6.2 to 6.3) | | |
| Exploratory p value vs ADA | 0.001 | 0.99 | | |

*Hierarchical testing according to prespecified, US Food and Drug Administration-reviewed, statistical analysis plan. Patients who had missing values were defined as non-responders, and NRI was employed for both primary and key secondary analyses.
†Difference in response rates vs placebo or ADA for categorical outcomes; least-squares mean difference vs placebo or ADA for continuous outcomes.
‡Exploratory p value without multiplicity adjustment.
ACR20, American College of Rheumatology criteria 20% decrease from baseline; ADA, adalimumab; DAS28(CRP), Disease Activity Score in 28 joints with C reactive protein; FACIT-F, Functional Assessment of Chronic Illness Therapy-Fatigue; FIL100, filgotinib 100 mg; FIL200, filgotinib 200 mg; HAQ-DI, Health Assessment Questionnaire-Disability Index; mTSS, van der Heijde modified total Sharp score; NRI, non-responder imputation; PBO, placebo; SF-36 PCS, Short Form 36 Physical Component Summary.

achieve non-inferiority versus adalimumab for this measure (p=0.054) (table 2).

The remaining key secondary endpoints were not adjusted for multiplicity and are presented as exploratory analyses (table 2). ACR50/70 responses at week 12 were higher following FIL200 (47.2%/26.1%), FIL100 (36.5%/18.5%) or adalimumab (35.1%/14.2%) compared with placebo (19.8%/6.7%) (figure 2B,C). Response rates for DAS28(CRP) ≤3.2 at week 12 were higher in both filgotinib dose arms and placebo (table 2). Patients receiving filgotinib achieved higher rates of

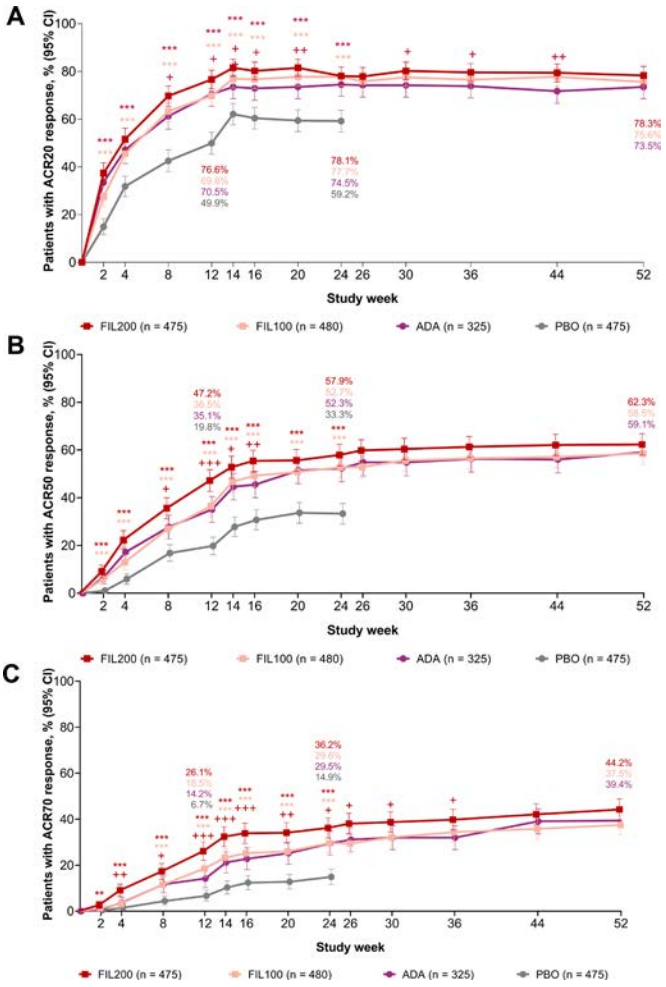


**Figure 2** Proportions of patients achieving (A) ACR20, (B) ACR50 and (C) ACR70 through week 52. Error bars show 95% CI. Additional statistical details are available in online supplemental table S3 and all response rates in online supplemental table S7. **p<0.01, ***p<0.001 versus PBO, not adjusted for multiplicity and should be considered exploratory except for ACR20 for FIL200 and FIL100 versus PBO at week 12. $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 versus ADA, not adjusted for multiplicity and should be considered exploratory. ACR20/50/70, 20%/50%/70% improvement from baseline by the American College of Rheumatology core criteria; ADA, adalimumab; FIL100, filgotinib 100 mg; FIL200, filgotinib 200 mg; PBO, placebo.

remission and low disease activity across several composite disease measures (DAS28(CRP), CDAI, SDAI, Boolean remission) versus placebo at weeks 12 and 24 (figure 4A,B). Filgotinib efficacy was sustained through week 52 (figures 2A–C and 4A,B, online supplemental tables S3 and S4, figure S3).

Changes from baseline in ACR and DAS28(CRP) components at week 12 were generally consistent with the primary and key secondary efficacy outcomes, although the effect of FIL versus adalimumab or placebo treatment was more pronounced for high-sensitivity CRP compared with other measures (online supplemental table S5). However, in post-hoc exploratory analyses, FIL200 was non-inferior to adalimumab for CDAI low disease activity and remission at weeks 12 and 24 (online supplemental table S3). In a subanalysis of proportion of patients achieving ACR20 at week 12 across countries, the placebo response rate ranged from 36.8% to 59.2% and was highest in group B (predominantly Eastern Europe) and group C (Mexico and Argentina) (online supplemental table S6).

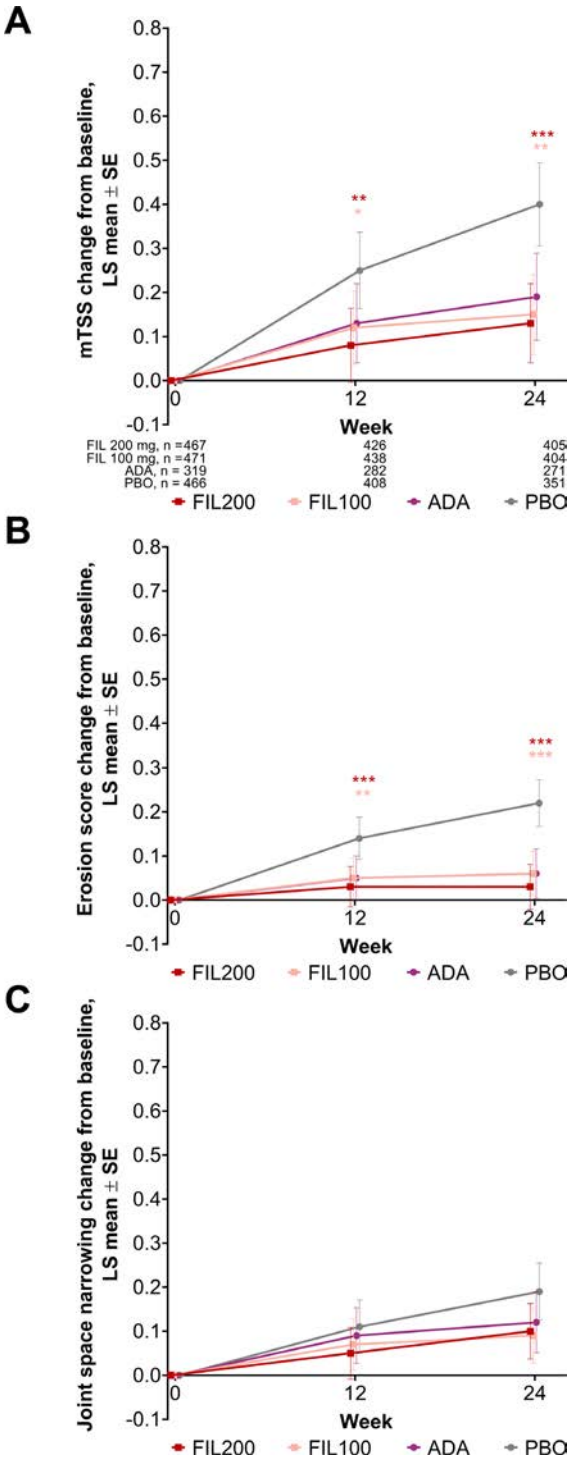


**Figure 3** Radiographic progression through week 24. (A) mTSS change from baseline, (B) erosion score change from baseline and (C) joint space narrowing change from baseline. Data from campaign A (through week 24) are shown. Supporting data are shown in online supplemental table S4. Patient numbers at each time point in (B) and (C) are the same as for (A). Error bars represent the SE of the LS mean. *p<0.05, **p<0.01, ***p<0.001 versus PBO, not adjusted for multiplicity and should be considered exploratory except for mTSS change from baseline following FIL200 and FIL100 versus PBO at week 24. Difference for mTSS change from baseline at week 24 following treatment with FIL200 or FIL100 versus ADA was explored and was not significant for either dose. ADA, adalimumab; FIL100, filgotinib 100 mg; FIL200, filgotinib 200 mg; LS, least-squares; mTSS, van der Heijde modified total Sharp score; PBO, placebo.

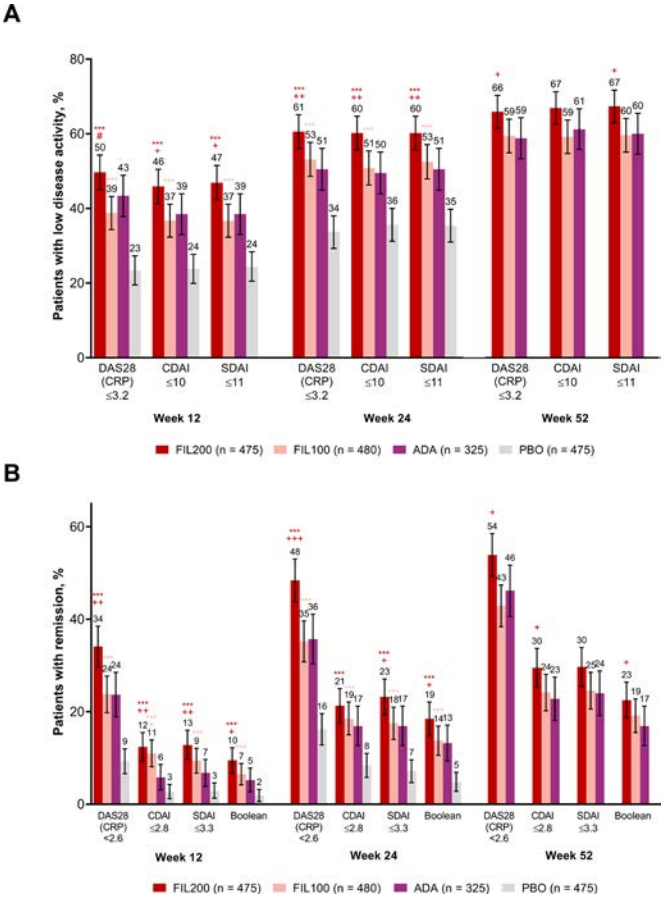


**Figure 4** Proportions of patients achieving (A) low disease activity and (B) DAS28(CRP) <2.6 or remission at weeks 12, 24 and 52. Error bars show 95% CI. Additional statistical details are available in online supplemental table S3. *p<0.05, **p<0.01, ***p<0.001 versus placebo, not adjusted for multiplicity and should be considered exploratory except for FIL200 and FIL100 versus placebo for DAS28(CRP) <2.6 at week 12. #Non-inferior versus adalimumab. +p<0.05, ++p<0.01, +++p<0.001 versus ADA, not adjusted for multiplicity and should be considered exploratory. ADA, adalimumab; Boolean, Boolean remission; CDAI, Clinical Disease Activity Index; DAS28(CRP), Disease Activity Score in 28 joints with C reactive protein; FIL100, filgotinib 100 mg; FIL200, filgotinib 200 mg; PBO, placebo; SDAI, Simplified Disease Activity Index.

## Safety

Treatment-emergent AEs (TEAEs) are presented in table 3. The incidence of serious TEAEs during the active-controlled period through week 52 was similar among all original active treatment arms and in patients rerandomised from placebo to filgotinib. During the placebo-controlled period, malignancy (excluding non-melanoma skin cancer) was reported in five patients: one (0.2%), one (0.3%) and three (0.6%) patients receiving FIL100, adalimumab and placebo, respectively. Venous thromboembolism (VTE) was reported in three patients: one (0.2%) receiving FIL200 and two (0.4%) receiving placebo. Adjudicated MACE occurred in four patients: one (0.2%) receiving FIL100, one (0.3%) receiving adalimumab and two (0.4%) receiving placebo. All patients with VTE and MACE had at least one risk factor, and no patient with deep vein thrombosis (DVT) or pulmonary embolism had a platelet count measurement above $600\times10^9$/L.

Through week 24, death was reported in five patients: two (0.4%) receiving FIL200 (both attributed to septic shock), one (0.2%) receiving FIL100 (myocardial infarction) and two (0.4%) receiving placebo (one toxic reaction to amoxicillin/clavulanic acid and one non-TEAE septic shock). Four additional deaths occurred in the active-controlled period: one patient receiving FIL200 (alveolitis), one receiving adalimumab (sepsis), one placebo-treated patient rerandomised to FIL200 (acute DVT) and one placebo-treated patient rerandomised to FIL100 (primary varicella). Additional details of the DVT-associated and primary varicella-associated deaths are provided in online supplemental results.

Overall, infectious and serious infectious TEAEs occurred more frequently in patients receiving filgotinib or adalimumab versus placebo through week 24. Serious infections occurring in >2 patients were pneumonia (13 patients), cellulitis (3 patients) and bronchitis (3 patients). Through week 24, herpes zoster (excluding primary varicella) occurred in all treatment arms in 0.4% of patients receiving either filgotinib dose or placebo and in 0.6% of patients receiving adalimumab. Through week 52, serious infections occurred in 2.7%, 2.7% and 3.1% and herpes zoster occurred in 1.3%, 0.8% and 0.6% of patients receiving FIL200, FIL100 and adalimumab, respectively. In 14% of patients randomised in Asia (online supplemental figure S2), the frequency of herpes zoster was 1%, 3% and 0% for patients receiving FIL200, FIL100 and adalimumab, respectively, through week 52, and 2% in placebo-treated patients through week 24. Both reported opportunistic infections were in patients receiving adalimumab: one patient with *Pneumocystis jirovecii* pneumonia before week 24 and one patient with active *Mycobacterium tuberculosis* after week 24.

Grade 3/4 changes in laboratory values are shown in table 4. Mean haemoglobin levels were stable or increased across all treatment arms, with no imbalance in individual decreased haemoglobin events or grade 3 changes. Decreases in neutrophil and lymphocyte levels were seen in filgotinib-treated and adalimumab-treated patients. Grade ≥3 lymphopaenia and neutropaenia were more frequent in patients receiving filgotinib versus placebo. The majority of white cell count abnormalities were grade 1/2, not associated with infection, and resolved at follow-up testing. No grade ≥3 changes in platelet counts were observed. Higher mean creatinine levels were observed in patients receiving filgotinib versus adalimumab or placebo. Grade 3/4 serum creatinine elevations were reported in three patients: one receiving FIL100 and two receiving placebo, all before week 24. Mean creatine kinase and low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol levels were increased in patients treated with filgotinib versus placebo, without meaningful change in the LDL to HDL cholesterol ratio.

## DISCUSSION

The FINCH 1 study assessed filgotinib, an oral JAK-1-preferential inhibitor, to address the unmet needs for RA treatment in MTX-IR patients. Two doses of filgotinib were compared with adalimumab and placebo, all with background MTX. Both filgotinib doses were superior to placebo for ACR20 response and hierarchical key secondary endpoints evaluating signs and symptoms, physical function and structural damage. Although conclusions are limited for tests without multiplicity adjustment, proportions of patients achieving various measures of low disease activity and remission were generally consistent with DAS28(CRP) <2.6 and DAS28(CRP) ≤3.2 response results.

These phase III results confirm those of two phase II studies investigating filgotinib with or without MTX versus placebo in MTX-IR patients[23 24] and a phase III study (FINCH 2) in bDMARD-refractory patients,[5] and are consistent with the

**Table 3** Treatment-emergent adverse events through week 24 and week 52

| | PBO-controlled period (weeks 0–24) | | | | Weeks 0–52 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | PBO | | |
| | FIL200 (n=475) | FIL100 (n=480) | ADA (n=325) | PBO (n=475) | FIL200 (n=475) | FIL100 (n=480) | ADA (n=325) | On FIL200 period (n=190) | On FIL100 period (n=191) | On PBO period (n=475) |
| TEAEs, n (%) | | | | | | | | | | |
| Any TEAE | 287 (60.4) | 287 (59.8) | 186 (57.2) | 252 (53.1) | 352 (74.1) | 350 (72.9) | 239 (73.5) | 92 (48.4) | 97 (50.8) | 254 (53.5) |
| TE SAE | 21 (4.4) | 24 (5.0) | 14 (4.3) | 20 (4.2) | 35 (7.4) | 40 (8.3) | 22 (6.8) | 7 (3.7) | 8 (4.2) | 21 (4.4) |
| TEAE leading to treatment discontinuation | 15 (3.2) | 9 (1.9) | 13 (4.0) | 15 (3.2) | 26 (5.5) | 15 (3.1) | 18 (5.5) | 6 (3.2) | 2 (1.0) | 15 (3.2) |
| Deaths | 2 (0.4) | 1 (0.2) | 0 | 2 (0.4) | 3 (0.6) | 1 (0.2) | 1 (0.3) | 1 (0.5) | 1 (0.5) | 2 (0.4) |
| TEAEs in >5% of patients* | | | | | | | | | | |
| Nasopharyngitis | 31 (6.5) | 29 (6.0) | 15 (4.6) | 25 (5.3) | 43 (9.1) | 48 (10.0) | 24 (7.4) | 7 (3.7) | 6 (3.1) | 25 (5.3) |
| URTI | 25 (5.3) | 33 (6.9) | 17 (5.2) | 14 (2.9) | 41 (8.6) | 49 (10.2) | 21 (6.5) | 8 (4.2) | 6 (3.1) | 14 (2.9) |
| ALT increased | 13 (2.7) | 15 (3.1) | 14 (4.3) | 11 (2.3) | 17 (3.6) | 25 (5.2) | 22 (6.8) | 7 (3.7) | 3 (1.6) | 11 (2.3) |
| AST increased | 9 (1.9) | 14 (2.9) | 11 (3.4) | 9 (1.9) | 12 (2.5) | 20 (4.2) | 18 (5.5) | 8 (4.2) | 3 (1.6) | 9 (1.9) |
| Nausea | 19 (4.0) | 10 (2.1) | 4 (1.2) | 7 (1.5) | 26 (5.5) | 16 (3.3) | 6 (1.8) | 4 (2.1) | 1 (0.5) | 7 (1.5) |
| Urinary tract infection | 11 (2.3) | 8 (1.7) | 8 (2.5) | 5 (1.1) | 19 (4.0) | 20 (4.2) | 17 (5.2) | 10 (5.3) | 8 (4.2) | 6 (1.3) |
| TEAEs of special interest | | | | | | | | | | |
| Infectious AEs | 133 (28.0) | 128 (26.7) | 88 (27.1) | 105 (22.1) | 206 (43.4) | 194 (40.4) | 129 (39.7) | 45 (23.7) | 39 (20.4) | 106 (22.3) |
| Serious infectious AEs | 8 (1.7) | 8 (1.7) | 8 (2.5) | 4 (0.8) | 13 (2.7) | 13 (2.7) | 10 (3.1) | 1 (0.5) | 2 (1.0) | 4 (0.8) |
| Herpes zoster | 2 (0.4) | 2 (0.4) | 2 (0.6) | 2 (0.4) | 6 (1.3) | 4 (0.8) | 2 (0.6) | 2 (1.1) | 1 (0.5) | 2 (0.4) |
| Hepatitis B or C | 0 | 0 | 1 (0.3) | 0 | 1 (0.2) | 1 (0.2) | 1 (0.3) | 1 (0.5) | 1 (0.5) | 0 |
| Opportunistic infections | 0 | 0 | 1 (0.3) | 0 | 0 | 0 | 2 (0.6) | 0 | 0 | 0 |
| Active tuberculosis | 0 | 0 | 0 | 0 | 0 | 0 | 1 (0.3) | 0 | 0 | 0 |
| MACE† | 0 | 1 (0.2) | 1 (0.3) | 2 (0.4) | 0 | 2 (0.4) | 1 (0.3) | 1 (0.5) | 1 (0.5) | 2 (0.4) |
| Malignancy | | | | | | | | | | |
| Excluding NMSC | 0 | 1 (0.2) | 1 (0.3) | 3 (0.6) | 2 (0.4) | 2 (0.4) | 2 (0.6) | 0 | 0 | 3 (0.6) |
| NMSC | 0 | 0 | 0 | 0 | 1 (0.2) | 1 (0.2) | 0 | 0 | 0 | 0 |
| VTE† | 1 (0.2) | 0 | 0 | 2 (0.4) | 1 (0.2) | 0 | 1 (0.3) | 1 (0.5) | 0 | 2 (0.4) |
| GI perforation | 0 | 0 | 0 | 0 | 1 (0.2) | 0 | 0 | 0 | 0 | 0 |

*TEAEs occurring in >5% of patients in a single treatment arm during either study period.
†Positively adjudicated.
ADA, adalimumab; AE, adverse event; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FIL100, filgotinib 100 mg; FIL200, filgotinib 200 mg; GI, gastrointestinal; MACE, major adverse cardiac event; NMSC, non-melanoma skin cancer; PBO, placebo; SAE, serious AE; TE, treatment-emergent; TEAE, treatment-emergent AE; URTI, upper respiratory tract infection; VTE, venous thromboembolism.

results for other JAKis in MTX-IR patients with RA.[18 25 26] FIL200 efficacy was statistically non-inferior to adalimumab for a proportion of patients with DAS28(CRP) ≤3.2 at week 12, a treat-to-target checkpoint,[2 15] and remained non-inferior in exploratory analyses of CDAI low disease activity and remission, suggesting direct effects of JAK inhibition on high-sensitivity CRP did not impact FIL200 treatment effect. FIL100 did not attain statistical significance for non-inferiority to adalimumab for DAS28(CRP) ≤3.2 at week 12 in hierarchical testing, resulting in loss of multiplicity adjustment for subsequent comparisons and limiting possible conclusions. Filgotinib efficacy generally compared favourably with adalimumab, consistent with a recent systematic review on the efficacy of bDMARDs and JAKis in RA.[27]

Filgotinib benefits must be considered in the context of risks. In this study, serious TEAEs and discontinuations due to TEAEs were similar among treatment arms through week 24. Safety data remained consistent over the entire 52-week study. Adjudicated MACE and VTE were observed in all treatment arms at frequencies similar to reported background rates in patients with RA[28 29]; VTE remains a concern for the JAKi class.[30] Infections were increased in patients treated with FIL200 versus placebo, with similar rates of serious infections across active treatment groups. The frequency of herpes zoster was low and similar across all groups through week 24; the number of uncomplicated cases increased slightly after week 24 in the filgotinib versus adalimumab treatment arms. The low frequency of herpes zoster does not appear attributable to geography; the proportion of FINCH 1 patients enrolled in Asian countries (14%) was comparable relative to similar JAKi studies (3%–18%).[25 26 31] No cases of opportunistic infection or tuberculosis were observed in filgotinib-treated patients. Rates of AEs in filgotinib-treated patients were consistent with or below those from a recent meta-analysis on JAKi treatment in RA.[32]

Filgotinib was associated with decreases in neutrophil, lymphocyte and platelet counts and increases in lipid, creatine kinase and creatinine levels, as previously reported for filgotinib and other JAKis.[5 18 23–26] There were small numerical differences in frequencies of grade 3/4 neutropaenia and lymphopaenia in patients treated with filgotinib versus placebo. Treatment with filgotinib was associated with small increases in fasting total, LDL and HDL cholesterol without affecting fasting LDL to HDL ratio, consistent with the hypothesis that JAKi treatment suppresses elevated cholesterol ester catabolism in patients with active RA and normalises their cholesterol levels towards the range in healthy volunteers.[33]

**Table 4** Laboratory values and grade ≥3 abnormalities through week 24 and week 52

| | PBO-controlled period (weeks 0–24) | | | | Weeks 0–52 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | PBO | | |
| | FIL200 (n=475) | FIL100 (n=480) | ADA (n=325) | PBO (n=475) | FIL200 (n=475) | FIL100 (n=480) | ADA (n=325) | On FIL200 period (n=190) | On FIL100 period (n=191) | On PBO period (n=475) |
| Haemoglobin, g/L | 2 (11) | 1 (10) | 2 (10) | 0 (9) | 5 (11) | 3 (11) | 5 (10) | 5 (9) | 2 (9) | NA |
| Grade 3, n (%) | 2 (0.4) | 3 (0.6) | 2 (0.6) | 4 (0.9) | 4 (0.8) | 5 (1.0) | 3 (0.9) | 0 | 0 | 4 (0.9) |
| Neutrophils, $10^9$/L | −1.0 (1.9) | −0.9 (2.0) | −1.2 (2.0) | −0.2 (1.9) | −1.0 (2.0) | −0.9 (1.9) | −1.3 (2.3) | −0.8 (1.8) | −0.5 (1.7) | NA |
| Grade 3 or 4, n (%) | 5 (1.1) | 5 (1.0)* | 1 (0.3) | 2 (0.4) | 5 (1.1) | 6 (1.3) | 1 (0.3) | 0 | 1 (0.5) | 2 (0.4) |
| Lymphocytes, $10^9$/L | −0.1 (0.6) | −0.1 (0.6) | 0.3 (0.6) | −0.1 (0.5) | −0.2 (0.6) | −0.1 (0.5) | 0.4 (0.6) | −0.1 (0.5) | −0.0 (0.6) | NA |
| Grade 3 or 4†, n (%) | 11 (2.3)* | 6 (1.3) | 2 (0.6) | 3 (0.6) | 15 (3.2) | 11 (2.3) | 3 (0.9) | 4 (2.1) | 2 (1.1) | 3 (0.6) |
| Platelets, $10^9$/L | −30 (61.0) | −28 (62.4) | −34 (63.8) | −8 (65.3) | −26 (66.8) | −31 (56.6) | −31 (70.9) | −17 (59.2) | −7 (65.2) | NA |
| ALT, U/L | 6 (23.8) | 4 (20.7) | 6 (19.2) | 2 (19.2) | 6 (33.0) | 6 (23.7) | 6 (18.7) | 5 (25.3) | 2 (18.3) | NA |
| Grade 3 or 4, n (%) | 3 (0.6) | 4 (0.8) | 6 (1.9) | 5 (1.1) | 9 (1.9) | 8 (1.7) | 8 (2.5) | 2 (1.1) | 0 | 5 (1.1) |
| AST, U/L | 6 (16.8) | 5 (14.0) | 4 (13.2) | 2 (14.3) | 7 (22.7) | 6 (14.5) | 4 (12.6) | 6 (18.9) | 3 (15.3) | NA |
| Grade 3 or 4, n (%) | 3 (0.6) | 2 (0.4) | 2 (0.6) | 1 (0.2) | 6 (1.3) | 3 (0.6) | 3 (0.9) | 1 (0.5) | 0 | 1 (0.2) |
| Creatinine, mg/dL | 0.1 (0.1) | 0.1 (0.1) | 0.0 (0.1) | 0.0 (0.1) | 0.1 (0.1) | 0.1 (0.1) | 0.0 (0.1) | 0.1 (0.1) | 0.0 (0.1) | NA |
| Grade 3 or 4, n (%) | 0 | 1 (0.2) | 0 | 2 (0.4) | 0 | 1 (0.2) | 0 | 0 | 0 | 2 (0.4) |
| Creatine kinase, U/L | 54 (89.5) | 34 (64.4) | 9 (70.1) | 4 (78.6) | 56 (92.3) | 37 (63.9) | 15 (77.0) | 57 (163.6) | 26 (46.5) | NA |
| Grade 3 or 4, n (%) | 4 (0.8)‡ | 2 (0.4)* | 1 (0.3) | 3 (0.6) | 6 (1.3) | 3 (0.6) | 1 (0.3) | 1 (0.5) | 0 | 3 (0.6) |
| LDL cholesterol, mg/dL§ | 15 (29.1) | 12 (25.9) | 7 (21.7) | 5 (23.4) | 24 (27.6) | 20 (26.8) | 12 (25.0) | 13 (29.6) | 10 (22.7) | NA |
| % change | 16 (29.2) | 13 (27.7) | 9 (20.5) | 7 (23.6) | 25 (29.3) | 21 (28.5) | 12 (22.6) | 13 (22.9) | 11 (21.3) | NA |
| HDL cholesterol, mg/dL§ | 12 (14.9) | 5 (12.8) | 3 (11.8) | −1 (11.0) | 13 (14.4) | 7 (13.3) | 4 (11.0) | 12 (11.7) | 6 (14.3) | NA |
| % change | 21 (25.7) | 11 (22.0) | 7 (20.6) | 0 (20.5) | 24 (26.5) | 14 (23.4) | 9 (20.1) | 24 (22.6) | 11 (26.3) | NA |
| LDL:HDL ratio§ | −0.1 (0.6) | 0.1 (0.6) | 0.0 (0.5) | 0.1 (0.7) | 0.0 (0.6) | 0.1 (0.6) | 0.1 (0.5) | −0.2 (0.6) | 0.0 (0.5) | NA |
| % change | −0.6 (31.1) | 6.4 (36.4) | 4.5 (23.6) | 10.3 (29.2) | 3.8 (30.8) | 9.5 (29.5) | 6.0 (24.5) | −6.5 (23.0) | 2.6 (23.6) | NA |

Absolute values are presented as mean (SD) change from baseline at weeks 24 and 52 unless otherwise specified.
Severity was graded using Common Terminology Criteria for Adverse Events Version 4.03.
*Grade 4 in one patient.
†Lymphocytes decreased.
‡Grade 4 in two patients.
§Fasting values; not available for all patients.
ADA, adalimumab; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FIL100, filgotinib 100 mg; FIL200, filgotinib 200 mg; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NA, not assessed; PBO, placebo.

### Limitations

The study excluded patients with prior bDMARD failure, so data cannot be extrapolated to bDMARD-experienced patients; filgotinib was previously compared with placebo in this population.[5] Generalisability to patients with less active RA is potentially limited because the study enrolled patients with moderate-to-severe disease. Placebo treatment was limited to 24 weeks due to ethical concerns. An elevated placebo response was observed, consistent with RA trial data showing increasing placebo rates over the last 20 years.[34] In the present study, placebo response rates were especially high in geographical group B (predominantly Eastern Europe) and group C (Mexico and Argentina); as these groups comprised 65% of randomised patients, the regional differences contributed substantially to the overall placebo response rate. Nearly 50% of placebo-treated patients achieving study endpoints present a challenge to differentiating active agents from placebo. The study was not powered to compare AEs between arms, so no definitive conclusions about safety can be reached. Additional safety data will come from the integrated safety analysis across all phase II and III filgotinib trials, long-term extension study (ClinicalTrials.gov NCT03025308) and future registries.

## CONCLUSIONS

In MTX-IR patients with active RA, filgotinib plus MTX reduced RA signs and symptoms, improved physical function and inhibited progression of structural joint damage. This study demonstrated non-inferiority of FIL200 plus MTX, but not FIL100 plus MTX, to adalimumab plus MTX, based on DAS28(CRP) low disease activity. Overall, filgotinib showed a favourable benefit-to-risk profile and both doses were well tolerated.

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**Data availability statement** Data are available upon reasonable request. Anonymised individual patient data will be shared upon request for research purposes dependent upon the nature of the request, the merit of the proposed research, the availability of the data and the intended use. The full data sharing policy for Gilead Sciences can be found at https://www.gilead.com/about/ethics-and-code-of-conduct/policies.



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CLINICAL SCIENCE

# Modern treatment approach results in low disease activity in 90% of pregnant rheumatoid arthritis patients: the PreCARA study

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## ABSTRACT

**Objectives** In patients with rheumatoid arthritis (RA), high disease activity impairs fertility outcomes and increases the risk of adverse pregnancy outcomes. The aim of this study was to determine the feasibility of a modern treatment approach, including treat-to-target (T2T) and the prescription of tumour necrosis factor (TNF) inhibitors, in patients with RA with a wish to conceive or who are pregnant.

**Methods** Patients were derived from the Preconception Counseling in Active RA (PreCARA) cohort. Patients with a wish to conceive or who are pregnant were treated according to a modified T2T approach, in which the obvious restrictions of pregnancy were taken into account. Results of the PreCARA study were compared with results of the Pregnancy-induced Amelioration of Rheumatoid Arthritis (PARA) study, a historic reference cohort on RA during pregnancy. Patients in the PARA cohort were treated according to the standards of that time (2002–2010). Differences in disease activity over time between the two cohorts were tested using a linear mixed model.

**Results** 309 patients with RA were included in the PreCARA study, 188 children were born. 47.3% of the patients used a TNF inhibitor at any time during pregnancy. Mean disease activity over time in the PreCARA cohort was lower than in the reference cohort ($p<0.001$). In the PreCARA cohort, 75.4% of the patients were in low disease activity (LDA) or remission before pregnancy increasing to 90.4% in the third trimester, whereas in the PARA cohort, these percentages were 33.2% and 47.3%, respectively.

**Conclusions** This first study on a modern treatment approach in pregnant patients with RA shows that LDA and remission are an attainable goal during pregnancy, with 90.4% of patients achieving this in the third trimester.

## Key messages

**What is already known about this subject?**

- In patients with rheumatoid arthritis (RA), high disease activity is associated with a prolonged time to pregnancy and is an independent risk factor for lower birth weight of the offspring.
- Tumour necrosis factor (TNF) inhibitors are considered safe during pregnancy; however, it is not known how many patients require treatment with TNF inhibitors during pregnancy.

**What does this study add?**

- In this first study on a modern treatment approach during pregnancy, we showed that low disease activity (LDA) and remission are a feasible goal, with 90.4% of the patients in LDA in the third trimester of pregnancy.

**How might this impact on clinical practice or future developments?**

- In patients with RA with a wish to conceive or who are pregnant, clinicians should strive for remission or LDA.
- The effect of a modern treatment approach on fertility outcomes and pregnancy outcomes should be the focus of further studies.

## INTRODUCTION

Rheumatoid arthritis (RA) impairs fertility and pregnancy outcomes.[1] High disease activity in patients with RA is associated with a prolonged time to pregnancy[2] and is an independent risk factor for lower birth weight.[3]

Over the last decades, the treatment of RA has evolved: early diagnosis, immediate initiation of disease-modifying antirheumatic drugs (DMARDs), several new approved drugs and a treat-to-target (T2T) approach aiming for remission have resulted in better outcomes for patients.[4–6] All of these developments are fundamental aspects of both the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) treatment guidelines.[7 8]

Tumour necrosis factor (TNF) inhibitors have revolutionised the treatment in RA. Treatment with TNF inhibitors and/or a combination of DMARDs are considered key elements of a T2T approach.[5 9] Most TNF inhibitors are considered safe during pregnancy,[10 11] resulting in the European Medicines Agency (EMA) advising to use certolizumab pegol if clinically needed during pregnancy and adalimumab, etanercept and infliximab if clearly needed during pregnancy.[12] A drawback of prescribing TNF inhibitors during pregnancy is active transport of these biologics over the placenta into the fetal circulation. This occurs as early as week 18 of gestation.[1] Therefore, the EULAR points to consider and ACR guidelines conditionally advise to discontinue treatment with most TNF inhibitors before the third trimester of pregnancy. These guidelines advise that certolizumab pegol can

be continued throughout pregnancy.[10 13] To date, the effect of stopping TNF inhibitors during pregnancy on disease activity is not well established, and what treatment strategy should be followed after stopping TNF inhibitors during pregnancy is unknown.

The efficacy of T2T was demonstrated in previous studies; however, whether this approach is feasible in pregnant patients with RA is unknown. The primary aim of our study was to evaluate the feasibility of a modified T2T approach aiming for remission or low disease activity (LDA) in patients with RA with a wish to conceive or who are pregnant. The secondary aims were to determine the percentage of patients that require treatment with TNF inhibitors during pregnancy, and to investigate the effect of stopping TNF inhibitors during pregnancy on disease activity.

## METHODS

### Patient population and data collection

Patients were derived from the Preconception Counseling in Active RA (PreCARA) cohort (first inclusion 2011). The PreCARA cohort is an ongoing, prospective cohort study on inflammatory rheumatic diseases and pregnancy. Available data up to 1 October 2020 was used for analysis. The PreCARA study is performed in one tertiary referral hospital (Erasmus MC, Rotterdam) and registered on clinicaltrials.gov with reference number NCT01345071. For the current analysis, patients with RA who delivered and who had at least one visit post partum were used.

Patients were preferably included in the PreCARA study before they got pregnant. Study visits were scheduled every 3 months before conception, during each trimester, and at 6, 12 and 26 weeks post partum. At every visit, patients underwent joint examination, filled in questionnaires, blood was drawn and data on disease activity and frequencies and dosages of conventional synthetic DMARDs (csDMARDs) and biologic DMARDs (bDMARDs) were collected. Information on relevant medical history and previous medication use were collected at inclusion.

### PreCARA treatment protocol

Patients in the PreCARA cohort were treated according to a modified T2T approach aimed at remission. In this protocol, the obvious restrictions of pregnancy, previous response on treatment, previous experienced side effects and patients preference were taken into account. Treatment was, if needed, intensified according to the T2T treatment approach at every study visit. In the PreCARA protocol, first, sulfasalazine and/or hydroxychloroquine were started. Followed by the addition of prednisone (preferably in a maximum daily dosage of 7.5 mg) and/or a TNF inhibitor, preferably certolizumab pegol. Patients were allowed to get pregnant using the TNF inhibitor on which they enrolled in the cohort. TNF inhibitors were stopped during pregnancy at the gestational age as advised by the EULAR,[10] and a switch to certolizumab pegol or prednisone was considered.

### Data analysis

Disease activity was calculated using the Disease Activity Score with three variables: 28 swollen and tender joint count and C reactive protein (CRP) (DAS28CRP).[14 15] We stratified disease activity states according to recommendations of the EULAR: remission (DAS28CRP≤2.6), LDA (2.6<DAS28CRP≤3.2), intermediate disease activity (3.2<DAS28CRP≤5.1) and high disease activity (DAS28CRP>5.1).[16]

In line with previous literature, we assessed increase in disease activity between 6 and 12 weeks post partum based on the 'reversed' EULAR response criteria.[17]

Results of the PreCARA study were compared with the results of the Pregnancy-induced Amelioration of Rheumatoid Arthritis (PARA) study,[17 18] a historic reference cohort on RA during pregnancy with a similar study design (inclusion 2002–2010). Patients in the PARA cohort were visited at home and were treated by their own rheumatologist according to the standards of that time for pregnancy, mainly using sulfasalazine, prednisone or no medication. Treatment in this time period was characterised by cautious approach due to insufficient information with regard to breast feeding, gonadotoxic effects and long-term effects in children exposed to immunosuppressive drugs in utero.[19]

### Statistical analysis

Descriptive statistics are presented as numbers (n) and percentages (%). Values are given as mean±SD or median±IQR. We tested categorical data using $\chi^2$ and Fisher's exact tests, continuous data using (paired) t-test, analysis of variance and Wilcoxon rank. A two-sided p value of $<0.05$ was considered significant.

Differences in disease activity over time between the cohorts were tested using linear mixed models with unstructured covariance and random variation within individuals and between individuals. Subgroup analysis for the disease course over time for the use of TNF inhibitors during pregnancy is performed by using linear mixed models with unstructured covariance and random variation within individuals and between individuals. Patients who used a TNF inhibitor at any point during pregnancy were considered TNF inhibitor users during pregnancy. All statistical analyses were performed using Stata V.15 (StataCorp-LP).

### Ethics

This study was approved by the Erasmus MC ethics review board in compliance with Declaration of Helsinki. All patients gave their informed consent.

### Patient and public involvement

Patients were involved in the design of the cohorts. We obtained input from patients in the design of the questionnaires, cohort materials and cohort management. We carefully assessed the burden on participating patients. We intend to share the results to participating patients and will appropriately disseminate the results.

## RESULTS

A total of 587 patients with an inflammatory rheumatic disease, of which 309 women had RA, were included in the PreCARA cohort. 188 children were born (4 twins). A detailed description of the demographics of these women and a description of patients in the PARA cohort are given in table 1.

### Medication use during pregnancy

Table 2 shows the medication used in the PreCARA cohort. Eleven patients (6.0%) did not use any DMARDs during pregnancy. Sulfasalazine, hydroxychloroquine, prednisone and certolizumab pegol were the most commonly used DMARDs. The median daily dosage of prednisone in the third trimester of pregnancy was 5 mg (IQR 5–7.5 mg), 19.0% of the patients used a dosage of >7.5 mg at at least one timepoint during pregnancy. The median daily dosage, for the same period, of hydroxychloroquine was 200 mg (IQR 200–400 mg) and of sulfasalazine was 2000 mg (IQR 1000–2000 mg).

**Table 1** Clinical and demographic features of patients with rheumatoid arthritis included in the PreCARA cohort (n=184) and PARA cohort (n=253) that were used for the current data analysis

| Variable | PreCARA cohort | PARA cohort | P value |
|---|---|---|---|
| Mean age at delivery, years (SD) | 32.8 (3.9) | 32.7 (3.8) | 0.88 |
| Median disease duration at first visit, years (IQR) | 6.8 (3.7–10.7) | 4.9 (2.2–9.7) | 0.009 |
| Erosive disease, n (%) | 52 (28.3) | 161 (63.7) | <0.001 |
| Rheumatoid factor positive and/or ACPA positive, n (%) | 164 (89.1) | 176 (71.8) | <0.001 |
| Nulliparity, n (%) | 81 (44.0) | 126 (49.8) | 0.23 |
| Education level, years of education (SD) | 15.9 (3.5) | 15.0 (3.0) | 0.02 |
| Number of different DMARDs prescribed prior to inclusion in the cohort (IQR) | 3 (2–4) | 2 (1–3) | <0.001 |
| Number of different csDMARDs prescribed prior to inclusion in the cohort (IQR) | 2 (2–3) | 2 (1–2) | <0.001 |
| Number of different bDMARDs prescribed prior to inclusion in the cohort (IQR) | 1 (0–2) | 0 (0–0) | <0.001 |

ACPA, anti-citrullinated protein antibody; bDMARDs, biologic disease-modifying antirheumatic drugs; csDMARDs, conventional synthetic disease-modifying antirheumatic drugs; DMARDs, disease-modifying antirheumatic drugs.

### TNF-inhibitor use during pregnancy

Eighty-seven patients (47.3%) used a TNF inhibitor at any time during pregnancy. The most frequently used TNF inhibitor was certolizumab pegol. A total of 26 patients stopped treatment with a TNF inhibitor during pregnancy: adalimumab n=4, infliximab n=7, etanercept n=13, certolizumab pegol n=4. After stopping their TNF, inhibitor, 17 patients (65.4%) used prednisone in the third trimester of pregnancy. Thirteen patients with RA switched their type of TNF inhibitor during pregnancy: switch from adalimumab to certolizumab pegol, n=4; switch from etanercept to certolizumab pegol n=5; switch from infliximab to certolizumab pegol n=4. The median number of weeks of gestation when treatment with infliximab was stopped was 15.3 weeks (IQR 12.7–20.3 weeks), for adalimumab this was 18.4 weeks (IQR 16.9–19.5 weeks), for etanercept 23.4 weeks (IQR 9.9–26.9 weeks) and for certolizumab pegol 35.6 weeks (IQR 26.3–37.4 weeks).

In the third trimester of pregnancy, TNF inhibitors (number of patients that used TNF inhibitors in the third trimester=56) were in 62.5% of the patients combined with sulfasalazine, in 46.4% of the patients with hydroxychloroquine and in 35.7% with prednisone. Twenty-five patients (44.6%) used both sulfasalazine and hydroxychloroquine combined with their TNF inhibitor (with or without prednisone). In patients that did not use a TNF inhibitor (n=116) in the third trimester, sulfasalazine, hydroxychloroquine and prednisone were frequently used in combination. Further, 30.2% of the patients (n=35) used sulfasalazine, hydroxychloroquine and prednisone in combination, while 32.8% of the patients (n=38) used sulfasalazine and hydroxychloroquine without prednisone.

### Medication use in the historic reference cohort

In this cohort of patients, 41.2% did not use any DMARDs during pregnancy (table 3). Patients in the PARA cohort were usually on stable medication: 85% of the patients used the same medication in the first trimester of pregnancy compared with the prepregnancy visit. Prednisone and sulfasalazine were most frequently prescribed during pregnancy. The median daily dosage of prednisone was 7.5 mg (IQR 5–10 mg), 70.6% of the patients used a dosage of >7.5 mg at at least one timepoint during pregnancy. Sulfasalazine was used by 63 (25.7%) patients in the third trimester, in 2 (3.2%) patients sulfasalazine was combined with hydroxychloroquine and in 23 (36.5%) patients with prednisone.

### Disease activity during pregnancy

Disease activity did not change during pregnancy and postpartum in the PreCARA-cohort (figure 1).

**Table 2** The percentage of patients in the PreCARA cohort using certain medication during pregnancy (total number of patients=184)

| Medication | Last visit before pregnancy n (%) N=116 | 1st trimester visit n (%) N=167 | 2nd trimester visit n (%) N=174 | 3rd trimester visit n (%) N=172 | 6 weeks postpartum visit n (%) N=170 | 12 weeks postpartum visit n (%) N=153 | 26 weeks postpartum visit n (%) N=125 |
|---|---|---|---|---|---|---|---|
| Methotrexate | 2 (1.7) | 0 | 0 | 0 | 27 (15.9) | 34 (22.2) | 31 (24.8) |
| Leflunomide | 0 | 0 | 0 | 0 | 1 (0.6) | 2 (1.3) | 3 (2.4) |
| Hydroxychloroquine | 77 (66.4) | 96 (57.5) | 94 (54.0) | 93 (54.1) | 97 (57.1) | 88 (57.5) | 70 (56.0) |
| Sulfasalazine | 76 (65.6) | 103 (61.7) | 104 (59.8) | 103 (59.8) | 104 (61.2) | 95 (62.1) | 79 (63.2) |
| Prednisone | 53 (45.7) | 69 (41.3) | 67 (38.5) | 72 (41.9) | 67 (39.4) | 60 (39.2) | 46 (36.8) |
| Azathioprine | 1 (0.9) | 3 (1.8) | 3 (1.7) | 3 (1.7) | 1 (0.6) | 2 (1.3) | 1 (0.8) |
| Certolizumab pegol | 31 (26.7) | 38 (22.8) | 48 (27.6) | 50 (29.1) | 47 (27.7) | 46 (30.1) | 38 (30.4) |
| Adalimumab | 8 (6.9) | 8 (4.8) | 0 | 0 | 5 (2.9) | 6 (3.9) | 7 (5.6) |
| Etanercept | 19 (16.4) | 20 (12.0) | 19 (10.9) | 6 (3.5) | 19 (11.2) | 22 (14.4) | 16 (12.8) |
| Infliximab | 11 (9.5) | 11 (6.6) | 4 (2.3) | 0 | 1 (0.6) | 2 (1.3) | 1 (0.8) |
| Tocilizumab | 2 (1.7) | 0 | 0 | 0 | 4 (2.4) | 4 (2.6) | 6 (4.8) |
| Golimumab | 0 | 0 | 0 | 0 | 0 | 0 | 1 (0.8) |
| Abatacept | 0 | 0 | 0 | 0 | 1 (0.6) | 2 (1.3) | 2 (1.6) |

**Table 3** The percentage of patients in the PARA cohort, a historic reference cohort (2002–2010), using certain medication during pregnancy (total number of patients=253)

| Medication | Before pregnancy visit n (%) N=124 | 1st trimester visit n (%) N=213 | 2nd trimester visit n (%) N=232 | 3rd trimester visit n (%) N=245 | 6 weeks postpartum visit n (%) N=239 | 12 weeks postpartum visit n (%) N=240 | 26 weeks postpartum visit n (%) N=222 |
|---|---|---|---|---|---|---|---|
| Methotrexate | 0 | 0 | 0 | 0 | 45 (18.8) | 73 (30.4) | 89 (40.0) |
| Leflunomide | 0 | 0 | 0 | 0 | 0 | 3 (1.3) | 4 (1.8) |
| Hydroxychloroquine | 8 (6.5) | 5 (2.3) | 5 (2.2) | 4 (1.6) | 10 (4.2) | 19 (7.9) | 18 (8.1) |
| Sulfasalazine | 42 (33.9) | 61 (28.6) | 65 (28.0) | 63 (25.7) | 64 (26.8) | 73 (30.4) | 70 (31.5) |
| Prednisone | 52 (41.9) | 80 (37.6) | 87 (37.5) | 87 (35.5) | 85 (35.6) | 89 (37.1) | 78 (35.1) |
| Azathioprine | 2 (1.6) | 1 (0.5) | 1 (0.4) | 1 (0.4) | 3 (1.3) | 2 (0.8) | 2 (0.9) |
| Adalimumab | 0 | 0 | 0 | 0 | 5 (2.1) | 7 (2.9) | 12 (5.4) |
| Infliximab | 0 | 0 | 0 | 0 | 1 (0.4) | 2 (0.8) | 3 (1.4) |
| Etanercept | 0 | 0 | 0 | 0 | 7 (2.9) | 14 (5.8) | 13 (5.9) |

The medication that is not listed in this table was not prescribed during this study period.

Mean DAS28CRP before pregnancy in the historic reference cohort was 3.73 (SD 1.18) and decreased during pregnancy to DAS28CRP 3.35 (SD 1.12) in the third trimester. Disease activity increased in the postpartum period, the highest observed DAS28CRP 3.78 was at 12 weeks post partum (SD 1.28) (figure 1).

Disease activity over time in the PreCARA cohort was statistically significantly lower than in the historic reference cohort (p<0.001). Also, mean disease activity at every different timepoint in the PreCARA cohort was statistically significant lower (p<0.001).

The percentage of patients in remission or LDA in the PreCARA cohort was significantly higher at all timepoints during follow-up compared with the PARA cohort (p<0.001) (figure 2). In the PreCARA cohort, the total number of patients in remission and LDA increased from 64.8% and 75.4% at inclusion to 76.1% and 90.4%, respectively, in the third trimester. The number of patients in remission remained stable post partum. The percentage of patients in different disease activity states was different between the PreCARA cohort and the PARA cohort at all timepoints (p<0.001) (figure 2).

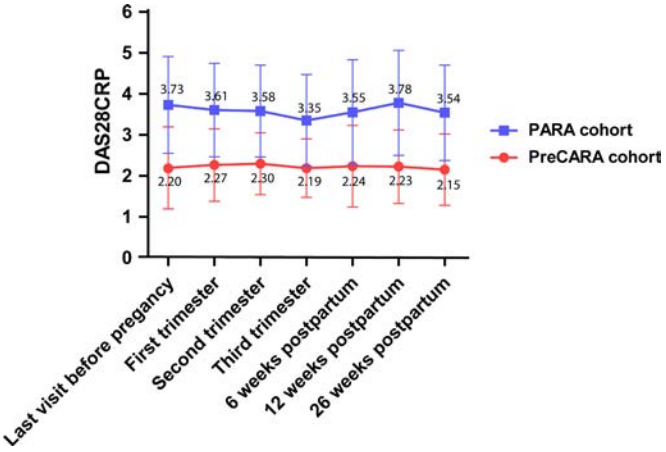


**Figure 1** DAS28CRP (mean, SD) scores over time for the PreCARA cohort (modern treatment approach cohort) and the PARA cohort (historic reference cohort). The x-axis displays specific timepoints before, during and after pregnancy, and the y-axis represents mean (SD) disease activity. Mean disease activity over time in the PreCARA cohort was lower than in the reference cohort (p<0.001).

### TNF-inhibitor use and disease activity during pregnancy

Stratified analysis showed no statistically significant difference in disease activity in the third trimester of pregnancy between patients that switched their TNF inhibitor to certolizumab pegol during pregnancy (n=13, DAS28CRP 2.17 (SD 0.73)) versus patients that stopped their TNF inhibitor and used prednisone (n=17, DAS28CRP 2.63 (SD 0.69)) versus patients that used certolizumab pegol throughout pregnancy (n=30, DAS28CRP 2.18 (SD 0.63)), versus patients that stopped their TNF inhibitor and did not use certolizumab pegol nor prednisone (n=8, DAS28CRP 2.23 (SD 0.67)), p=0.13.

Disease activity over time did not differ between patients who used a TNF inhibitor during pregnancy (any use during pregnancy) and patients who did not (p=0.14) (online supplemental figure 1).

### Disease activity increase post partum

Not one patient in the PreCARA cohort experienced a severe increase in disease activity post partum, 12.2% of the patients in this cohort experienced a moderate increase. These rates were 5.7% (vs PreCARA cohort, p=0.01) and 21.0%(vs PreCARA cohort, p=0.18) in the PARA cohort, respectively.

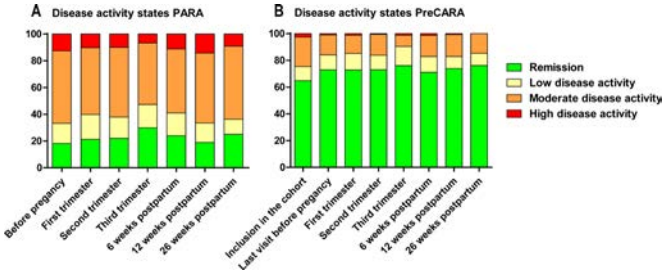


**Figure 2** Bar charts showing disease activity states DAS28CRP scores for the PARA cohort (historic reference cohort) (A) and the PreCARA cohort (modern treatment approach cohort) (B). The x-axis displays the specific timepoints before, during and after pregnancy, and the y-axis shows the percentage of patients in the different disease activity states. The percentage of patients in moderate or high disease activity was higher at all timepoints in the historic reference cohort compared with the modern treatment approach cohort (p<0.001).

## DISCUSSION

Until recently, rheumatologists assumed that almost all patients with RA reach a state of remission during pregnancy independent of treatment; however, more literature shows that over half of the patients still has active disease during pregnancy.[1 17 20] This highlighted the need for improved care. Our study was the first to evaluate a T2T approach, with the use of TNF inhibitors, low dose prednisone and a combination of DMARDs, in patients with RA with a wish to conceive or get pregnant. Our results show that entering pregnancy in LDA or remission, as advised by ACR guidelines, is attainable when applying T2T. Over 80% of the patients in our study was in LDA at their last visit before pregnancy. Moreover, we showed that applying a T2T approach results in LDA during pregnancy and post partum in a vast majority of patients with RA.

Half of the patients in our study were able to get in LDA or remission using only csDMARDs or prednisone. In a large percentage of the patients, csDMARDs were prescribed in combination. The percentage of patients on prednisone during pregnancy was comparable between our modern treatment approach cohort and the historic reference cohort. However, the dosage of prednisone that was used during pregnancy was considerably lower in the modern treatment approach cohort. In this cohort, it was chosen to preferably prescribe a maximum dosage of 7.5 mg to limit the risk of fertility problems, premature birth, gestational diabetes and high blood pressure since higher doses of prednisone are associated with these complications during pregnancy.[1 21]

We showed that TNF inhibitors were efficacious during pregnancy, no significant difference in disease activity over time between patients that used a TNF inhibitor during pregnancy and patients who used csDMARDs were observed. Patients that were included in our cohort were allowed to get pregnant using their own TNF inhibitor in order to prevent an increase in disease activity by switching therapy. We did, however, observe a larger percentage of patients using certolizumab pegol at inclusion in our cohort (21.2%) than one can expect from the usual Dutch RA patient population.[22] This could be caused by a switch to certolizumab pegol already before referral to our specialised clinic, since literature shows no to minimal placental transfer of certolizumab pegol during pregnancy.[23] During pregnancy, TNF inhibitors were stopped at the gestational age advised by the EULAR. Due to reports on high bioavailability of infliximab during pregnancy, it was later chosen to stop infliximab preferably before week 16 of gestation in line with British Society of Rheumatology guidelines.[24] After stopping a TNF inhibitor during pregnancy, a switch to certolizumab pegol or prednisone was considered to prevent a possible increase in disease activity. Based on expert opinion, certolizumab pegol was arbitrarily stopped at 38 weeks of gestational age in order to minimise maternal infectious complications during delivery. This expert opinion was formed based on guidelines to withhold treatment with a TNF inhibitor before surgery.[25] TNF inhibitors could be restarted 1 week after a vaginal delivery and 2 weeks after a caesarean section. After delivery, there was no specific preference for one certain TNF inhibitor. However, patients got counselling on breast feeding and many preferred certolizumab pegol due to its robust pharmacokinetic data for use during breast feeding.[26] According to guidelines, no woman that breast fed used methotrexate. Children that were exposed to TNF inhibitors in utero were vaccinated in line with the Dutch national vaccination policy, in which the first live inactivated vaccine is administered at 14 months. No exceptions for any of the TNF inhibitors were made.

We observed, based on a low number of observations, no statistically significant difference in disease activity between patients that switched TNF-inhibitor treatment during pregnancy and patients that stopped TNF-inhibitor treatment all together. However, this observation is confounded by indication: TNF inhibitors were stopped only in those patients in complete remission after careful consideration of the treating physician and in consultation with the patient. Although TNF inhibitors were stopped, many patients used other medication like prednisone. These results show that physicians are able to distinguish between those patients that have calm disease during pregnancy in which TNF inhibitors can be stopped and patients that, despite having LDA, do require a switch in medication during pregnancy to prevent an increase in disease activity. Our results should not be interpreted as if TNF inhibitors can be stopped during pregnancy without an increased risk of increase in disease activity.

Patients with RA have an increased risk of a flare in disease activity after delivery,[1 20] not one patient in our modern treatment approach cohort experienced a severe increase in disease activity post partum. In the absence of well-defined criteria, we used criteria based on the 'reversed' EULAR response criteria. However, we should note that RA flares are complex, and comprehend more than an increase in disease activity as measured by a physician.[27]

Also, mean disease activity post partum was not different from mean disease activity during pregnancy. This indicates that applying T2T and an immediate restart of medication after delivery may help to prevent an increase in disease activity post partum.

The PreCARA study was designed as an observational study reflecting daily clinical practice in a specialised centre for arthritis and systemic autoimmune disorders and pregnancy. The selection of patients was therefore different from randomised controlled trials (RCTs), like the TICORA trial,[6] on which the most evidence on T2T is based. Comparing our results with the results of these RCTs might not be appropriate. In previously published studies on T2T in daily clinical practice, the percentage of patients in remission after an extensive follow-up period varies between 52% and 62.6%.[28 29] The percentage of patients in remission in our study increased from 62.8% at inclusion in the cohort to 74.4% in the third trimester of pregnancy. And although our study cannot be compared directly to these studies, it underscores that in pregnant patients with RA, a T2T approach is feasible too.

Some limitations of our study need to be considered. We compared the results of our modern treatment approach cohort with the results of a historic cohort. Patient characteristics in this historic cohort are slightly different compared with the current patient population. Second, our study could have suffered from selection bias. Our study was performed in one tertiary referral centre, which could have resulted in an over-representation of patients with more severe disease. The significant difference in percentage of patients that had RF or anti-citrullinated protein antibody (ACPA) antibodies in the PreCARA cohort could indicate that this type of bias has occurred. Yet, we showed that even in these patients with more severe disease, LDA during pregnancy is attainable. Furthermore, based on the nature of our study, it was impossible to show that either T2T or new targeted therapies such as TNF inhibitors or combination therapy, or all were responsible for the improved disease outcomes during pregnancy.

We presented in our study only those patients who got pregnant. It is reasonable to speculate that there is an over-representation of patients in LDA or remission in the current study, since active disease is associated with a longer time to pregnancy.[2] However, for those patients that did not get pregnant, the mean DAS28CRP of all visits during their wish to conceive was 2.36 (SD 1.00). Therefore, we conclude that selection bias based on disease activity is not a relevant factor in our study.

Our study has several strengths. This is the first study to prospectively collect results of a T2T approach in a large cohort of pregnant patients with RA. And, the study was performed in only one tertiary

referral hospital, which limited the variation on management of the disease between healthcare professionals. Moreover, the results of the current cohort will allow us to study the effect of T2T and TNF inhibitors on fertility outcomes and pregnancy outcomes in patients with RA in future studies.

The findings of our study should be applied in daily clinical practice. We advise clinicians to apply a T2T approach, including prescribing TNF inhibitors, in all patients with a wish to conceive and during pregnancy. We showed that patients can get pregnant with the TNF inhibitor they already used before pregnancy, and TNF inhibitors can be switched during pregnancy, without an increase in disease activity. Moreover, we advise pregnancy counselling and regular visits during pregnancy and post partum like performed in our specialised hospital. This extra care will contribute to the improved disease outcomes like we observed in our study.

In conclusion, we showed that a modern treatment approach results in LDA or remission in 90% of pregnant patients with RA. Therefore, LDA or remission should also be strived for in this group of patients, despite the obvious restrictions on medication use during pregnancy.

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**Contributors** All authors met the authorship criteria, they had a substantial contribution to the conception or design of the work (HS, ER, JMH, RJEMD) or the acquisition (HW, LK-vK, JMH, RJEMD), analysis (HS, RJEMD), or interpretation of data for the work (all authors) and were involved in revising a draft of this work, gave final approval of this version to be published, and are accountable for all aspects of the work in ensuring accuracy and integrity.

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**Competing interests** None declared.

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**Patient consent for publication** Obtained.

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TRANSLATIONAL SCIENCE

# JAK selectivity and the implications for clinical inhibition of pharmacodynamic cytokine signalling by filgotinib, upadacitinib, tofacitinib and baricitinib

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## ABSTRACT

**Objective** Janus kinase inhibitors (JAKinibs) are efficacious in rheumatoid arthritis (RA) with variable reported rates of adverse events, potentially related to differential JAK family member selectivity. Filgotinib was compared with baricitinib, tofacitinib and upadacitinib to elucidate the pharmacological basis underlying its clinical efficacy and safety.

**Methods** In vitro JAKinib inhibition of signal transducer and activator of transcription phosphorylation (pSTAT) was measured by flow cytometry in peripheral blood mononuclear cells and whole blood from healthy donors and patients with RA following cytokine stimulation of distinct JAK/STAT pathways. The average daily pSTAT and time above 50% inhibition were calculated at clinical plasma drug exposures in immune cells. The translation of these measures was evaluated in ex vivo-stimulated assays in phase 1 healthy volunteers.

**Results** JAKinib potencies depended on cytokine stimulus, pSTAT readout and cell type. JAK1-dependent pathways (interferon (IFN)α/pSTAT5, interleukin (IL)-6/pSTAT1) were among the most potently inhibited by all JAKinibs in healthy and RA blood, with filgotinib exhibiting the greatest selectivity for JAK1 pathways. Filgotinib (200 mg once daily) had calculated average daily target inhibition for IFNα/pSTAT5 and IL-6/pSTAT1 that was equivalent to tofacitinib (5 mg two times per day), upadacitinib (15 mg once daily) and baricitinib (4 mg once daily), with the least average daily inhibition for the JAK2-dependent and JAK3-dependent pathways including IL-2, IL-15, IL-4 (JAK1/JAK3), IFNγ (JAK1/JAK2), granulocyte colony stimulating factor, IL-12, IL-23 (JAK2/tyrosine kinase 2) and granulocyte-macrophage colony-stimulating factor (JAK2/JAK2). Ex vivo pharmacodynamic data from phase 1 healthy volunteers clinically confirmed JAK1 selectivity of filgotinib.

**Conclusion** Filgotinib inhibited JAK1-mediated signalling similarly to other JAKinibs, but with less inhibition of JAK2-dependent and JAK3-dependent pathways, providing a mechanistic rationale for its apparently differentiated efficacy:safety profile.

## Key messages

**What is already known about this subject?**

- Janus kinase (JAK) inhibitors (JAKinibs) have emerged as an important new class of oral therapy for the treatment of rheumatoid arthritis (RA).
- Despite showing similar clinical efficacy, the reported incidence rates of some adverse events of special interest vary among the JAKinibs.
- The relationship between JAK isoform selectivity and inhibition of distinct cytokine responses at clinical plasma exposures of these JAKinibs could provide a mechanistic basis for their relative efficacy and safety profiles.

**What does this study add?**

- This study is the first to combine in vitro inhibition of cytokine responses in whole blood with clinical pharmacokinetics of filgotinib, baricitinib, tofacitinib and upadacitinib to model daily cytokine-mediated pharmacodynamic profiles in healthy individuals and patients with RA.
- These data demonstrate that, compared with upadacitinib, tofacitinib and baricitinib, filgotinib had similar calculated daily average inhibition of JAK1-dependent pathways activated by interleukin 6 and interferon α, but the least inhibition of JAK2-dependent and JAK3-dependent signalling.
- The observed inhibition of JAKinibs on cytokine signalling was highly nuanced, and it was observed to be dependent on cytokine stimulus, STAT (signal transducers and activators of transcription) substrate and cell type, indicating that the differential cytokine profiles may provide a mechanistic rationale for reported efficacy and safety.

## INTRODUCTION

The Janus kinase (JAK) and signal transducers and activators of transcription (STAT) proteins constitute the JAK-STAT pathways, which are essential to immune regulation. Signalling through the pathways is initiated when a cytokine binds its cell-surface receptor, activating receptor-associated JAKs and phosphorylating STAT proteins. The STATs dimerise and migrate to the nucleus to induce and/or maintain immune responses via transcriptional regulation.

The JAK family consists of JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2). They bind intracellularly to dimeric cytokine receptor chains in pairs that vary by receptor.[1 2] Depending on which JAK is activated, the functional effects vary. Specific JAK pairs are implicated in diverse functions that regulate inflammation,[3] haematopoiesis[2 4–8] and

## Key messages

**How might this impact on clinical practice or future developments?**

- This study demonstrates that JAKinibs have unique, differential effects on specific cytokine signalling pathways and supports that preferential JAK1 activity is sufficient to drive RA efficacy, while JAK2 and JAK3 inhibition may increase the incidence of adverse events of special interest.
- These data can be used to build mechanistic correlations between cytokine inhibition and rates of adverse events observed with real-world JAKinib use.

immune homeostasis (figure 1).[9–14] The JAK-STAT pathways also play essential roles in immune-mediated pathology, including rheumatoid arthritis (RA).[6 15] Uncontrolled cytokine expression drives chronic inflammation; inadequately treated, this leads to systemic illness, joint destruction and deformity that characterise RA.

JAK inhibitors (JAKinibs) have emerged as an important new class of oral therapy in RA.[1 6] Baricitinib,[16] tofacitinib[17 18] and upadacitinib[19] are currently approved in the USA, European Union, Japan and other countries. Filgotinib is a novel JAKinib and has recently been approved in the European Union and Japan.[20] Filgotinib forms an active human metabolite, GS-829845/G254445,[21] that contributes to its pharmacological activity.[22] Studies suggest that JAK1 inhibition might be largely responsible for the efficacy of JAKinibs in RA.[23 24] Biochemically, all these JAKinibs show the greatest potency at inhibiting JAK1, with varying levels of selectivity for other JAK isoforms.[21 25–29] While these inhibitors have similar efficacy in patients with RA, reported rates of adverse events (AEs) differ, including: increased incidences of herpes zoster (HZ), serious infections, venous thromboembolism, decreased natural killer (NK) cell numbers, thrombocytopaenia and anaemia (online supplemental table 1).[16 18–20] Genetic evidence has linked JAK2 to erythropoiesis and thrombopoiesis, JAK3 to lymphocyte proliferation and immune homeostasis, and TYK2 to antiviral responses.[6 30 31] Therefore, differences in JAKinib selectivity for cytokine signalling via distinct JAK pairs may provide a mechanistic rationale for reported differences in safety profiles.

The objective of this study was to calculate the daily clinical pharmacodynamic (PD) inhibition profiles of cytokine signalling for filgotinib, baricitinib, tofacitinib and upadacitinib to compare their inhibition and selectivity. To accomplish this, we used a combination of in vitro cellular cytokine assays in human peripheral blood mononuclear cells (PBMCs) and whole blood (WB), coupling these results with the clinical RA plasma exposures of each JAKinib. Furthermore, we confirmed these results through ex vivo PD data obtained from blood samples from phase 1 healthy volunteers administered filgotinib.

## METHODS

Detailed experimental procedures are depicted in figure 2 and described in the online supplemental materials.

## RESULTS

### JAK isoforms differentially contribute to JAK-STAT pathway activity

JAKinibs showed dose-dependent inhibition of cytokine-stimulated phosphorylated STAT (pSTAT) levels in CD4+ and CD8+ T-cells, monocytes, NK cells, neutrophils and B-cells in human WB (tables 1 and 2, online supplemental figures 1–4) and PBMCs (online supplemental tables 2 and 3). For a given cell type, STAT substrate and cytokine stimulus, potency differences were observed between JAKinibs. The potencies measured in WB were consistently weaker than those assessed in PBMCs (tables 1

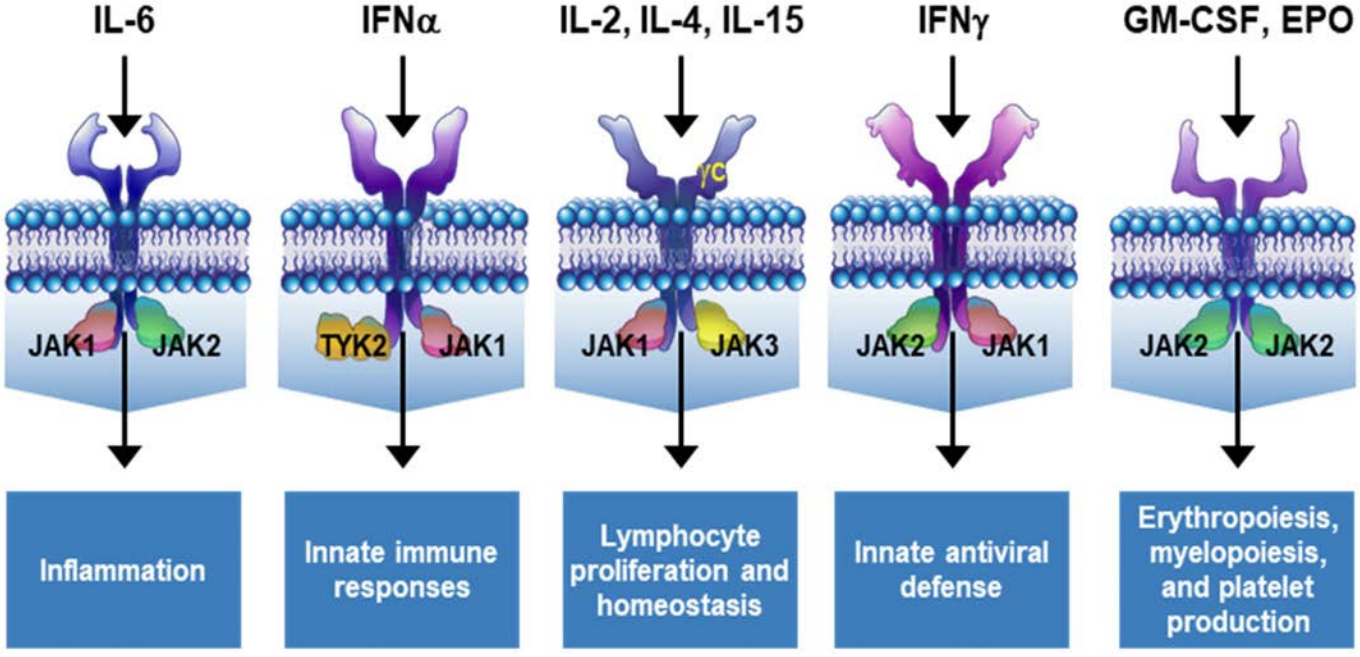



**Figure 1** Cytokine receptors are associated with distinct JAK pairing patterns. The JAK isoforms involved in each pathway vary according to the specific cytokine receptor and dictate downstream outcomes. Figure adapted from Winthrop.[49] EPO, erythropoietin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; JAK, Janus kinase; TYK2, tyrosine kinase 2.

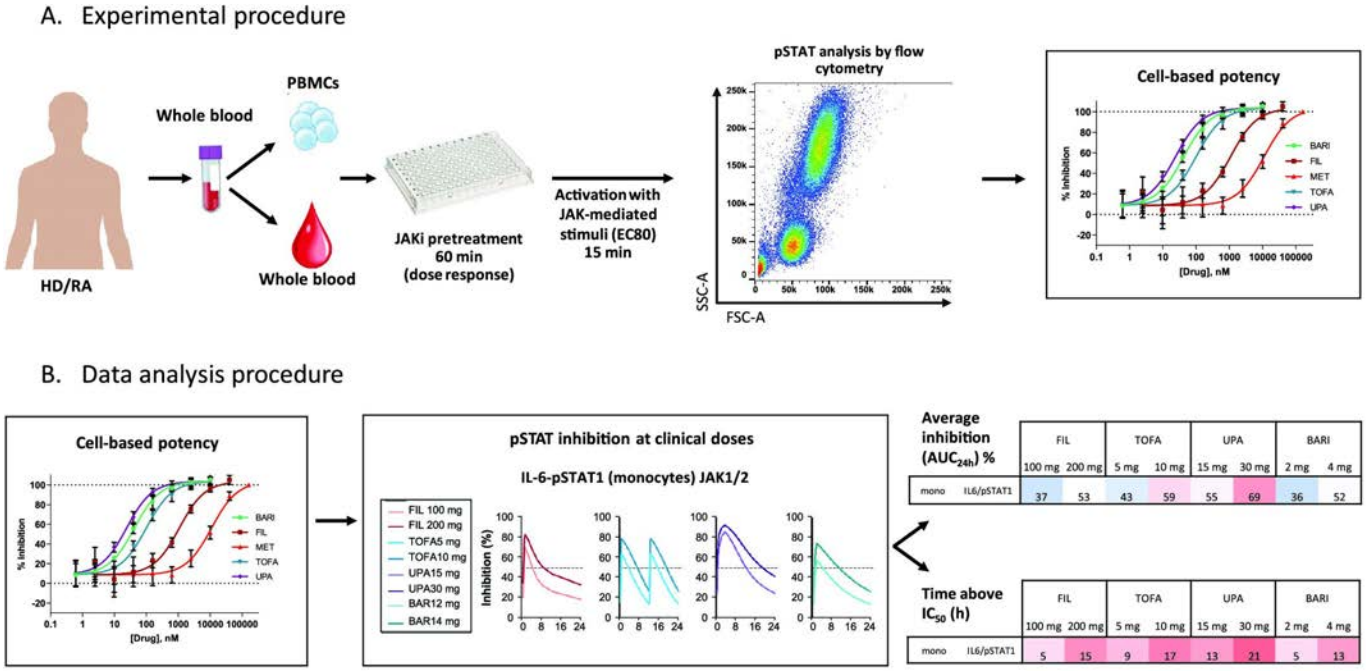



**Figure 2** (A) Experimental design and (B) data analysis procedures. Detailed methods are included in online supplemental materials. AUC-24h, area under the curve 0–24 hours; BARI, baricitinib; FIL, filgotinib; FSC-A, forward scatter area; HD, healthy donors; $IC_{50}$, half maximum inhibitory concentration; IL, interleukin; JAK, Janus kinase; JAKi, JAK inhibitor; MET, major metabolite of filgotinib (GS-829845); mono, monocytes; PBMCs, peripheral blood mononuclear cells; pSTAT, phosphorylated signal transducer and activator of transcription; RA, rheumatoid arthritis; SSC-A, side scatter area;TOFA, tofacitinib; UPA, upadacitinib.

and 2, online supplemental tables 2 and 3). This discrepancy was accounted for by compound plasma binding (online supplemental figure 5).

Cell type impacted the measured JAKinib potency to inhibit cytokine signalling. For example, comparing WB CD4+ cells and monocytes, there was an approximately threefold difference for the JAK1/TYK2-dependent interferon (IFN) α-stimulated pSTAT1 for each JAKinib ($p<0.001$), whereas for B and NK cells, the potencies were comparable (tables 1 and 2, online supplemental table 4). For each cytokine evaluated, cell type affected JAKinib potencies with varying magnitudes of significance.

JAKinib potencies were dependent on the STAT substrate phosphorylated in response to a given stimulus. In healthy donors, inhibition was consistently 7-fold to 11-fold greater for JAK1/JAK2-driven interleukin (IL)-6/pSTAT1 than for

**Table 1** JAKinib $IC_{50}$ values in CD4+ T-cells, monocytes and NK cells from whole blood assays

| | CD4+ T-cells | | | | | Monocytes | | | | | NK cells | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BARI | FIL | MET | TOFA | UPA | BARI | FIL | MET | TOFA | UPA | BARI | FIL | MET | TOFA | UPA |
| **Stimulation/pSTAT** | **$IC_{50}$, nM** | | | | | | | | | | | | | | |
| JAK2/JAK2 or JAK2/TYK2-dependent cytokines | | | | | | | | | | | | | | | |
| G-CSF/pSTAT3 | | | NS | | | 81 | 4977 | 50 215 | 292 | 81 | | | NS | | |
| GM-CSF/pSTAT5 | | | NS | | | 127 | 9916 | 102 910 | 510 | 74 | | | NS | | |
| IL-12/pSTAT4 | | | NS | | | NS | | | | | 269 | 10 351 | 221 777 | 1216 | 364 |
| JAK1/JAK2/TYK2-dependent cytokines | | | | | | | | | | | | | | | |
| IFNα/pSTAT1 | 50 | 1096 | 17 161 | 98 | 30 | 192 | 4560 | 91 078 | 393 | 83 | 131 | 2440 | 41 161 | 256 | 117 |
| IFNα/pSTAT3 | 39 | 871 | 12 644 | 80 | 24 | 40 | 991 | 15 793 | 86 | 17 | 30 | 675 | 8620 | 60 | 25 |
| IFNα/pSTAT5 | 28 | 638 | 9587 | 49 | 17 | 25 | 613 | 9518 | 51 | 11 | 34 | 507 | 7484 | 50 | 26 |
| IFNγ/pSTAT1 | | | NS | | | 74 | 4138 | 62 374 | 228 | 58 | | | NS | | |
| IL-6/pSTAT1 | 29 | 783 | 5637 | 63 | 27 | 39 | 1011 | 10 019 | 84 | 22 | | | NS | | |
| IL-6/pSTAT3 | 274 | 5435 | 62 680 | 644 | 225 | 161 | 3527 | 49 109 | 368 | 100 | | | NS | | |
| JAK1/JAK3-dependent cytokines | | | | | | | | | | | | | | | |
| IL-2/pSTAT5 | 40 | 988 | 14 079 | 40 | 21 | | | NS | | | 80 | 2153 | 23 824 | 87 | 56 |
| IL-4/pSTAT6 | 73 | 1458 | 39 420 | 77 | 36 | 53 | 1337 | 36 537 | 116 | 32 | 40 | 869 | 20 984 | 45 | 25 |
| IL-15/pSTAT5 | 38 | 967 | 14 326 | 39 | 21 | | | NS | | | 83 | 2044 | 27 540 | 93 | 58 |

G-CSF, GM-CSF, IFNα, IFNγ, IL-6, IL-2, IL-4 and IL-15 reported $IC_{50}$ values are based on the average of duplicates from 7–10 healthy volunteer whole blood.
BARI, baricitinib; FIL, filgotinib; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; $IC_{50}$, half maximum inhibitory concentration; IFN, interferon; IL, interleukin; JAK, Janus kinase; JAKinib, Janus kinase inhibitor; MET, major metabolite of filgotinib (GS-829845); NK, natural killer cell; NS, not sampled; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

**Table 2** JAKinib $IC_{50}$ values in B-cells, neutrophils and CD8+ T-cells from whole blood assays

| | B-cells | | | | | Neutrophils | | | | | CD8+ T-cells | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | BARI | FIL | MET | TOFA | UPA | BARI | FIL | MET | TOFA | UPA | BARI | FIL | MET | TOFA | UPA |
| Stimulation/pSTAT | $IC_{50}$, nM | | | | | | | | | | | | | | |
| JAK2/JAK2 or JAK2/TYK2-dependent cytokines | | | | | | | | | | | | | | | |
| G-CSF/pSTAT3 | | | NS | | | 404 | 16717 | 158111 | 1245 | 369 | | | NS | | |
| GM-CSF/pSTAT5 | | | NS | | | 66 | 3436 | 40925 | 143 | 21 | | | NS | | |
| IL-23/pSTAT3* | | | NS | | | NS | | | | | 210 | 15040 | 138638 | 970 | 368 |
| JAK1/JAK2/TYK2-dependent cytokines | | | | | | | | | | | | | | | |
| IFNα/pSTAT1 | 156 | 2957 | 54052 | 322 | 112 | | | NS | | | 80 | 1809 | 27730 | 163 | 56 |
| IFNα/pSTAT3 | 28 | 588 | 9777 | 62 | 20 | 47 | 833 | 13438 | 66 | 18 | 35 | 795 | 10617 | 73 | 23 |
| IFNα/pSTAT5 | 23 | 436 | 7524 | 44 | 16 | | | NS | | | 27 | 607 | 8231 | 46 | 17 |
| IFNγ/pSTAT1 | 22 | 900 | 12820 | 61 | 21 | 78 | 3137 | 42649 | 152 | 46 | | | NS | | |
| JAK1/JAK3-dependent cytokines | | | | | | | | | | | | | | | |
| IL-2/pSTAT5 | | | NS | | | | | NS | | | 32 | 809 | 11046 | 33 | 21 |
| IL-4/pSTAT6 | 295 | 6426 | 164309 | 356 | 162 | 71 | 1200 | 37069 | 106 | 37 | 47 | 1022 | 25900 | 51 | 26 |
| IL-15/pSTAT5 | | | NS | | | | | NS | | | 56 | 1459 | 19958 | 56 | 32 |

G-CSF, GM-CSF, IFNα, IFNγ, IL-2, IL-4 and IL-15 reported $IC_{50}$ values are based on the average of duplicates from 6–10 healthy volunteer whole blood.
*Memory CD8+ T-cells.
BARI, baricitinib; FIL, filgotinib; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; $IC_{50}$, half maximum inhibitory concentration; IFN, interferon; IL, interleukin; JAK, Janus kinase; JAKinib, Janus kinase inhibitor; MET, major metabolite of filgotinib (GS-829845); NS, not sampled; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

IL-6/pSTAT3 ($p<0.001$), potentially implicating that JAK1 is predominantly mediating phosphorylation of STAT1 and JAK2 is mediating STAT3 (tables 1 and 2, online supplemental table 5). Similarly, JAKinib inhibition of JAK1/TYK2-mediated IFNα-driven pSTAT5 and pSTAT3 was more potent than pSTAT1, again potentially demonstrating the reported reliance on TYK2 for regulating STAT1 phosphorylation.[32]

JAKinibs showed differences in inhibition of different cytokines using the same JAK pair. Filgotinib showed an approximately fourfold potency shift for JAK1/JAK2-dependent IL-6/pSTAT1 compared with IFNγ/pSTAT1 in monocytes ($p<0.001$) (table 1, online supplemental table 6). For other JAKinibs, potency differences were observed, although with less magnitude. These data highlight that cytokine receptors using identical JAK pairs may have differential reliance on a JAK isoform for mediating signalling.

## JAKinibs demonstrate distinct cellular selectivity

Selectivity between JAKinibs was analysed by measuring inhibition of cytokine signalling via each JAK pair in WB monocytes or NK cells (table 1). JAKinibs most potently inhibited the JAK1/TYK2-dependent IFNα/pSTAT5. Measured half maximum inhibitory concentration ($IC_{50}$) values for other cytokine responses were normalised to this value to control for intrinsic JAKinib potency differences (figure 3). All JAKinibs showed selectivity within approximately fivefold on JAK1-dependent pathways including IL-6 (JAK1/JAK2) and IL-15 (JAK1/JAK3). Conversely, JAKinibs showed differential selectivity against JAK2-mediated pathways. Filgotinib, GS-829845, tofacitinib and upadacitinib showed more than fivefold selectivity versus JAK2-dependent granulocyte colony stimulating factor (G-CSF)-driven or granulocyte-macrophage colony-stimulating factor (GM-CSF)-driven signalling, with filgotinib demonstrating the greatest selectivity against JAK2 compared with JAK1 (IFNα). Baricitinib showed lower JAK1 selectivity (≤5.1-fold for JAK1 versus non-JAK1 pathways; figure 3).

## JAKinibs show differentiated JAK1-selective pharmacological profiles at clinical plasma exposures

Representative cytokine/pSTAT pathway inhibition profiles were modelled over a 24-hour period at clinically relevant drug

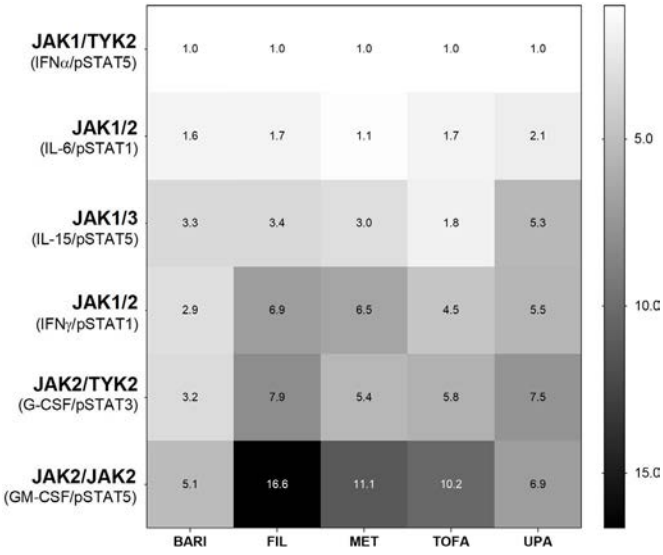



**Figure 3** Distinct JAKinib cellular selectivity for JAK heterodimeric cytokine signalling. Mean fold selectivity for each JAK–dimer pair normalised to inhibition of JAK1/TYK2 pathway (IFNα/pSTAT5 in monocytes). Cytokine/pSTAT pairs: JAK1/TYK2 (IFNα/pSTAT5 in monocytes); JAK1/2 (IL-6/pSTAT1 in monocytes); JAK1/3 (IL-15/pSTAT5 in NK cells); JAK1/2 (IFNγ/pSTAT1 in monocytes); JAK2/TYK2 (G-CSF/pSTAT3 in monocytes); and JAK2/2 (GM-CSF/pSTAT5 in monocytes). BARI, baricitinib; FIL, filgotinib; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; IFN, interferon; IL, interleukin; JAK, Janus kinase; JAKinib, JAK inhibitor; MET, major metabolite of filgotinib (GS-829845); NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

plasma concentrations in patients with RA at low and high JAKinib doses evaluated in the phase 3 clinical programmes (figure 4).[33–35]

The predicted average inhibition and time above 50% inhibition ($IC_{50}$ coverage) of the JAK1/JAK2-mediated IL-6/pSTAT1 (figure 4A) and JAK1/TYK2-mediated IFNα/pSTAT5 (figure 4B) are similar among JAKinibs at doses reported to have similar overall American College of Rheumatology efficacy responses (filgotinib 200 mg once daily; baricitinib 4 mg once daily; tofacitinib 5 mg two times per day; and upadacitinib 15 mg once daily) (online supplemental table 1). For IL-6/pSTAT1, all JAKinibs showed similar inhibition at therapeutic doses. For IFNα/pSTAT5, filgotinib had comparable daily inhibition as baricitinib and tofacitinib, and slightly less than upadacitinib. Some differences were noted in the time above 50% inhibition among JAKinibs. Thus, globally, the four JAKinibs demonstrate comparable $IC_{50}$ coverage and percentage of pSTAT inhibition at therapeutic doses for these 2 JAK1-driven pathways.

On other JAK1/JAK2-dependent signalling pathways (IFN-γ-induced pSTAT1) in representative cell types, filgotinib 200 mg displayed comparable or lower inhibition and time above 50% inhibition compared with baricitinib 4 mg, tofacitinib 5 mg and upadacitinib 15 mg (figure 4C,D). At these same doses, the average daily inhibition of JAK1/JAK3-dependent IL-4/pSTAT6 with filgotinib and baricitinib was comparable, but significantly lower than with tofacitinib and upadacitinib ($p<0.001$). Time above 50% inhibition was ≥3 times longer for tofacitinib and upadacitinib compared with filgotinib 200 mg ($p<0.001$) (figure 4D). Similarly, the average daily inhibition and time above 50% inhibition of IFNγ/pSTAT1 were lowest for filgotinib and tofacitinib, compared with baricitinib and upadacitinib ($p<0.001$) (figure 4C).

For JAK1-independent pathways, filgotinib 200 mg showed similar average daily inhibition of JAK2/TYK2-dependent G-CSF/pSTAT3 signalling as tofacitinib, but lower than baricitinib or upadacitinib (figure 4E). On the JAK2/JAK2-dependent GM-CSF/pSTAT5 pathway, upadacitinib and baricitinib showed ≥3-fold greater inhibition than filgotinib 200 mg ($p<0.001$), with only upadacitinib achieving >50% inhibition during the dose interval ($p<0.001$) (figure 4F). At clinical doses of upadacitinib, there were greater inhibition and time above 50% inhibition for JAK2-dependent and JAK3-dependent pathways signalling via G-CSF, GM-CSF, IFNγ and IL-4 compared with filgotinib 200 mg ($p<0.001$) (figure 4C-F).

Taken together, these data demonstrate that filgotinib preferentially inhibits JAK1 pathways at therapeutic doses, with upadacitinib showing the least selectivity at clinical doses. As reported, baricitinib displays JAK1/JAK2 selectivity[26] and tofacitinib mainly inhibits JAK1 and JAK3.

### In vitro study predictability and translation to clinical PD effects

The in vitro predicted inhibition of IL-6/pSTAT1 and GM-CSF/pSTAT5 in healthy donor blood was confirmed in ex vivo blood measurements from healthy volunteers orally dosed with filgotinib 200 mg. Average inhibitions of IL-6/pSTAT1 and GM-CSF/pSTAT5 with filgotinib were 78% and 36%, respectively (figure 5A). Subtracting the placebo response, there was a good concordance between the measured IL-6 and GM-CSF daily inhibition (68% and 15%, respectively) to the in vitro predicted inhibitions (64% and 6%, respectively; figure 5A), directly demonstrating the clinical relevance of the WB cellular modelling calculations.

To determine if inhibition of cytokine responses in blood of healthy individuals was an appropriate surrogate for RA responses, we directly compared inhibition of cytokine pathways in a small number of blood samples of healthy and RA donors. Using these measured values, the predicted daily cytokine inhibition profile for IFNα/pSTAT5 in CD4+ cells by JAKinibs was comparable in healthy and RA blood (figure 5B). Across pathways, generally comparable average daily inhibitions were predicted for JAKinibs in healthy and RA blood (figure 5C), suggesting that measurements in healthy blood could be translated to predictive inhibitions in patients with RA.

### Pharmacological profiles of JAKinibs across cell populations and cytokine stimuli at clinical plasma exposures

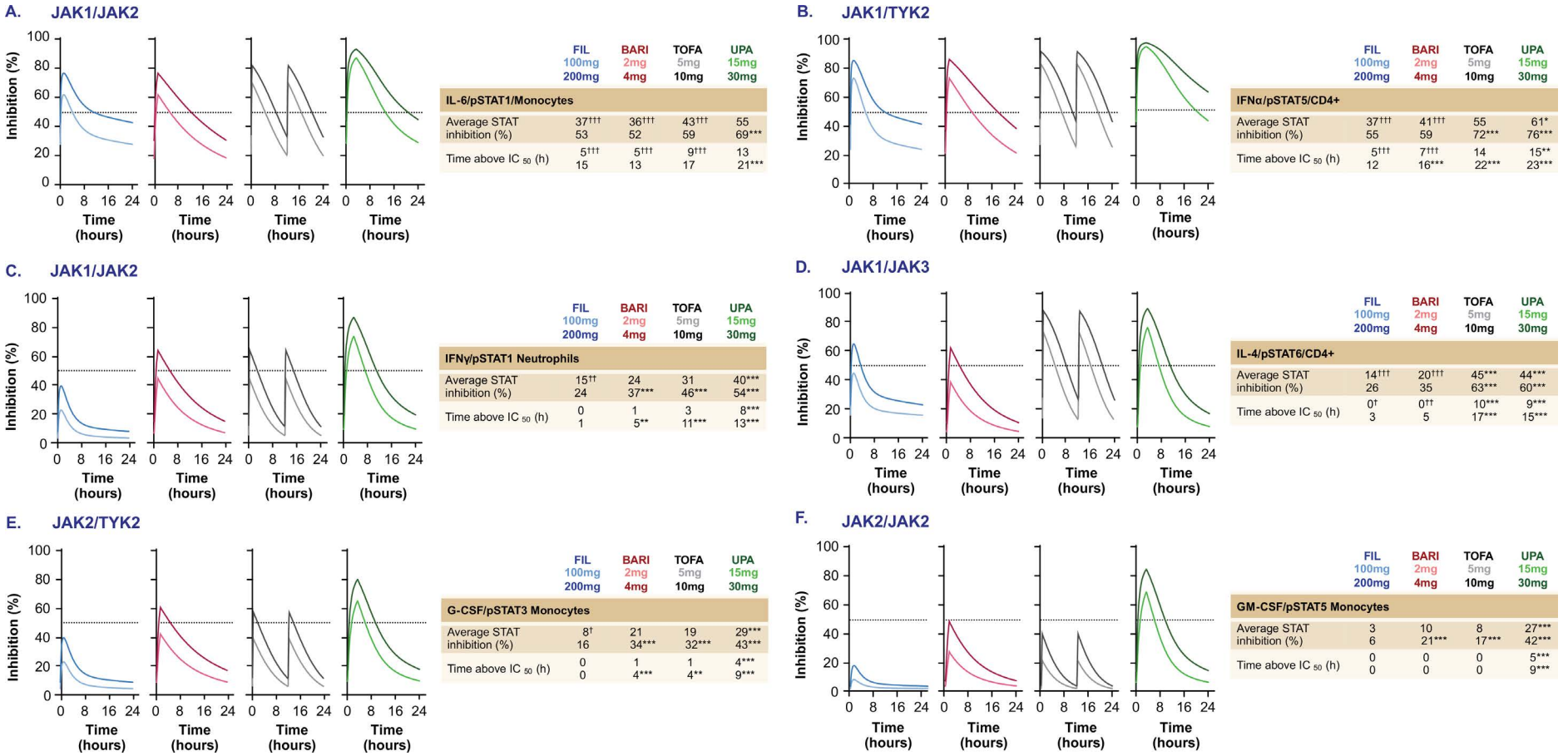
Clinical PD inhibition curves of cytokine signalling mediated by distinct JAK pairs were modelled (online supplemental figures 6–9). Average inhibition and time above 50% inhibition are summarised at clinical doses (online supplemental table 7).

For JAK1/TYK2, IFNα/pSTAT5 signalling showed average inhibitions >50% across all cell populations tested (figure 6A,B). Comparable levels of average daily inhibition were generally observed across cell populations, with upadacitinib showing greater inhibition in monocytes and CD4+ cells versus filgotinib ($p<0.05$). The magnitude of inhibition was lower for IFNα-driven pSTAT3 and pSTAT1, but all JAKinibs showed similar potency shifts. The weaker inhibition of IFNα/pSTAT1 (<50%) by all JAKinibs potentially indicates more TYK2 dependence for STAT1 phosphorylation.[32]

For JAK1/JAK2, IL-6/pSTAT1 signalling was inhibited by all JAKinibs, whereas there was half the inhibition of IL-6/pSTAT3 (figure 6A,C). Differences in average daily inhibition by JAKinibs between cell types were observed, but overall filgotinib showed comparable or greater inhibition of IL-6/pSTAT1, while tofacitinib showed the least ($p<0.001$). In contrast, for the IFNγ/pSTAT1 pathway (also mediated by JAK1/JAK2), upadacitinib and baricitinib showed greater inhibition of this pathway compared with filgotinib ($p<0.001$). There was a cell type-dependent difference in the magnitude of inhibition, with the greatest daily inhibition observed in B-cells and roughly half as much in neutrophils and monocytes, indicating a potentially greater reliance on JAK2 relative to JAK1 in mediating IFNγ inhibition in specific cells.

For JAK1/JAK3, filgotinib consistently showed the least inhibition of common γ-chain cytokines (IL-2, IL-15 and IL-4) (figure 6A,D). Greater daily inhibition of IL-2-mediated and IL-15-mediated pSTAT5 followed tofacitinib > upadacitinib > baricitinib > filgotinib. There were significant differences between JAKinib daily inhibition that were dependent on cell type, but tofacitinib and upadacitinib reproducibly showed greater inhibition in CD4+ and CD8+ cells than filgotinib ($p<0.001$). Tofacitinib, upadacitinib and baricitinib showed greater inhibition of IL-4/pSTAT5 than filgotinib in all cell types evaluated ($p<0.05$).

For JAK2/TYK2, the JAKinibs had a reduced effect on JAK2-mediated signalling, with <40% average inhibition of G-CSF/pSTAT3 in monocytes and neutrophils and <20% average inhibition of IL-12/pSTAT4 in NK cells and IL-23/pSTAT3 in CD8+ memory T-cells, compared with the JAK1-mediated pathways (figure 6A,E). Upadacitinib and baricitinib showed significantly greater inhibition in monocytes compared with filgotinib. In the remaining cell types, inhibition by JAKinibs was approximately 25%–50% of that in monocytes, and a trend for lower inhibition by filgotinib compared with other JAKinibs was observed.




| A. JAK1/JAK2 | FIL 100mg 200mg | BARI 2mg 4mg | TOFA 5mg 10mg | UPA 15mg 30mg |
|---|---|---|---|---|
| IL-6/pSTAT1/Monocytes | | | | |
| Average STAT inhibition (%) | 37†††<br>53 | 36†††<br>52 | 43†††<br>59 | 55<br>69*** |
| Time above $IC_{50}$ (h) | 5†††<br>15 | 5†††<br>13 | 9†††<br>17 | 13<br>21*** |

| B. JAK1/TYK2 | FIL 100mg 200mg | BARI 2mg 4mg | TOFA 5mg 10mg | UPA 15mg 30mg |
|---|---|---|---|---|
| IFNα/pSTAT5/CD4+ | | | | |
| Average STAT inhibition (%) | 37†††<br>55 | 41†††<br>59 | 55<br>72*** | 61*<br>76*** |
| Time above $IC_{50}$ (h) | 5†††<br>12 | 7†††<br>16*** | 14<br>22*** | 15**<br>23*** |

| C. JAK1/JAK2 | FIL 100mg 200mg | BARI 2mg 4mg | TOFA 5mg 10mg | UPA 15mg 30mg |
|---|---|---|---|---|
| IFNγ/pSTAT1 Neutrophils | | | | |
| Average STAT inhibition (%) | 15††<br>24 | 24<br>37*** | 31<br>46*** | 40***<br>54*** |
| Time above $IC_{50}$ (h) | 0<br>1 | 1<br>5** | 3<br>11*** | 8***<br>13*** |

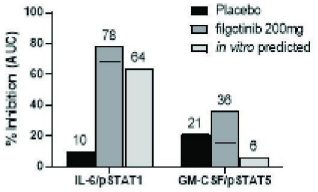
| D. JAK1/JAK3 | FIL 100mg 200mg | BARI 2mg 4mg | TOFA 5mg 10mg | UPA 15mg 30mg |
|---|---|---|---|---|
| IL-4/pSTAT6/CD4+ | | | | |
| Average STAT inhibition (%) | 14†††<br>26 | 20†††<br>35 | 45***<br>63*** | 44***<br>60*** |
| Time above $IC_{50}$ (h) | 0†<br>3 | 0††<br>5 | 10***<br>17*** | 9***<br>15*** |

| E. JAK2/TYK2 | FIL 100mg 200mg | BARI 2mg 4mg | TOFA 5mg 10mg | UPA 15mg 30mg |
|---|---|---|---|---|
| G-CSF/pSTAT3 Monocytes | | | | |
| Average STAT inhibition (%) | 8†<br>16 | 21<br>34*** | 19<br>32*** | 29***<br>43*** |
| Time above $IC_{50}$ (h) | 0<br>0 | 1<br>4*** | 1<br>4** | 4***<br>9*** |

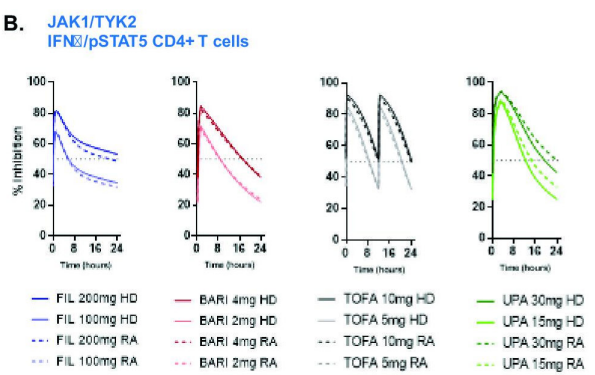
| F. JAK2/JAK2 | FIL 100mg 200mg | BARI 2mg 4mg | TOFA 5mg 10mg | UPA 15mg 30mg |
|---|---|---|---|---|
| GM-CSF/pSTAT5 Monocytes | | | | |
| Average STAT inhibition (%) | 3<br>6 | 10<br>21*** | 8<br>17*** | 27***<br>42*** |
| Time above $IC_{50}$ (h) | 0<br>0 | 0<br>0 | 0<br>0 | 5***<br>9*** |

**Figure 4** Predicted cytokine inhibition profiles of JAKinibs at clinical doses. Pharmacodynamic pSTAT inhibition for JAKinibs at rheumatoid arthritis clinical doses over a 24-hour dose interval at steady state for selected cytokine stimulations based on in vitro blood measurements (n=7–10). (A) JAK1/JAK2 (IL-6/pSTAT1 in monocytes); (B) JAK1/TYK2 (IFNα/pSTAT5 in CD4+ T-cells); (C) JAK1/JAK2 (IFNγ/pSTAT1 in neutrophils); (D) JAK1/JAK3 (IL-4/pSTAT6 in CD4+ T-cells); (E) JAK2/TYK2 (G-CSF/pSTAT3 in monocytes); (F) JAK2/JAK2 (GM-CSF/pSTAT5 in monocytes). Dashed lines show 50% target inhibition. The average daily STAT inhibition and the number of hours per day JAKinib concentrations above 50% inhibition are indicated. FIL values include contribution from the major metabolite of FIL. *P<0.05, **P<0.01, ***P<0.001 higher vs FIL (200 mg); †P<0.05, ††P<0.01, †††P<0.001 lower vs FIL (200 mg). BARI, baricitinib; FIL, filgotinib; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; $IC_{50}$, half maximal inhibitory concentration; IFN, interferon; IL, interleukin; JAK, Janus kinase; JAKinib, JAK inhibitor; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

**A.**




**B.**




**C.**

| | | | RA donors | | | | | | | | Matched HD | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | FIL | | TOFA | | UPA | | BARI | | FIL | | TOFA | | UPA | | BARI | |
| | Cell type | Stimuli/Readout | 100mg | 200mg | 5mg | 10mg | 15mg | 30mg | 2mg | 4mg | 100mg | 200mg | 5mg | 10mg | 15mg | 30mg | 2mg | 4mg |
| JAK1/TYK2 | monocytes | IFNα/pSTAT5 | 42 | 60 | 58 | 73 | 61 | 75 | 41 | 59 | 49 | 65 | 62 | 75 | 66 | 79 | 47 | 63 |
| | CD8+T cells | IFNα/pSTAT5 | 40 | 57 | 58 | 73 | 55 | 69 | 39 | 56 | 49 | 66 | 64 | 78 | 55 | 69 | 44 | 60 |
| | B cells | IFNα/pSTAT5 | 45 | 63 | 59 | 74 | 60 | 74 | 46 | 63 | 47 | 66 | 61 | 76 | 56 | 72 | 48 | 66 |
| | CD4+T cells | IFNα/pSTAT5 | 43 | 60 | 59 | 73 | 60 | 73 | 43 | 60 | 45 | 63 | 61 | 76 | 54 | 69 | 43 | 61 |
| | CD8+T cells | IFNα/pSTAT3 | 44 | 61 | 55 | 70 | 55 | 70 | 44 | 61 | 44 | 61 | 58 | 72 | 50 | 65 | 43 | 59 |
| | CD4+T cells | IFNα/pSTAT3 | 42 | 59 | 55 | 70 | 57 | 71 | 42 | 59 | 43 | 60 | 57 | 72 | 50 | 65 | 43 | 60 |
| | NK | IFNα/pSTAT3 | 46 | 63 | 54 | 69 | 49 | 64 | 40 | 56 | 43 | 60 | 57 | 71 | 51 | 66 | 46 | 63 |
| | B cells | IFNα/pSTAT3 | 38 | 57 | 52 | 68 | 54 | 69 | 41 | 58 | 38 | 58 | 58 | 75 | 47 | 64 | 42 | 61 |
| | monocytes | IFNα/pSTAT3 | 42 | 60 | 55 | 71 | 60 | 74 | 43 | 60 | 35 | 54 | 61 | 76 | 57 | 73 | 48 | 67 |
| | CD4+T cells | IFNα/pSTAT1 | 38 | 51 | 44 | 58 | 48 | 61 | 35 | 48 | 34 | 48 | 46 | 60 | 40 | 53 | 34 | 47 |
| | CD8+T cells | IFNα/pSTAT1 | 25 | 38 | 34 | 47 | 34 | 47 | 24 | 36 | 33 | 46 | 42 | 56 | 36 | 48 | 31 | 43 |
| | B cells | IFNα/pSTAT1 | 24 | 34 | 27 | 38 | 28 | 39 | 20 | 29 | 20 | 30 | 24 | 35 | 24 | 34 | 17 | 26 |
| | NK | IFNα/pSTAT1 | 18 | 30 | 25 | 39 | 21 | 33 | 15 | 26 | 19 | 32 | 32 | 48 | 29 | 43 | 19 | 32 |
| | monocytes | IFNα/pSTAT1 | 15 | 24 | 20 | 32 | 28 | 40 | 14 | 24 | 13 | 23 | 20 | 31 | 24 | 36 | 13 | 22 |
| JAK1/JAK2 | CD4+T cells | IL-6/pSTAT1 | 33 | 48 | 53 | 67 | 52 | 65 | 32 | 47 | 42 | 57 | 52 | 66 | 49 | 63 | 43 | 58 |
| | CD4+T cells | IL-6/pSTAT3 | 28 | 46 | 33 | 50 | 39 | 54 | 27 | 44 | 13 | 26 | 25 | 42 | 23 | 38 | 14 | 28 |
| | B cells | IFNγ/pSTAT1 | 28 | 44 | 42 | 59 | 44 | 60 | 39 | 56 | 30 | 48 | 46 | 63 | 47 | 62 | 46 | 64 |
| | monocytes | IFNγ/pSTAT1 | 6 | 14 | 16 | 30 | 29 | 45 | 15 | 29 | 7 | 15 | 20 | 36 | 30 | 47 | 21 | 38 |
| JAK1/JAK3 | CD4+T cells | IL-15/pSTAT5 | 22 | 38 | 51 | 69 | 49 | 66 | 24 | 41 | 19 | 35 | 54 | 71 | 47 | 63 | 26 | 43 |
| | CD8+T cells | IL-15/pSTAT5 | 15 | 30 | 45 | 64 | 40 | 58 | 18 | 34 | 14 | 27 | 44 | 63 | 38 | 54 | 18 | 33 |
| | NK | IL-15/pSTAT5 | 11 | 22 | 33 | 52 | 25 | 40 | 11 | 22 | 14 | 27 | 43 | 62 | 30 | 46 | 15 | 28 |
| | CD8+T cells | IL-2/pSTAT5 | 31 | 47 | 59 | 74 | 57 | 72 | 35 | 52 | 29 | 46 | 60 | 76 | 52 | 68 | 32 | 49 |
| | CD4+T cells | IL-2/pSTAT5 | 25 | 41 | 53 | 69 | 53 | 69 | 27 | 44 | 21 | 36 | 53 | 70 | 51 | 68 | 25 | 41 |
| | NK | IL-2/pSTAT5 | 16 | 31 | 45 | 64 | 36 | 53 | 17 | 32 | 17 | 32 | 49 | 68 | 39 | 56 | 19 | 35 |
| | NK | IL-4/pSTAT5 | 22 | 36 | 55 | 71 | 49 | 65 | 28 | 44 | 39 | 54 | 62 | 76 | 59 | 73 | 33 | 49 |
| | CD8+T cells | IL-4/pSTAT6 | 18 | 30 | 53 | 70 | 48 | 64 | 26 | 43 | 26 | 41 | 60 | 75 | 56 | 71 | 31 | 47 |
| | monocytes | IL-4/pSTAT6 | 19 | 34 | 44 | 63 | 56 | 73 | 33 | 52 | 25 | 41 | 50 | 68 | 56 | 72 | 37 | 56 |
| | CD4+T cells | IL-4/pSTAT6 | 19 | 33 | 50 | 67 | 50 | 65 | 25 | 41 | 19 | 33 | 54 | 71 | 51 | 67 | 25 | 41 |
| | B cells | IL-4/pSTAT6 | 3 | 6 | 11 | 23 | 16 | 29 | 4 | 9 | 4 | 9 | 13 | 25 | 18 | 31 | 4 | 10 |
| JAK2/TYK2 | monocytes | G-CSF/pSTAT3 | 17 | 30 | 21 | 34 | 28 | 42 | 19 | 32 | 20 | 33 | 35 | 51 | 40 | 55 | 32 | 48 |

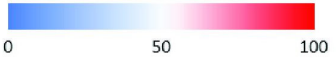



**Figure 5** Cytokine-induced pSTAT inhibition in the samples of patients with RA and in ex vivo stimulated blood from phase 1 studies of healthy volunteers. (A) Measured average inhibition of ex vivo stimulated pSTAT1 (IL-6/CD4+ T-cells) and pSTAT5 (GM-CSF/monocytes) over a 24-hour period in healthy volunteers receiving FIL (200 mg once daily) or placebo or from in vitro calculated values. The black bar indicates placebo-adjusted inhibition. FIL values include contribution of GS-829845. (B) Calculated pSTAT5 inhibition for JAKinibs at RA clinical doses over a 24-hour dose interval at steady state for IFNα/pSTAT5 in CD4+ T-cells in HDs (n=2) and patients with RA (n=3) based on in vitro measurements. (C) Heatmap of average daily percent STAT inhibition (area under the curve (AUC)–24 hours) by JAKinibs at clinical doses in patients with RA and matched HDs (based on in vitro whole blood measurements; n=2–3). BARI, baricitinib; FIL, filgotinib; GM-CSF, granulocyte macrophage colony stimulating factor; HDs, healthy donors; IFN, interferon; IL, interleukin; JAK, Janus kinase; JAKinib, JAK inhibitor; NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; RA, rheumatoid arthritis; SD, standard deviation; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

A.

| Signaling Pair | Cell Type | Stimuli Readout | FIL 100 mg | FIL 200 mg | TOFA 5 mg | TOFA 10 mg | UPA 15 mg | UPA 30 mg | BARI 2 mg | BARI 4 mg |
|---|---|---|---|---|---|---|---|---|---|---|
| JAK1/TYK2 | NK | IFNα/pSTAT5 | 45† | 62 | 54 | 69* | 51† | 65 | 37† | 53† |
| | B cells | IFNα/pSTAT5 | 45† | 62 | 58 | 74* | 63 | 77* | 46† | 63 |
| | CD8+ T cells | IFNα/pSTAT5 | 41† | 59 | 57 | 73* | 61 | 75* | 42† | 60 |
| | NK | IFNα/pSTAT3 | 41† | 58 | 51† | 68* | 51† | 67* | 40† | 58 |
| | monocytes | IFNα/pSTAT5 | 37† | 56 | 55 | 71* | 71* | 83* | 43† | 62 |
| | B cells | IFNα/pSTAT3 | 37† | 55 | 49 | 67* | 57 | 72* | 41† | 59 |
| | CD4+ T cells | IFNα/pSTAT5 | 37† | 55 | 55 | 72* | 61* | 76* | 41† | 59 |
| | CD8+ T cells | IFNα/pSTAT3 | 34† | 52 | 45† | 63* | 54 | 69* | 36† | 54 |
| | neutrophils | IFNα/pSTAT3 | 32† | 48 | 49 | 65* | 60* | 74* | 31† | 47 |
| | CD4+ T cells | IFNα/pSTAT1 | 31† | 45 | 41 | 55* | 49 | 62* | 32† | 45 |
| | CD8+ T cells | IFNα/pSTAT1 | 30† | 43 | 37 | 51* | 43 | 56* | 29† | 42 |
| | CD4+ T cells | IFNα/pSTAT3 | 30† | 47 | 43 | 61* | 53 | 69* | 32† | 50 |
| | monocytes | IFNα/pSTAT3 | 27† | 43 | 43 | 61* | 63* | 77* | 33† | 51* |
| | B cells | IFNα/pSTAT1 | 17† | 27 | 22 | 32* | 26 | 37* | 17† | 26 |
| | NK | IFNα/pSTAT1 | 17† | 28 | 23 | 35* | 24 | 35* | 16† | 26 |
| | monocytes | IFNα/pSTAT1 | 16† | 24 | 22 | 31* | 33* | 43* | 17† | 25 |
| JAK1/JAK2 | CD4+ T cells | IL-6/pSTAT1 | 49† | 65 | 51† | 65 | 52† | 66 | 44† | 59 |
| | monocytes | IL-6/pSTAT1 | 37† | 53 | 43† | 59 | 55 | 69* | 36† | 52 |
| | monocytes | IL-6/pSTAT3 | 5† | 13 | 10 | 21* | 21* | 36* | 6† | 11 |
| | B cells | IFNγ/pSTAT1 | 34† | 49 | 51 | 67* | 56 | 70* | 51 | 67* |
| | neutrophils | IFNγ/pSTAT1 | 15† | 25 | 31 | 46* | 40* | 54* | 24 | 37** |
| | monocytes | IFNγ/pSTAT1 | 8† | 15 | 20 | 35* | 33* | 49* | 20 | 35 |
| JAK1/JAK3 | CD8+ T cells | IL-2/pSTAT5 | 34† | 51 | 65* | 79* | 56 | 71* | 37† | 55 |
| | CD4+ T cells | IL-2/pSTAT5 | 28† | 45 | 61* | 76* | 57* | 72* | 32† | 49 |
| | NK | IL-2/pSTAT5 | 15† | 29 | 41* | 60* | 33 | 49* | 17† | 31 |
| | CD4+ T cells | IL-15/pSTAT5 | 30† | 48 | 66* | 80* | 61* | 76* | 35† | 53 |
| | CD8+ T cells | IL-15/pSTAT5 | 18† | 33 | 53* | 71* | 46* | 63* | 23† | 40* |
| | NK | IL-15/pSTAT5 | 13† | 26 | 40* | 59* | 31 | 48* | 15† | 29 |
| | NK | IL-4/pSTAT6 | 25† | 40 | 58* | 74* | 53* | 68* | 34 | 51* |
| | CD8+ T cells | IL-4/pSTAT6 | 21† | 35 | 55* | 71* | 51* | 67* | 29 | 46* |
| | neutrophils | IL-4/pSTAT6 | 18† | 29 | 39* | 56* | 47* | 62* | 24 | 39* |
| | monocytes | IL-4/pSTAT6 | 14† | 26 | 34* | 52* | 46* | 63* | 25 | 42* |
| | CD4+ T cells | IL-4/pSTAT6 | 14† | 26 | 45* | 63* | 44* | 60* | 20 | 35* |
| | B cells | IL-4/pSTAT6 | 3 | 7 | 14* | 26* | 17* | 28* | 5 | 11 |
| JAK2/TYK2 | monocytes | G-CSF/pSTAT3 | 8† | 17 | 19 | 32* | 29* | 43* | 21 | 34* |
| | neutrophils | G-CSF/pSTAT3 | 2 | 4 | 4 | 9 | 8 | 15* | 4 | 8 |
| | NK | IL-12/pSTAT4 | 6† | 10 | 8 | 13 | 12 | 19* | 10 | 17* |
| | CD8+ m T cells | IL-23/pSTAT3 | 3 | 7 | 6 | 12** | 8 | 15* | 9 | 17* |
| JAK2/JAK2 | neutrophils | GM-CSF/pSTAT5 | 15† | 25 | 33* | 47* | 56* | 69* | 26 | 39* |
| | monocytes | GM-CSF/pSTAT5 | 3 | 6 | 8 | 17* | 27* | 42* | 10 | 21* |

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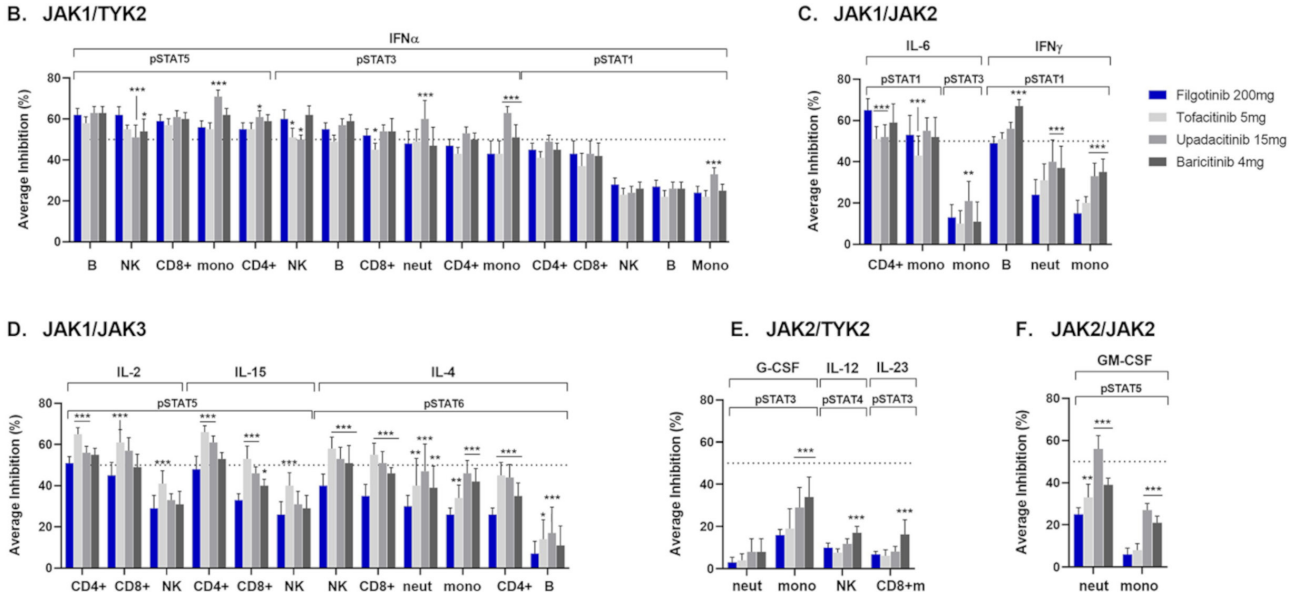


**Figure 6** Calculated cytokine inhibition of JAK-STAT signalling pathways at clinical doses with similar efficacy. (A) Calculated average target inhibition (AUC-24h±SD) over a 24-hour dose interval for selected JAKinibs for a given stimuli/cell type/pSTAT based on in vitro measurements in whole blood from healthy donors (n=7–10). *P<0.05 higher vs FIL 200 mg; †P<0.05 lower vs FIL 200 mg. (B–F) Calculated average target inhibition (AUC-24h±SD) over a 24-hour dose interval for selected JAKinibs at clinical doses with similar efficacy for a given stimulus/cell type/pSTAT based on in vitro measurements in whole blood from healthy donors (n=7–10). The clinical doses represented were FIL (200 mg), TOFA (5 mg), UPA (15 mg) and BARI (4 mg). (B) JAK1/TYK2, (C) JAK1/JAK2, (D) JAK1/JAK3, (E) JAK2/TYK2 and (F) JAK2/JAK2; FIL includes contribution of GS-829845. Dashed lines show 50% target inhibition. *P<0.05, **P<0.01, ***P<0.001 vs FIL (200 mg). AUC-24h, area under the curve 0–24 hours; BARI, baricitinib; FIL, filgotinib; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; IFN, interferon; IL, interleukin; JAK, Janus kinase; JAKinib, JAK inhibitor; mono, monocyte; neut, neutrophil; NK, natural killer; pSTAT, phosphorylated signal transducer and activator of transcription; TOFA, tofacitinib; TYK2, tyrosine kinase 2; UPA, upadacitinib.

For JAK2/JAK2, inhibition of the GM-CSF/pSTAT5 pathway was significantly greater with tofacitinib, upadacitinib and baricitinib in neutrophils ($p<0.01$) and for upadacitinib and baricitinib in monocytes ($p<0.001$) compared with filgotinib (figure 6F). These data are consistent with the observation that upadacitinib and baricitinib inhibit JAK2 signalling at clinically relevant exposures, as seen for IFNγ/pSTAT1 (figure 6A,C) and G-CSF/pSTAT3 (figure 6A,E).

## DISCUSSION

JAKinibs have similar efficacy in RA with distinct safety profiles, potentially reflecting differential JAK isoform selectivity. Enzymatically, JAKinibs have the greatest potency on JAK1, an isoform predominantly involved in inflammatory and innate immune responses.[21 22 26–29] Given that cytokine signalling involves JAK pairs, consideration solely of JAK1 enzymatic inhibition is insufficient to capture the differential effects of heterogeneous cytokine activation. Inhibition of JAK2 and JAK3 may contribute to AEs, given their involvement in regulating immune cell proliferation and homeostasis. In this study, we demonstrated that cytokine signals were differentially inhibited by filgotinib, tofacitinib, upadacitinib and baricitinib in human PBMCs and WB that translated to differential PD inhibition clinically, revealing unique JAKinib pharmacological selectivity profiles. Our key finding is that filgotinib inhibits JAK1-mediated IFNα and IL-6 similar to other JAKinibs at doses demonstrating similar efficacy, but exhibits reduced inhibition of JAK2-dependent and JAK3-dependent pathways, potentially explaining the pharmacological basis for the reported differentiated safety profile.

In cellular assays, JAKinibs showed the greatest potency at inhibiting JAK1-dependent IFNα and IL-6 pathways and are calculated to have comparable average daily inhibition clinically, highlighting a shared JAK1 PD profile. These results are consistent with previously reported data for JAKinibs in PBMCs and blood.[25 27] To our knowledge, this is the first comparison of JAKinib cytokine signalling inhibition of blood from healthy subjects and patients with RA, revealing an overall similar potency and predicted inhibition clinically across a panel of cytokines. Interestingly, IL-6/pSTAT3 inhibition in healthy donors was roughly half that of IL-6/pSTAT1, but in RA donors the predicted daily inhibition was comparable, indicating that some JAKinib responses in RA may not be adequately characterised using healthy donors as a surrogate. Additional studies would be required to verify these initial observations. As IL-6 and IFNα are well-established drivers of autoimmunity,[36–38] and IL-6 is a clinically validated target in RA,[39] the observation that all JAKinibs strongly suppress IL-6 and IFNα signalling with comparable potency implicates these pathways as primary drivers of efficacy in RA.[25 27]

In contrast to the comparable IFNα and IL-6 inhibition, JAKinibs showed significant differences in other JAK1-dependent pathways, reflecting the interplay between cell type, JAK pairing and STAT substrate downstream of different cytokine stimuli. Nuances in cytokine inhibitory profiles for JAKinibs emerged. For example, filgotinib, baricitinib and upadacitinib showed more than threefold weaker inhibition of JAK1/JAK3-dependent IL-15/pSTAT5 pathway relative to IFNα/pSTAT5, whereas tofacitinib showed only a 1.8-fold reduction. Filgotinib showed 6.9-fold weaker activity on JAK1/JAK2-dependent IFNγ/pSTAT1 relative to IFNα/pSTAT5. At clinical exposures, these intrinsic potency differences were amplified. Consistently, there were lower average inhibition and less time above 50% inhibition for filgotinib and baricitinib compared with tofacitinib and upadacitinib on JAK1/JAK3-dependent pathways. On the JAK1/JAK2-dependent IFNγ/pSTAT1 signalling, filgotinib showed lower inhibition than other JAKinibs in all cell types evaluated. This observation contrasts with the comparable inhibition on JAK1/JAK2-dependent IL-6/pSTAT1, indicating a stronger dependence of JAK1 in mediating IL-6/pSTAT1, as previously reported.[6 40] Conversely, IFNγ/pSTAT1 may be more reliant on JAK2, suggesting that, at clinical exposures, upadacitinib and baricitinib inhibit JAK2 more than filgotinib and tofacitinib. The lower inhibition of IFNγ/pSTAT1 compared with IL-6/pSTAT1 in CD14+ monocytes for JAKinibs is consistent with data reported by Dowty *et al*;[25] however, in that study filgotinib showed comparable inhibition with other JAKinibs. This may be related to the use of a single average concentration to determine target coverage, as opposed to an integrated PD area under the curve approach, or use of saturating levels of cytokine stimulus.[25]

JAK2-dependent cytokine signalling pathways were less inhibited than JAK1-dependent pathways for all JAKinibs, with filgotinib showing the least inhibition, including in neutrophils. The low level of inhibition of G-CSF/pSTAT3 in neutrophils is consistent with that observed by others in granulocytes.[25] JAKinibs are associated with serious infections,[16 18–20] so minimising inhibition in neutrophils may maintain antipathogenic function. These data are consistent with the observation that filgotinib is the least potent compound for inhibition of IFNγ/pSTAT1, which is dependent on JAK2.

The data for JAKinib inhibition of JAK2-dependent and JAK3-dependent cytokine responses corroborate previous findings that JAKinibs selectively inhibit cytokines that signal via JAK1 versus JAK2, and tofacitinib and upadacitinib have greater relative inhibition of JAK3-dependent common γ-chain cytokine receptor pathways compared with baricitinib or filgotinib at clinical doses.[24 25 27] Furthermore, our data demonstrate that filgotinib has greater selectivity for cytokine signalling via JAK1 versus JAK2 than baricitinib and upadacitinib. The relatively low clinical inhibition of JAK2-dependent GM-CSF, IL-12, IL-23 and G-CSF is consistent with previous findings that filgotinib did not inhibit these pathways.[25] Despite these shared findings, there were notable differences in reported results. Dowty *et al*[25] claim 'limited differentiation' of JAKinib selectivity; however, it was not statistically powered for comparison, nor did they provide a clinical PD profile of cytokine inhibition. Their approach used an average clinical JAKinib concentration at similarly efficacious therapeutic doses.[25] As inhibitory activity is not linear over a concentration range, methodology using a single average concentration may minimise PD differences, potentially obscuring clinical safety and efficacy implications. Currently, it is unknown what level of target inhibition or time above 50% inhibition could have clinical impact on safety, and subtle differences may be meaningful.

Our data indicate that JAKinibs have clinically differentiated PD profiles that mechanistically may underlie incidence rates of presumed class-effect AEs.[41–48] Selectivity for JAK1 can drive RA efficacy, whereas increasing inhibition of JAK2-dependent and JAK3-dependent pathways may elicit safety liabilities. This is predicted from genetic deletion and clinical data, showing that JAK2 is essential for erythropoiesis, myelopoiesis and platelet production, and JAK3 is critical for lymphocyte proliferation and homeostasis.[49] Compared with other JAKinibs, filgotinib had less inhibition of JAK1-mediated IFNγ (JAK1/JAK2) and IL-2, IL-15 and IL-4 (JAK1/JAK3), JAK2-mediated G-CSF, IL-12 and IL-23 (JAK2/TYK2), and GM-CSF (JAK2/JAK2). The reduced inhibition of JAK2-dependent and JAK3-dependent

cytokine signalling by filgotinib may underlie its lower reported impact on homeostatic immune functions that control NK cells, platelet numbers, anaemia, lymphocyte numbers, infection and HZ (online supplemental table 1).[49–51]

This study has limitations. We established that JAKinib effects are context-dependent and influenced by parameters including cytokine stimulus, STAT substrate and cell type. The interdependence of these parameters needs to be considered when extrapolating these data. We evaluated cytokines that signal via distinct JAK pairs and generalised the results to other cytokines using the same JAK pairs. Our results, however, indicate that the inhibitory effect of each JAKinib is dependent on the specific cytokine stimulus, STAT substrate and cell type, so the association of pathway inhibition with clinical impact would require cell-specific cytokine evaluation. To validate whether the findings in healthy controls would predict cellular effects at clinical exposures, we measured ex vivo effects of IL-6/pSTAT1 and GM-CSF/pSTAT5 in healthy volunteers dosed with filgotinib and showed good agreement with the in vitro-derived extent of inhibition. Although we studied JAKinib cytokine inhibition in blood from patients with RA, the number of donors and the breadth of the cytokine panel were limited. The validity of extrapolating these findings to a broad spectrum of cytokine responses in patients with RA requires further evaluation. While this study provides a potential mechanistic basis for the differential JAKinib rates of AEs from meta-analyses, confirmation would involve head-to-head clinical evaluations.

In conclusion, we demonstrated that, based on in vitro cellular assays and clinical pharmacokinetics, filgotinib is predicted to have a differentiated cytokine PD profile in the clinical setting compared with other JAKinibs. JAK1-dependent IL-6 and IFNα pathways were comparably inhibited by JAKinib doses that have similar efficacy in RA, but filgotinib demonstrated reduced JAK2 and JAK3 activity, providing a potential mechanistic basis for the reported differences in its safety profiles.

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**Contributors** PGT and JAD designed the research. PGT, JAD and FC analysed the data and performed statistical analyses. BM, RG and AM analysed clinical PK and PD data. All authors approved the final manuscript.

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**Competing interests** PGT, BM, FC, AM and JAD are employees of Gilead Sciences, Inc. RG is an employee of Galapagos SASU.

**Patient consent for publication** Not required.

**Ethics approval** The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and Good Clinical Practice guidelines, and was approved by the Independent Ethics Committee or Institutional Review Board. The study was performed in compliance with the ethical principles of good clinical practice and according to the ICH Harmonised Tripartite Guideline. Patients provided written informed consent to participate in the study and had the right to withdraw at any time.

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

**Data availability statement** All data relevant to the study are included in the article or uploaded as supplementary information. Anonymised individual patient data will be available upon request to qualified external researchers 6 months after FDA and European Medicines Agency approval per Gilead's Clinical Trial Disclosure and Data Transparency Policy as posted at https://www.gilead.com/research/disclosure-and-transparency.



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TRANSLATIONAL SCIENCE

# Genetic variants shape rheumatoid arthritis-specific transcriptomic features in CD4$^+$ T cells through differential DNA methylation, explaining a substantial proportion of heritability

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## ABSTRACT

**Objective** CD4$^+$ T cells have been suggested as the most disease-relevant cell type in rheumatoid arthritis (RA) in which RA-risk non-coding variants exhibit allele-specific effects on regulation of RA-driving genes. This study aimed to understand RA-specific signatures in CD4$^+$ T cells using multi-omics data, interpreting inter-omics relationships in shaping the RA transcriptomic landscape.

**Methods** We profiled genome-wide variants, gene expression and DNA methylation in CD4$^+$ T cells from 82 patients with RA and 40 healthy controls using high-throughput technologies. We investigated differentially expressed genes (DEGs) and differential methylated regions (DMRs) in RA and localised quantitative trait loci (QTLs) for expression and methylation. We then integrated these based on individual-level correlations to inspect DEG-regulating sources and investigated the potential regulatory roles of RA-risk variants by a partitioned-heritability enrichment analysis with RA genome-wide association summary statistics.

**Results** A large number of RA-specific DEGs were identified (n=2575), highlighting T cell differentiation and activation pathways. RA-specific DMRs, preferentially located in T cell regulatory regions, were correlated with the expression levels of 548 DEGs mostly in the same topologically associating domains. In addition, expressional variances in 771 and 83 DEGs were partially explained by expression QTLs for DEGs and methylation QTLs (meQTLs) for DEG-correlated DMRs, respectively. A large number of RA variants were moderately to strongly correlated with meQTLs. DEG-correlated DMRs, enriched with meQTLs, had strongly enriched heritability of RA.

**Conclusion** Our findings revealed that the methylomic changes, driven by RA heritability-explaining variants, shape the differential expression of a substantial fraction of DEGs in CD4$^+$ T cells in patients with RA, reinforcing the importance of a multidimensional approach in disease-relevant tissues.

## Key messages

**What is already known about this subject?**

- Rheumatoid arthritis (RA) has a highly polygenic genetic architecture, with nearly 120 reported RA susceptibility loci and a large number of unidentified RA loci.
- The majority of disease variants are located in non-coding elements, most of which significantly overlap with CD4$^+$ T cell regulatory elements. In addition, many genes in RA loci are involved in CD4$^+$ T cell pathways.

**What does this study add?**

- This study provides the landscapes of transcriptomic and methylomic features in RA CD4$^+$ T cells, with catalogues of quantitative trait loci for expression and methylation.
- The integrative approaches using individual-level genetic, epigenetic and transcriptomic data with recent Korean genome-wide RA association statistics dissected the regulatory sources for differentially expressed genes in RA CD4$^+$ T cells, newly suggesting that the RA-risk variant-driven methylation changes result in the differential expression of a large number of the genes in RA CD4$^+$ T cells.

**How might this impact on clinical practice or future developments?**

- Our findings contribute to a better understanding of the CD4$^+$ T cell alterations underlying RA association of non-coding variants and identify disease-relevant gene candidates that may be used for novel therapeutic targets.

## INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease causing chronic symmetrical polyarthritis of large and small joints and mostly occurs in women between 30 and 50 years of age.[1] The causes of RA are not yet fully understood but a family-based genetic approach estimated an overall genetic heritability of RA to be up to 65%.[2] Genome-wide association studies (GWASs) revealed a highly polygenic genetic aetiology of RA, identifying RA-associated common variants in ~120 susceptibility loci.[3–5] Most (~90%) of the RA-risk association signals in RA loci come from non-coding variants. The biological functions of most non-coding RA-risk variants have been largely

unknown,[6] but bioinformatic analyses using non-coding RA-risk variants and cell type-specific features (eg, regulatory annotations and cellular pathways) were highly fruitful in narrowing down disease-relevant cell types in RA pathogenesis.

Several studies reported that the non-coding variants, which were associated with autoimmune disorders, were significantly enriched within enhancers and around expression quantitative trait loci (eQTLs) in immune cell types,[7–10] implying a possible allele-specific regulatory effect of non-coding variants in disease-relevant cell types in the pathogenesis of autoimmune disorders. Several studies on the RA-risk non-coding variants highlighted the importance of $CD4^+$ T cells in RA. The RA-risk variants are strongly enriched in cell type-specific annotations including enhancers, histone modification marks and transcription factor-binding sites (TFBSs) in $CD4^+$ T cells in RA pathogenesis.[5 7 9–12] Indeed, a large number of the genes within RA susceptibility loci are involved in the activation and differentiation pathways of $CD4^+$ T cells.[3 13–15]

Despite the significant enrichment of non-coding disease variants in cell-type specific annotations, only a minor fraction (10% to 20%) of the GWAS signals in autoimmune diseases including RA were directly explained by known eQTLs or TFBSs.[6 16] Therefore, a new approach is needed to understand how the disease variants exert regulatory effects on disease effector genes. Indirect regulatory effects of disease variants through epigenetic changes are likely to be undetectable in limited sample sizes of most eQTL analyses.

Here, we generated genomic, transcriptomic and epigenomic (DNA methylation) data from purified $CD4^+$ T cells in identical patients with RA and healthy controls. This study provides comprehensive landscapes of RA-specific transcriptomic and epigenomic signatures in $CD4^+$ T cells, identifies the variants associated with expression or methylation levels and integrates them with recent Korean GWAS data[4] to understand how RA heritability-explaining variants shape RA-specific differential expression in $CD4^+$ T cells on a genome-wide scale.

## METHODS

### Subjects and $CD4^+$ T cell isolation

A total of 122 study subjects consisting of 82 patients with RA and 40 healthy controls were recruited at Hanyang University Hospital for Rheumatic Diseases (Seoul, South Korea). Sample sizes in each omics data set are summarised in figure 1. All the subjects provided written informed consent for participation. After collecting ~16 cc peripheral blood mononuclear cells using BD Vacutainer CPT, $CD4^+$ T cells were purified within 3 hour (without freezing and thawing) using Invitrogen Dynabeads $CD4^+$ Isolation Kit, and genomic DNA and messenger RNA were extracted. The $CD4^+$ T cell purification method was internally verified to yield a high purity of $CD4^+$ T cells by fluorescence-activated cell sorting and all samples showed >90% purity of $CD4^+$ T cells in a methylation-based cell composition analysis (online supplemental figure S1). All patients with RA were diagnosed according to the American College of Rheumatology 1987 classification criteria for RA.[17] The characteristics of the study subjects at the time of blood sampling are provided in online supplemental table S1.

### Gene expression analysis

The gene expression level in $CD4^+$ T cells was measured using the Illumina HumanHT-12 v4 BeadChip. Normal exponential background correction and quantile normalisation were performed for each slide using limma.[18] A total of 9414 expression probes were retained after a general quality control (QC) procedure (see the details in online supplemental table S2). A batch effect was removed by ComBat implemented in sva[19] using batch variables (slide and array position; online supplemental figure S2). A multivariate linear model was applied using limma to identify differentially expressed genes (DEGs) in RA at a false discovery rate (FDR) threshold of 0.05, controlling for potential confounders (sex, age and T cell purity) and computing moderated statistics by empirical Bayes shrinkage.[18]

| | | | Methylome (n=122) | Transcripotme (n=103) | Genome (n=104) |
|---|---|---|---|---|---|
| | | | Methylation 450K BeadChip (n=122) or MBD-Seq (n=68) | HumanHT-12 v4 BeadChip | Genome-wide SNP array (KoreanChip) |
| | Cases | Controls | | | |
| Sample size (n) | 64 | 26 | ✓ | ✓ | ✓ |
| | 9 | 4 | ✓ | ✓ | |
| | 7 | 7 | ✓ | | ✓ |
| | 2 | 3 | ✓ | | |

**Figure 1** Summary of sample sizes and overlap in three omics data sets. The study subjects consisted of 82 patients with rheumatoid arthritis and 40 healthy controls. Methylome data were generated for all the subjects (n=122) using a methylation array; a subset of the same subjects was used to generate MBD-Seq methylome data. Transcriptome and genome data for subsets (n=103 and 104, respectively) were generated using array technologies. The sample sizes were additionally summarised according to the combination of available omics data sets. MBD-Seq, methyl-CpG-binding domain sequencing.

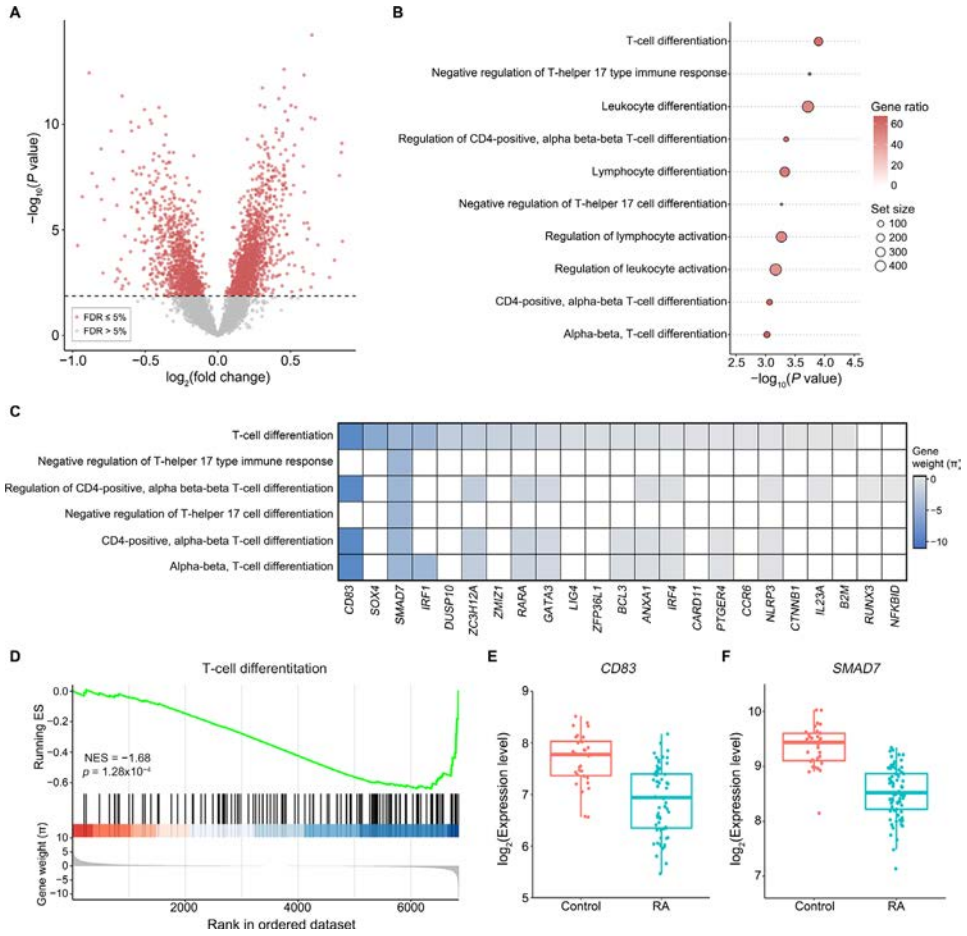


**Figure 2** DEGs in RA CD4$^+$ T cells and DEG-enriched gene sets. (A) The statistical significance level for differential expression (y-axis; a negative $\log_{10}$ scale) was plotted according to the $\log_2$-transformed fold change in expression in patients with RA compared with controls (x-axis). Significant data points above a significance threshold (depicted as a dashed line; FDR of 5%) are marked in red. (B) Dot plot represents the GO terms significantly enriching DEGs in a GSEA. The significance of enrichment is shown on the x-axis. The gene ratio indicates the ratio of the number of input genes to the total number of genes in the gene set (=set size). (C) Heat map shows the $\pi$ values of the genes strongly contributing to the enrichment in T cell pathways. (D) Enrichment plot was created for the GO term, T cell differentiation with normalised enrichment score (NES)=−1.68 and enrichment p value=$1.28\times10^{-4}$. The x-axis represents query genes ordered by their $\pi$ values (shown in the lower part of the plot). The upper part of the plot shows the running enrichment score (ES) that means the sum of ESs from the top ranked gene to a corresponding gene. Bars in the middle part indicate the location of gene members of the query GO term. (E and F) Box plots represent normalised expression levels of (E) *CD83* and (F) *SMAD7* on a $\log_2$ scale. The expressions of both genes were decreased in patients with RA compared with controls (fold change=−0.89 and $P_{FDR}=1.1\times10^{-9}$ for *CD83*; fold change=−0.83 and $P_{FDR}=6.0\times10^{-6}$ for *SMAD7*). DEGs, differentially expressed genes; FDR, false discovery rate; GSEA, gene set enrichment analysis; $P_{FDR,}$ FDR-corrected p values; RA, rheumatoid arthritis.

## Gene set enrichment analysis

For each gene tested in the DEG analysis, $\pi$-value[20] was calculated based on the $\log_2$-fold expression change ($K$) in patients with RA and its significance level ($p$), as follows:

$$\pi = \mathrm{K} \cdot \left(-\log_{10} \mathrm{p}\right)$$

If a gene was analysed by multiple probes, the probe with the lowest $p$ value for the differential expression was used in $\pi$ calculation. A $\pi$-based gene set enrichment analysis (GSEA) for immune-related biological process terms under GO:0002376 was performed using clusterProfiler.[21]

## DNA methylation analysis

DNA methylation data were generated from genomic DNAs in CD4$^+$ T cells using both methyl-CpG-binding domain sequencing (MBD-Seq) for profiling of DNA methylation on a whole-genome scale and the Illumina Infinium Human Methylation 450K BeadChip mostly for profiling of CpG sites around genic regions. In the MBD-Seq data analysis, reads were mapped to the human reference genome hg38 using bowtie2[22] and filtered out with <10 MAPQ values. Methylation peaks were called by MACS2[23] with default parameters and quantified by DiffBind[24] in 304 301 regions found in >1/3 in each group. Differentially methylated peaks (DMPeaks) were examined by DESeq2[25] with the same confounding factors used in the DEG analysis.

In methylation array data analysis, 413 718 CpG-targeting probes passed general QC filters (see the details in online supplemental table S3) and were analysed to identify differentially methylated probes (DMProbes) in RA using ChAMP.[26] Specifically, the fluorescence intensities of array probes were normalised by beta-mixture quantile normalisation[27] and transformed into M value[28] to avoid heteroscedasticity. We eliminated the batch effect using batch variables (data production time, slide and array position) by ComBat implemented in sva[19] (online supplemental figure S3). DMProbes between patients

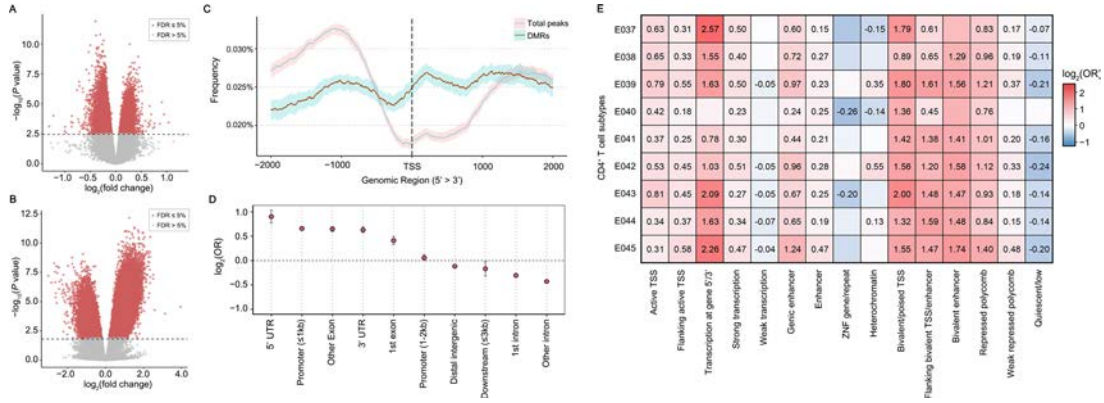


**Figure 3** DMRs and DMR-enriched genomic annotations in RA CD4$^+$ T cells. (A and B) Volcano plots were generated from the DMR analysis results using (A) methylation array and (B) MBD-Seq data. The negative $\log_{10}$-transformed statistical significance level for differential methylation (y-axis) was plotted according to $\log_2$-transformed fold change in methylation in patients with RA compared with controls (x-axis). Significant data points (DMProbes and DMPeaks) above a significance threshold (depicted as a dashed line; FDR of 5%) are marked in red. (C) Distribution of DMPeaks and total MBD-Seq peaks is shown based on the distance from TSSs. The 95% CIs estimated by bootstrapping are shown as shaded areas. (D) ORs for DMRs that are located in each genomic feature are shown with error bars indicating 95% CIs. (E) LOLA analysis results are summarised in the heat map highlighting T cell-specific ChromHMM chromatin states enriched with DMRs. Significant OR values (FDR ≤5%) are shown in the heat map. The Roadmap Epigenomics Project data was used in the analysis (E037, CD4$^+$ memory T cells; E038, CD4$^+$ naïve T cells; E039, CD4$^+$CD25$^-$CD45RA$^+$ T cells; E040, CD4$^+$CD25$^-$CD45RO$^+$ T cells; E041, stimulated CD4$^+$CD25$^-$IL17$^-$ T cells; E042, stimulated CD4$^+$CD25$^-$IL17$^+$ T cells; E043, CD4$^+$CD25$^-$ cells; E044, CD4$^+$CD25$^-$IL127$^-$ $T_{reg}$ cells; E045, CD4$^+$CD25$^-$IL127$^+$ T cells). DMPeaks, differentially methylated peaks; DMProbes, differentially methylated probes; DMRs, differential methylated regions; FDR, false discovery rate; MBD-Seq, methyl-CpG-binding domain sequencing; RA, rheumatoid arthritis; TSSs, transcription start sites.

with RA and controls were investigated under a multivariate linear model considering the same covariates used in the DEG analysis.

Differential methylation regions (DMRs; DMPeaks or DMProbes) with FDR-corrected p values ($P_{FDR}$) <0.05 were considered as significant.

### Profiling the genome-wide landscape of RA-specific DMPeaks

Genome-wide DNA methylation data from MBD-Seq were used to profile the overlap between genomic annotations and DMPeaks in RA. Genomic annotations for CD4$^+$ T cell-specific ChromHMM chromatin states,[29] gene-based positional annotations and TFBSs were retrieved from the Roadmap Epigenomics Project data[30] and ChIPseeker[31] and PAINTOR (https://github.com/gkichaev/PAINTOR_V3.0/wiki/2b.-Overlapping-annotations), respectively. The distribution of DMPeaks around transcription start sites (TSSs) was drawn using ChIPseeker.[31] A Fisher's exact test was performed to assess an enrichment of DMPeaks on a query annotation using LOLA[32] with all methylation peaks as background.

### Correlation analysis between expression level of DEGs and methylation level of DMR

The RA-specific DEGs and DMRs in the same topologically associating domain (TAD) of CD4$^+$ T cells[33] (available at https://osf.io/u8tzp) were tested for their individual-level correlation. In the region not characterised by any TADs, DMRs in a 2 kb region around the TSS of a DEG were used in the pairwise correlation analysis. Significant individual-level correlations between DMR and DEGs were identified in a linear regression controlling for the same covariates used in the DEG analysis, at a gene-level FDR of 5%.

### Analysis of genetic associations with the level of expression and methylation

Linear regression was performed to identify *cis*-QTLs adjusting for sex, age, T cell composition, disease status and the top five genotypic principal components using FastQTL[34] at peak-wise or probe-wise FDR of 5%. *Cis*-variants within 1 Mb of TSSs or methylated regions were used in the analyses. We employed QTLtools[35] to identify trans-QTLs ≥5 Mb away from each target site using the same model in cis-QTL analyses (p value threshold=$1\times10^{-10}$). We retained a subset of independent QTLs by linkage disequilibrium (LD) clumping ($r^2$ >0.2) in each gene or methylated region.

The methylation-mediated effects of methylation QTLs (meQTLs) on regulation of DEGs were assessed by a mediation analysis with quasi-Bayesian CIs[36] at a gene-wide FDR of 5%.

### Estimating heritability of RA partitioned by RA-specific DEGs or DMRs

The enrichment of heritability ($h^2$) of all variants in the identified annotations was estimated using stratified LD score regression[7] based on the 1KGP East Asian LD scores (https://data.broadinstitute.org/alkesgroup/LDSCORE/) and the RA association summary statistics of our recent Korean GWAS.[4] According to the observed distribution of eQTLs and meQTLs in this study, the regulatory genomic region enriched with eQTLs for DEGs or non-DEGs was defined as a 5 kb region around the TSS of each gene. Similarly, the genomic region enriched with meQTLs for DEG-expression-correlated DMRs, expression-uncorrelated DMRs or non-DMRs was defined as a methylation region of interest with a 5 kb buffer region.

Detailed methods of MBD-Seq data analysis, genotyping and whole-genome imputation, validation of eQTLs with public eQTLs, enrichment of QTLs on TFBSs and colocalisation test for RA association and QTL signals are described in online supplemental note.

## RESULTS

### Brief overview of the main analyses

This study consisted of four main analyses to understand RA-specific features in CD4$^+$ T cells at the level of genomics, methylomics and transcriptomics in a single cohort and to scope out the inter-omics relationship in regulating RA-specific DEGs in CD4$^+$ T cells. (1) An expression microarray analysis followed

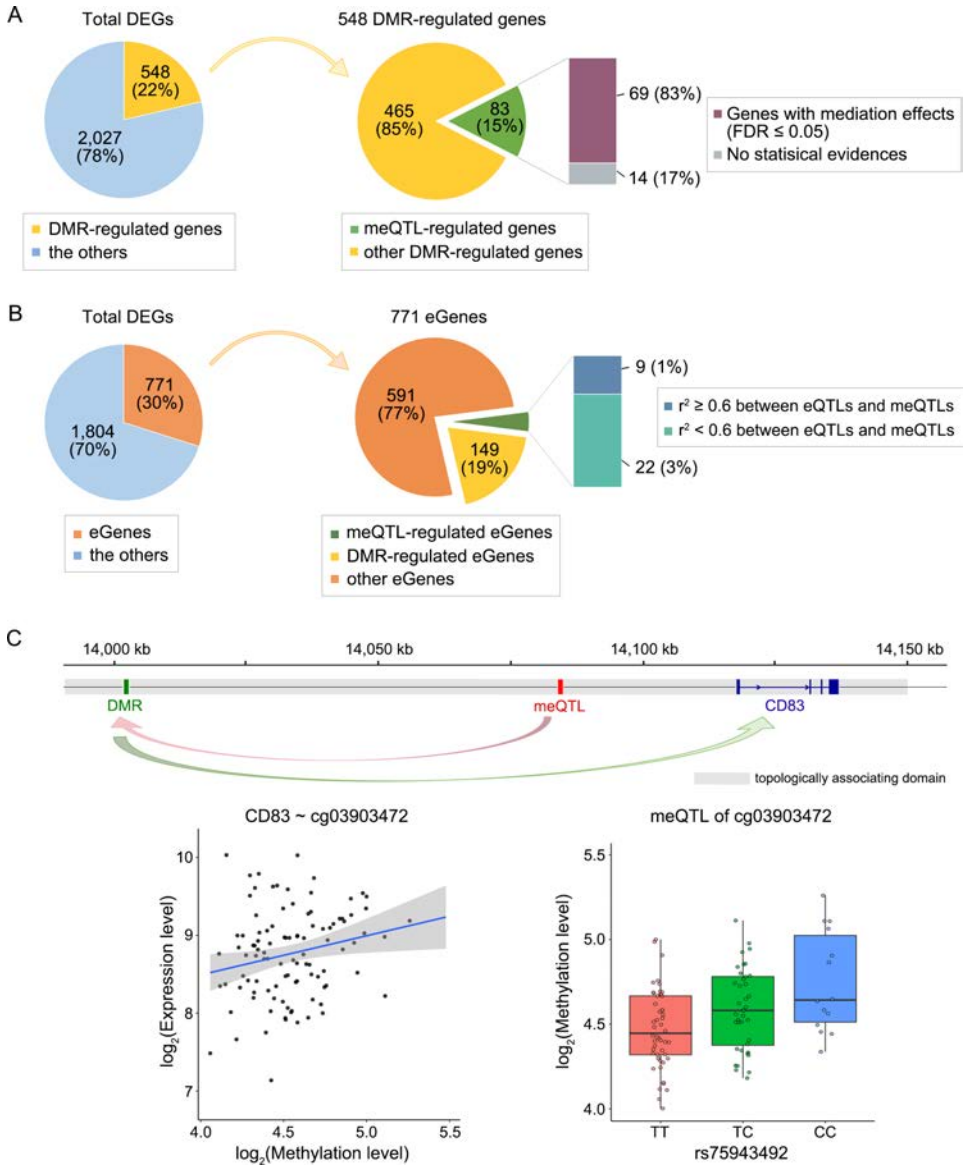


**Figure 4** Summary of regulatory features associated with differential expression in RA CD4$^+$ T cells. (A and B) The numbers of DEGs are shown according to potentially regulatory features; DMRs, *cis*-meQTLs or *cis*-eQTLs. (A) DMR-regulated genes refer to genes whose expression level was significantly correlated with the methylation level of nearby DMRs in the same topologically associating domain (TAD) or the promoter region. If *cis*-meQTLs were detected in the corresponding DMRs, the genes are referred as meQTL-regulated genes. The mediation effects of *cis*-meQTLs on DEGs were estimated using a mediation analysis with quasi-Bayesian CIs. (B) Genes that were regulated by *cis*-eQTLs in this study or other studies were defined as eGenes. The eGenes were stratified into meQTL-regulated and DMR-regulated eGenes according to methylation features associated with eGenes. (C) The expression level of *CD83* was regulated by the meQTL rs75943492, mediated by the differential methylation on the DMProbe cg03903472 (mediation effect=−0.12, $P_{FDR}$=0.01) in a TAD. The associations were significant between expression and methylation levels (beta=0.55, $P_{FDR}$=0.03) and between rs75943492 and methylation level (beta=0.16, $P_{FDR}$=0.02). DEGs, differentially expressed genes; DMProbe, differentially methylated probe; DMRs, differential methylated regions; eQTLs, expression quantitative trait loci; FDR, false discovery rate; meQTLs, methylation QTLs; $P_{FDR,}$ FDR-corrected pvalues; RA, rheumatoid arthritis.

by a GSEA was carried out to identify DEGs and DEG-enriched pathways in CD4$^+$ T cells in patients with RA. (2) DNA methylation analyses using both MBD-Seq and methylation arrays were conducted to determine genome-wide methylation profiles in CD4$^+$ T cells and RA-specific DMRs. (3) QTL analyses were performed by integrating genome-wide variant data with expression and methylation data in CD4$^+$ T cells. (4) An inter-omics analysis was performed to better understand plausible DEG-regulating mechanisms in RA CD4 +T cells and the potential contribution of RA heritability-explaining variants.

## RA-specific DEGs in CD4$^+$ T cells highlighting the differentiation and activation of CD4$^+$ T cells

Expression analyses identified 2575 DEGs in RA CD4$^+$ T cells, based on the differential fluorescence signal of 2785 expression probes. Among the identified DEGs, 1585 and 1200 DEGs were upregulated and downregulated in RA, respectively (figure 2A). In a GSEA, RA-specific DEGs were significantly enriched in 10 immune processes, most of which were T cell-related pathways including lymphocyte activation and CD4$^+$ T cell differentiation (figure 2B–D and online supplemental table S5). The

most significant DEGs included some known genes relevant to T-cell biology (eg, *CD83*,[37 38] *SMAD7*[39] and *IRF1*[40]; figure 2C, E and F). For example, the anti-inflammatory gene *CD83*[37] in an RA susceptibility locus showed an approximate twofold decrease in expression level in patients with RA compared with controls ($\log_2$ fold change=−0.89; $P_{FDR}$=1.1x10$^{-9}$; figure 2E) and belonged to most of the identified pathways including T-cell differentiation, contributing to the pathway enrichment with the largest size of $\pi$-value[20] ($\pi$=−11.0).

### RA-specific DMRs preferentially within regulatory regions

We used MBD-Seq and methylation microarrays to capture the genome-wide methylation architecture in CD4$^+$ T cells in RA, with a high resolution on genic CpG sites. We observed a high correlation between 300bp-bin MBD-Seq read counts and methylation probe intensities (Pearson's $r$=0.75 for cases, 0.71 for controls, (online supplemental figure S4). A total of 94 898 DMPeaks (30.4%; out of 304 301 peaks) and 28 786 DMProbes (7.0%; out of 413 718 probes) in RA were identified in MBD-Seq and microarray data, respectively (figure 3A,B). There was a significant overlap between DMPeaks and DMProbes (p=9.9×10$^{-94}$ in a Fisher's exact test for the region tested in both analyses), showing the concordant direction of methylation changes in >90% of co-localising DMPeak-DMProbe pairs.

The unbiased genome-wide methylation landscape through MBD-Seq revealed how the DNA methylation sites are distributed based on genomic annotation and emphasised the strong enrichment of RA-specific DMRs in the likely regulatory region around TSSs (figure 3C), including the 5' UTR (OR=1.87, p=1.34×10$^{-39}$) and proximal promoters (OR=1.58, p=2.29×10$^{-245}$; figure 3D and online supplemental table S6). Consistently, DMRs were significantly enriched in CD4$^+$ T cell-specific ChromHMM chromatin states[30] associated with transcription-activating, repressing or bivalent regions (figure 3E).

### Identification of QTLs

We identified 2125 *cis*-eQTLs for 682 expression probes, 120 424 meQTLs for 43 526 methylation probes and 23 690 *cis*-meQTLs for 11 998 methylation peaks within 1 Mb of corresponding TSSs or methylated regions. The detected *cis*-eQTLs and *cis*-meQTLs were very closely located to corresponding TSSs and methylated regions, respectively (online supplemental figure S5). Although the sample size and data type[41] might be insufficient to ensure statistical power to detect QTLs, we observed that the identified CD4$^+$ T cell *cis*-eQTLs were highly consistent with publicly available *cis*-eQTLs from the CD4$^+$ T cell RNA-Seq data (online supplemental figure S6). Similarly, by comparing with the publicly available lead meQTLs in whole blood,[42] we found consistent effect sizes in our study (online supplemental figure S7).

We further identified trans-eQTLs for 17 expression probes, 233 trans-meQTLs for 234 methylation probes and 21 trans-meQTLs for 21 methylation peaks outside 5 Mb of the TSSs or methylated sites.

RA variants in many susceptibility loci are correlated moderately to strongly with eQTLs or meQTLs (online supplemental table S7-9). For example, the *cis*-eQTL signals (rs8046707) of FBRS downregulated in RA were statistically showed $r^2$=0.42 with a lead RA variant rs12918327 in the same locus (posterior probability of a shared causal signal >50% in a colocalisation analysis).

In addition, we observed that QTLs were significantly enriched on binding sites of 109 transcription factors. Most of the identified transcription factors also significantly bound within DMRs at FDR of 5% (online supplemental table S10 and figure S8). Some top-ranked transcription factors that preferentially bind to QTLs and DMRs are known to be relevant to RA or T cell functions (eg, MAZ, a Myc-associated protein.[43])

### Methylation-mediation effects of meQTLs on RA-specific DEGs

We integrated the individual-level data of DEGs, DMRs and genome-wide genetic variants to understand the regulatory factors underlying the differential expression of DEGs in RA CD4$^+$ T cells.

A total of 548 RA-specific DEGs (22%) were significantly correlated with RA-specific DMRs in the same TADs or 2 kb windows around their TSSs, at a gene-level FDR of 5%, and eQTLs in this study and other CD4$^+$ T cell eQTL studies[44 45] were detected in 771 DEGs (figure 4A,B). Half of the DMR-methylation-correlated DEGs showed significantly negative correlations with the DNA methylation levels in DMRs, suggesting bivalency of DNA methylation in transcriptional regulation. Indeed, we found that DMRs were significantly localised in bivalent chromatin states bound to both activating and repressing epigenetic regulators (figure 3E). Among DMR-methylation-correlated DEGs, 83 DEGs were regulated by meQTLs. We statistically confirmed the presence of methylation-mediation effects of meQTLs on 69 DEGs (83.1%; out of the 83 DEGs).

Several known immune genes, involved in diverse immune pathways, were detected as potentially meQTL-regulated DEGs mediated by DNA methylation changes. For example, *CD83* was regulated by rs75943492, mediated by a DMProbe at 100 kb upstream of the TSS (mediation effect=0.12, $P_{FDR}$ <5%; figure 4C).

### Variants in differential expression-associated DMRs that explained significantly more heritability of RA

We observed DEGs in 79 loci out of 118 non-HLA RA-risk loci.[4 5 46] In addition, 43 loci with DEGs had DMRs correlated with expression levels of DEGs in CD4$^+$ T cells. Considering the highly enriched meQTLs and eQTLs around methylation sites and TSSs, respectively (online supplemental figure S5), we estimated RA heritability explained by all variants in the 5 kb regions around methylation sites or TSSs to examine the potential contribution of DMR-mediated DEG-regulating meQTLs and DEG-regulating eQTLs to RA susceptibility on a genome-wide scale. Strikingly, a relatively large fraction of RA heritability was explained by variants within RA-specific DMRs whose methylation levels correlated with the expression level of DEGs in RA CD4$^+$ T cells (15.4-fold more heritability than control variants, 8.6-fold more than variants in non-DMRs and 5.3-fold larger than variants in expression-uncorrelated DMRs; figure 5 and online supplemental table S11). The TSS regions with significantly more eQTLs explained a relatively large fraction of RA heritability but the enrichment estimate in DEGs (=33.4) was only 1.6-fold more than that in non-DEGs (=20.5). Although most of the disease association signals are reported to be little correlated with QTL drivers,[33] the genome-wide heritability partitioning analysis strongly suggests a potential regulatory effect of RA variants in DMRs that results in the differential expression of some disease-relevant genes in RA CD4$^+$ T cells and eventually leads to T cell alteration in patients with RA.

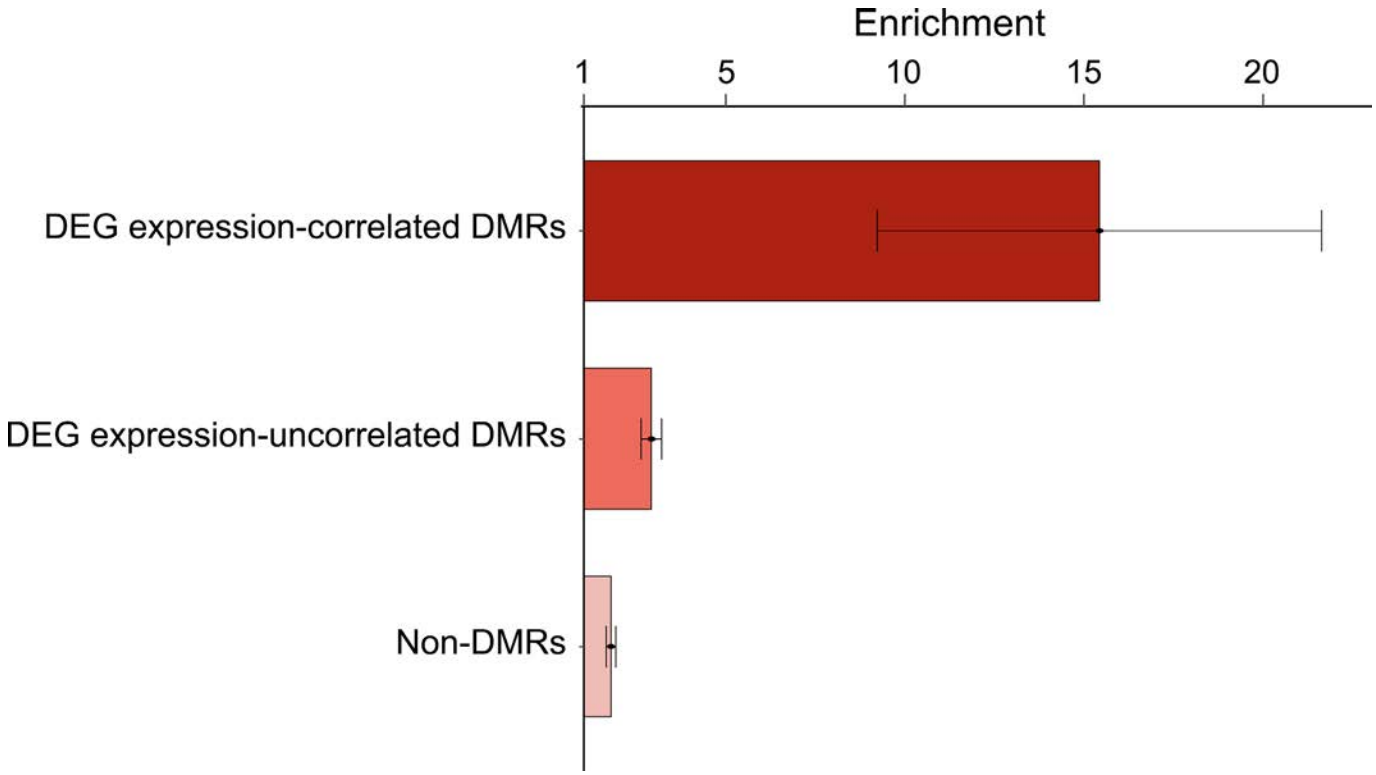


**Figure 5** Large enrichment of partitioned-heritability of RA in DEG expression-correlated DMRs. Enrichment of heritability of RA in three different types of DNA methylated regions was estimated using stratified LDSC. Strong enrichment was detected in DEG expression-correlated DMRs compared with methylated regions irrelevant to DEGs. The SEs of enrichment are shown as error bars. DEGs, differentially expressed genes; DMRs, differential methylated regions; RA, rheumatoid arthritis.

## DISCUSSION

This is the first multi-omics analysis for $CD4^+$ T cells in patients with RA to profile the plausible causal factors underlying RA-specific DEGs using genomic, transcriptomic and epigenetic data in a single cohort. This study identified the differential expression of several key immune regulator genes involved in the proliferation and differentiation of $CD4^+$ T cells. A large number of DMRs were identified in genome-wide and targeted methylation quantification approaches, suggesting that DMRs were preferentially located in highly regulatory elements in $CD4^+$ T cells or TFBSs. A large number of DEGs could be partially explained by meQTL-mediated DMRs or eQTLs and were located in RA susceptibility loci. Nevertheless, it is not common that RA-risk variants are genetically linked in known eQTLs,[33] suggesting a low statistical power in most eQTL analyses and the complex gene regulations involving multiple variants and indirect (epigenetic) regulation. We demonstrated the high enrichment of RA heritability in the region enriched with DMR-mediated DEG-regulating meQTLs.

Our results suggest that several key immune regulators (such as CD83, SMAD7 and GATA3) in $CD4^+$ T cells are involved in the T-cell alteration in RA. For example, we observed decreased expression of *CD83* in patients with RA possibly by a meQTL-mediated DMR. Deficiency of *CD83* in mice downregulates the differentiation of $T_{reg}$ cells[38] and leads to the proliferation of $CD4^+Foxp3^-$ T cells and the differentiation to $T_h1$ and $T_h17$ cells, enhancing the immune response.[37] As another example, *SMAD7*, encoding an inhibitor of TGF-β signalling in $T_{reg}$ differentiation,[39] was downregulated in RA $CD4^+$ T cells. Consistently, a recent study revealed the decreased expression of *SMAD7* in synovial tissues of patients with RA as well as severe joint inflammation in a *SMAD7*-knockout mouse model displaying imbalanced $T_h17/T_{reg}$ responses.[47] As a third example, *GATA3*, which is downregulated in RA $CD4^+$ T cells, is a master regulator of T cell function[48] and a promising target of non-steroidal drugs for the treatment of autoimmune diseases.[49–51]

In conclusion, our findings shed light on how genetic variants can shape the disease-specific transcriptomic signatures in $CD4^+$ T cells in patients with RA, illustrating the advantage of the same-sample inter-omics data analysis on disease-relevant tissues in dissecting the complex transcriptional regulation driven by genome-wide genetic variants.

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**Contributors** KK, SCB and BJK designed the study. SYB, HSL and SCB recruited and characterised patients with rheumatoid arthritis and controls. SYB, JHY, JMK, JBB, HSL, BJK, KK and SCB generated genetic, epigenetic and transcriptomic data. EH, JL, KK and SCB analysed the data and interpreted the results. EH, KK and SCB wrote the manuscript. All authors reviewed and approved the manuscript.

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**Data availability statement** Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as supplementary information. The summary statistics of DEGs, DMRs, eQTLs and meQTLs in this study are available at https://doi.org/10.5061/dryad.w0vt4b8pw.



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CLINICAL SCIENCE

# Role of joint damage, malalignment and inflammation in articular tenderness in rheumatoid arthritis, psoriatic arthritis and osteoarthritis

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## ABSTRACT

**Objectives** To determine whether clinical tenderness can be considered a sign of inflammatory joint activity in patients with rheumatoid arthritis (RA), osteoarthritis (OA) or psoriatic arthritis (PsA) and to assess other possible factors associated with tenderness.

**Methods** Patients diagnosed with RA, PsA and OA underwent clinical and ultrasound examination of wrists and finger joints. Radiographs of the hands were scored for erosions, joint space narrowing (JSN), osteophytes and malalignment. A binary damage score (positive if ≥1 erosion, JSN and/or presence of malalignment) was calculated. Differences in grey scale signs of synovitis and power Doppler (PD) between tender non-swollen (TNS) versus non-tender non-swollen (NTNS) joints were calculated. Disease duration was assessed,<2 years was regarded as early and >5 years as long-standing arthritis.

**Results** In total, 34 patients (9 early and 14 long-standing) from patients with RA, 31 patients (7 early and 15 long-standing) with PsA and 30 with OA were included. We found equal frequencies of PD signal between TNS and NTNS joints in RA (p=0.18), PsA (p=0.59) or OA (p=0.96). However, PD had a significant association with tenderness in early arthritis both in RA (p=0.02) and in PsA (p=0.02). The radiographic damage score showed significant association with tenderness in RA (p<0.01), PsA (p<0.01) and OA (p=0.04).

**Conclusion** Tenderness might not always be a sign of active inflammation in RA, PsA and OA. While tenderness in early arthritis may be more related to inflammation, established disease is better explained by joint damage and malalignment.

## Key messages

**What is already known about this subject?**
- Tender and swollen joint counts are part of disease activity scores for inflammatory arthritides.
- Joint swelling is associated with synovitis and development of radiographic damage.
- Whether tenderness in non-swollen joints can or should be regarded as a sign of inflammation, it is presently unclear.

**What does this study add?**
- We found tenderness in early arthritis to be associated to inflammation, while in established disease, it is better explained by joint damage and malalignment.

**How might this impact on clinical practice?**
- Tenderness in non-swollen joints in patients with long-standing rheumatoid arthritis or psoriatic arthritis should not automatically be regarded as a sign of active disease.

## INTRODUCTION

Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) are chronic inflammatory joint diseases, characterised by swelling, pain, stiffness of joints, as clinical signs of synovitis, and systemic inflammation also mirrored by elevated acute phase reactants.[1–3] Synovitis leads to joint destruction, characterised by bone erosions and cartilage loss.[4 5]

Joint swelling as assessed by clinical examination is generally regarded to denote the presence of synovitis and causes joint damage.[6] In contrast, tender joint counts (TJCs) exhibit a weaker association with radiographic progression and less signs of inflammation detected by various imaging modalities compared with swollen joints.[7–9] At the same time, the TJC has better interobserver reliability and higher sensitivity to change compared with the swollen joint count (SJC).[10] In the 28 joint disease activity score, the TJC even has a higher weight compared with the SJC.[8 10–12] All currently used composite disease activity indices, remission criteria and inclusion criteria into clinical drug trials generally include TJCs.

In the early phases of highly suspicious RA (eg, presence of joint swelling in one or more joints) and for classification of the disease, tenderness is regarded as a feature of inflammatory joint affection.[13] However, tenderness may have causes other than active inflammation, particularly in more established disease such as irreversible joint damage, psychological factors or central sensitisation in the medulla after long-lasting activation of pain signals.[7 14]

Similarly to RA, TJCs are part of most disease activity scores in PsA[15 16] while arthralgia alone in patients with psoriasis is insufficient to diagnose PsA clinically.[17 18] Tenosynovitis, synovitis and enthesitis were detected sonographically in patients

with psoriasis with or without arthralgia and found to be associated with the development of clinically evident PsA.[19 20]

Hand osteoarthritis (OA) is characterised by loss of articular cartilage, joint pain and joint deformation as opposed to synovial swelling.[21] Recent studies however showed that low grade inflammation triggered, for example, by mechanical stress is involved in the pathogenesis of OA.[22 23] Also, osteoarthritic joints are prone to secondary inflammation, which might lead or increase tenderness.[24]

Musculoskeletal ultrasound (US) has been reported to be a sensitive tool for the evaluation of inflammatory joint activity in RA and PsA.[25–29] Using US, joints can be assessed for signs of synovitis, that is, synovial hyperproliferation and synovial effusion detected by grey scale (GS) and hypervascularisation detected by colour or power Doppler (PD). In hand OA, synovitis detected by MRI and US was reported to be associated with pain and radiographic progression.[30 31]

It is presently unclear whether tenderness in non-swollen joints can or should be regarded as a sign of inflammation. The aim of this study was to evaluate, using US to detect subclinical inflammation, whether clinical tenderness in the absence of swelling may be considered a sign of inflammatory joint activity in patients with RA, PsA or OA. As a secondary aim, we wanted to detect other factors that may be associated with joint tenderness.

## METHODS

### Patients

Patients with RA diagnosed according to the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010 criteria,[32] patients with PsA diagnosed according to the CASPAR criteria[18] and patients with OA diagnosed according to the ACR 1990 criteria[33] were recruited from our outpatient clinic. Patients were included, if they had at least one proximal interphalangeal (PIP) or metacarpophalangeal (MCP) joint, which was tender and not swollen. All recruited patients with RA and PsA additionally were included in an observational database where standard variables including joint counts are routinely assessed every 3–5 months and prospectively documented. Details of the database have been published elsewhere.[34 35] For additional subanalyses, patients with RA and PsA were grouped according to disease duration as early disease (duration of less than 2 years) and long-standing disease (disease duration of more than 5 years).

We performed a sample size calculation to detect a difference of positive PD signals between tender non-swollen (TNS) joints and non-tender non-swollen (NTNS) joints of 8.3%. This cut-off was set according to the publication of Hammer *et al*,[36] where TNS and NTNS were PD positive in 17.7% and 9.4%, respectively. Alpha was set at 0.05 and beta at 0.8 for the sample size calculation. Therefore, 263 joints for each disease would be necessary. We aimed to recruit 30 patients for each disease, assuming that 22 joints per patient would yield enough TNS and NTNS to detect a difference of 17.7% versus 9.4%.

### Clinical examination

TJCs and SJCs of 22 joints (bilateral wrists, MCP joints 1–5 and PIP joints 1–5 were recorded of all patients by biometricians, health professionals with more than 5 years of experience performing daily joint counts in patients with arthritides, who were blind with regards to diagnosis as well as with regards to sonographic data. Particular care was taken to only count synovial swelling and not bony swelling. Additionally, a 28-joint count according to the Clinical Disease Activity Index (CDAI)[37] was assessed in RA and a 66/68 joint count according to the CDAI for psoriatic arthritis (cDAPSA)[38] was assessed in PsA. Joints which underwent operation or replacement were excluded from both the clinical and imaging analyses. The CDAI[37] was calculated for each patient with RA and the cDAPSA[38] was calculated for each patient with PsA to quantify disease activity. Furthermore, joints of patients with RA and PsA were tracked back for up to 12 years to identify the time point of last swelling of each joint.

### Ultrasound examination

All patients underwent an US examination of the same 22 joints of both hands on the day of the clinical examination. The examinations were performed on an ESAOTE Mylab Twice ultrasound unit equipped with a high-frequency linear transducer (6–18 MHz) by two rheumatologist sonographers with 3 years of experience (>300 examinations) in musculoskeletal US (IG, MP), who were blinded to clinical diagnosis and examination as well as radiographic data. Scanning was performed according to the EULAR standardised procedures.[39] Longitudinal and transverse scans were performed on the dorsal aspect of each joint using both B-mode (GS) and PD flow. The hand was examined palms down resting on the examination table. Wrist and MCP were examined in the neutral position, while the PIP joints were in slight flexion to ensure that the potentially present synovial hypertrophy is distinguished from the extensor apparatus attaching to the base of the middle phalanx. Copious amounts of gel were used and special care was taken not to apply too much pressure with the US transducer so as not to compress potential Doppler signal (online supplemental figure 1). Doppler gain was adjusted to the level just below random noise; pulse repetition frequency was set between 0.5 and 0.8 MHz.

GS and PD were both scored semiquantitatively (0–3) and as a combined score (EULAR-OMERACT combined synovitis scoring system)[40] according to the OMERACT definitions for sonographic pathology.[41] In case of conflicting grades, the higher grade was selected.

### Structural damage by radiography

X-rays of the hands at the time of the clinical and sonographic examination (±1 year) were evaluated for structural joint damage. Wrists, PIP and MCP joints were scored for erosions and joint space narrowing (JSN) according to the Sharp/van der Heijde method[42] in patients with RA and PsA and according to the Interphalangeal Osteoarthritis Radiographic Simplified (iOARS) scoring system[43] in patients with OA. Additionally, all wrists, PIP and MCP joints were scored for malalignment and osteophytes (presence/absence) in patients with RA, PsA as well as OA. Osteophytes were scored according to the iOARS scoring system.[43] Malalignment was scored as present/absent according to a published atlas of radiographic images[44] and defined as significant joint deviation. In order to assess the probability of tenderness in case of any joint damage, a binary damage score was used, in which a joint was regarded as damaged in case of either erosions (scored 1 or higher) or JSN (scored 1 or higher) or malalignment (presence).

### Statistics

Patient characteristics are described as percentages and frequencies for categorical variables or mean with SD for normally distributed continual variables. Differences in patient characteristics were assessed with Student's t-test or $\chi^2$ test, respectively. Differences in PD and GS signals and the EULAR-OMERACT combined US score between groups (RA vs OA, RA vs PsA, PsA

vs OA) as well as TNS versus NTNS were calculated by $\chi^2$ test. To further increase the power, we combined RA and PsA patients and performed the same analysis.

Kaplan-Meier estimates for the occurrence of the last time point of swelling were compared between TNS and NTNS joints.

To assess interobserver reliability, the recorded, anonymised images of 15 patients each with RA, OA and PsA were independently reviewed after 4 weeks by two rheumatologist experts in musculoskeletal US and scored for GS (0–3) signs of synovitis and PD (0–3) signal. Interobserver agreement was assessed by intraclass correlation. A value of 0–0.4 was interpreted as poor; 0.49–0.59 as fair; 0.60–0.74 as good and 0.75–1.00 as excellent reliability.

A binary logistic regression analysis adjusted for age and sex was performed to assess the association of damage score, JSN, erosions, osteophytes and malalignment with tenderness in non-swollen joints. Furthermore, multivariable logistic regression using a block-wise forward stepwise conditional approach was used to assess tenderness in non-swollen joints. Independent variables included in the analyses were age, sex and disease duration (for RA and PsA) (block 1), erosions, JSN, osteophytes and malalignment (block 2) and GS and PD (block 3).

Additionally, we separately performed logistic analyses adjusted for age and sex assessing the value of PD for TNS in split patient groups according to a disease duration of <2 years and >5 years.

A p value of <0.05 was regarded as significant. No correction for multiple testing was performed. All analyses were performed using SPSS software, V.25 (IBM, Armonk, New York, USA). This study was conducted in accordance with the declaration of Helsinki and approved by the ethics committee of the Medical University of Vienna (no: 1415/2015).

## RESULTS

### Patient characteristics

In total, 745 joints from patients with RA, 682 joints from patients with PsA and 657 joints from patients with OA were included in the study. The majority of RA patients (53.1%) was in moderate disease activity (CDAI of >10 and ≤22)[45] at the time of the US and the clinical examination. Patients with RA were seropositive in 61.8% (21/34). Most patients with PsA (65.5%) were in high disease activity (cDAPSA of >28).[38] The mean disease duration was 7.2±6.6 years for patients with RA and 7.4±6.3 years for patients with PsA (online supplemental table 1). Interobserver reliability for the US examination was excellent with 0.75 (95% CI 0.71 to 0.78) and 0.9 (95% CI 0.88 to 0.91) for GS and PD, respectively.

### Sonographic characteristics of tender non-swollen (TNS) joints

In patients with RA, 155/745 (20.8%) joints were TNS; in PsA and OA, these were 32.2% (219/682) and 19.5% (128/657), respectively. No PD signals at all were observed in the majority of TNS, namely, 85.8% of the TNS in RA, 90.9% TNS in PsA and 89.8% of TNS in OA (p=0.25). No significant difference was seen between PD findings in TNS versus NTNS in RA (14.2% vs 10.2%, respectively; p=0.18), in PsA (9.1% vs 8%, respectively; p=0.59) or in OA (10.2% vs 9.3%, respectively; p=0.96) (figure 1). Similarly, the combined RA and PsA group revealed no significant difference in PD signals between TNS and NTNS (11.2% vs 9.3%, p=0.3) (online supplemental figure 2).

GS synovitis (any grade) was detected more often in TNS joints of patients with PsA as compared with those of patients with RA (64.8% vs 54.2%, respectively; p=0.04); in patients with OA, 65.6% joints showed signs of GS synovitis (OA vs RA: p=0.05; OA vs PsA: p=0.39). TNS showed higher scores compared with NTNS in OA (65.6% vs 58.1%, respectively; p<0.01) but similar scores in PsA (64.8% vs 59.9%, respectively; p=0.10) and RA (54.2% vs 48.4%, respectively; p=0.17) (figure 2).

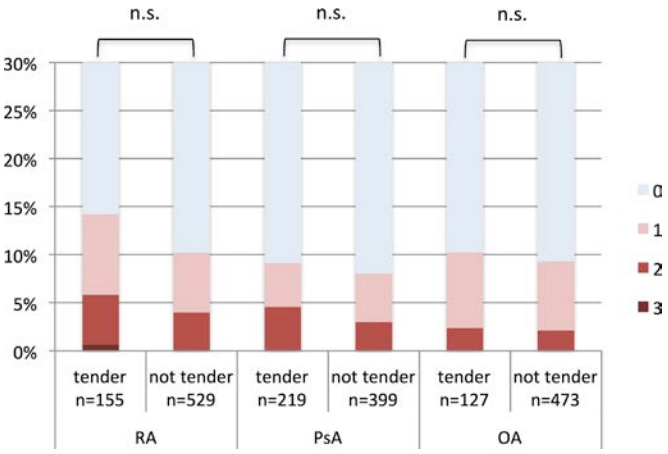


**Figure 1** Power Doppler signals (grade 0, 1, 2 or 3) in tender non-swollen joints (tender) versus non-tender non-swollen joints (not tender) in rheumatoid arthritis (RA), psoriatic arthritis (PsA) and osteoarthritis (OA). Difference between tender non-swollen and non-tender non-swollen joints was calculated for each disease by $\chi^2$ test.

The EULAR-OMERACT score revealed higher scores in OA compared with RA (p=0.02) and PsA (p=0.02) but no difference between RA and PsA (p=0.19). Similar scores were found between TNS and NTNS in RA (TNS: 54.5% vs 48.4%, p=0.14), PsA (TNS: 65.4% vs 60.2%, p=0.08) while TNS had significantly higher EULAR-OMERACT scores compared with NTNS in OA (TNS: 65.6% vs 58.2%, p<0.01) (figure 3).

### Tenderness as sign of past/preceding swelling

Kaplan-Meier analysis showed no difference in the time to last observed swelling between TNS and NTNS joints in patients with RA (62.1±3.3 vs 66.6±2 months, respectively; p=0.40) or PsA (101±6.2 vs 106.4±4.1 months, respectively; p=0.17) (online supplemental figure 3).

### Tender non-swollen (TNS) joints and structural damage

In RA, the mere presence of damage was associated with tenderness in non-swollen joints (OR 1.76, 95% CI 1.16 to 2.66, p<0.01). The binary logistic regression adjusted for age and sex showed an association of TNS with JSN (OR 1.12, 95% CI 1.00 to 1.25; p>0.05), but this was not significant (figure 4A). In PsA, we observed a similar association between tenderness and damage (OR 2.01, 95% CI 1.31 to 3.10; p<0.01), in particular, with malalignment (OR 4.17, 95% CI 1.48 to 11.81; p<0.01) and erosions (OR 1.49, 95% CI 1.05 to 2.10; p=0.03) (figure 4B). Similarly, in OA, the damage score (OR 1.89, 95% CI 1.03 to 3.46; p=0.04) was associated with tenderness. Furthermore, osteophytes (OR 3.52, 95% CI 2.04 to 6.14; p<0.01), malalignment (OR 5.65, 95% CI 2.16 to 14.76; p<0.01), erosions (OR 1.85, 95% CI 1.17 to 2.92; p<0.01) and JSN (OR 1.24, 95% CI 1.02 to 1.49; p=0.03) all had an association with tenderness in OA (figure 4C).

In swollen joints, the damage score showed neither association with tenderness in RA (OR 0.54, 95% CI 0.11 to 2.55; p=0.45) nor in PsA (OR 2.38, 95% CI 0.56 to 10.14; p=0.24).

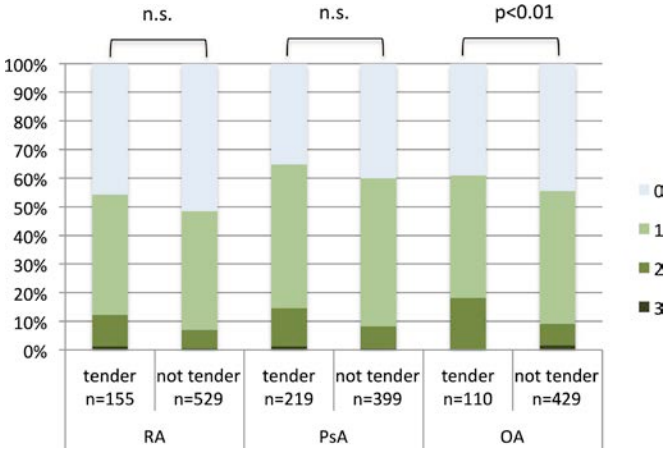


**Figure 2** Grey scale signals (grade 0, 1, 2 or 3) in tender non-swollen joints (tender) versus non-tender non-swollen joints (not tender) in rheumatoid arthritis (RA), psoriatic arthritis (PsA) and osteoarthritis (OA). Difference between tender non-swollen and non-tender non-swollen joints was calculated for each disease by $\chi^2$ test.

### Factors associated with tenderness in non-swollen joints

In non-swollen joints in RA, the block-wise multivariable regression analysis including age, sex and disease duration (block 1), erosions, JSN, osteophytes and malalignment (block 2) and GS and PD (block 3) resulted in exclusion of all variables except for sex (OR 0.40, 95% CI 0.19 to 0.84, p=0.02), JSN (OR 1.12, 95% CI 1.00 to 1.26, p=0.06) and disease duration (OR 1.03, 95% CI 1.00 to 1.07, p=0.07) with female sex being associated with tenderness. In PsA, only malalignment remained as a single variable after the analysis (OR 3.91 95% CI 1.29 to 11.87, p=0.02). In OA, osteophytes (OR 2.05, 95% CI 1.12 to 3.74, p=0.02), malalignment (OR=2.75, 95% CI 0.91 to 8.38, p=0.07), GS (OR 1.56, 95% CI 1.07 to 2.28, p=0.2) and PD (OR 1.05, 95% CI 0.46 to 2.38, p=0.91) remained as variables after the analysis (table 1).

### Tender non-swollen (TNS) joints in early disease

We included 174 joints in 9 patients with RA and 138 joints in 7 patients with PsA in the early disease group, respectively (duration of less than 2 years) and 278 joints in 14 patients with RA and 305 joints in 15 patients with PsA in the long-standing disease group, respectively (disease duration of more than 5 years). In patients with RA with disease duration of less than 2 years, presence of PD had a significant association with tenderness (OR 2.22, 95% CI 1.12 to 4.43, p=0.02) in a binary logistic regression adjusted for age and sex, in contrast to patients with RA with a disease duration of more than 5 years (OR 1.17, 95% CI 0.65 to 2.13, p=0.60). Similarly, in patients with PsA with a short disease duration of less than 2 years, higher PD coincided with a higher likelihood of tenderness (OR 3.26, 95% CI 1.21 to 8.81, p=0.02) while no significant results were

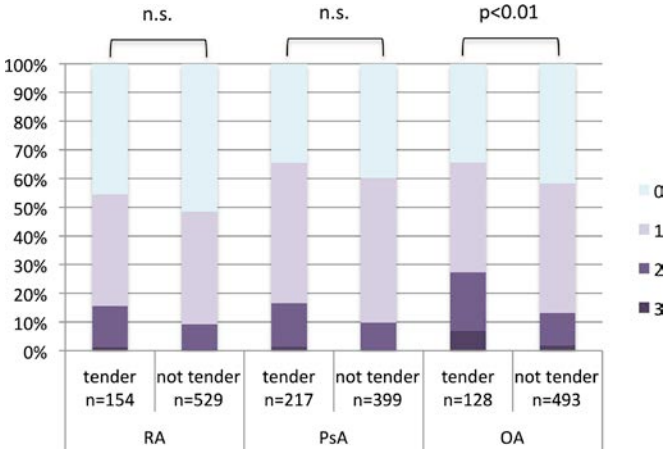


**Figure 3** EULAR-OMERACT combined ultrasound score (grade 0, 1, 2 or 3) in tender non-swollen joints (tender) versus non-tender non-swollen joints (not tender) in rheumatoid arthritis (RA), psoriatic arthritis (PsA) and osteoarthritis (OA). Difference between tender non-swollen and non-tender non-swollen joints was calculated for each disease by $\chi^2$ test.

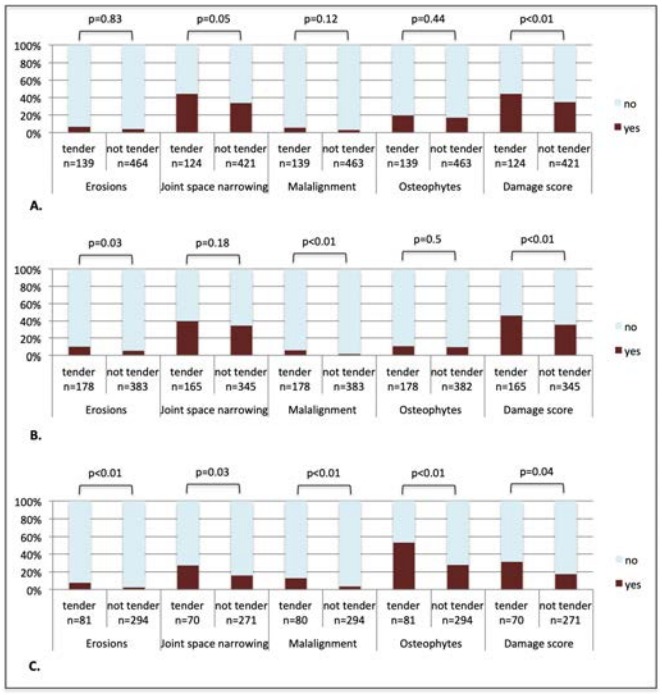


**Figure 4** Difference of detected erosions (0 vs ≥1), joint space narrowing (0 vs ≥1), malalignment (presence/ absence), osteophytes (presence/ absence) and damage score (0 vs ≥1) in patients with rheumatoid arthritis (A), psoriatic arthritis (B) and osteoarthritis (C). Association of erosions (semiquantitatively), joint space narrowing (semiquantitatively), malalignment, osteophytes and damage score with tenderness in non-swollen joints was calculated by age-adjusted and sex-adjusted binary logistic regression.

**Table 1** Remaining variables in the block-wise forward conditional multivariable regression analysis including age, sex and disease duration (block 1), erosions, joint space narrowing (JSN), osteophytes and malalignment (block 2) and Grey scale (GS) and power Doppler (PD) (block 3) for tenderness as the dependent variable in non-swollen joints

| Disease | Variable | OR |
|---|---|---|
| Rheumatoid arthritis | Sex | 0.40, 95% CI 0.19 to 0.84, p=0.02 |
| | JSN | 1.12, 95% CI 1.00 to 1.26, p=0.06 |
| | Disease duration | 1.03, 95% CI 1.00 to 1.07, p=0.07 |
| Psoriatic arthritis | Malalignment | 3.91 95% CI 1.29 to 11.87, p=0.02 |
| Osteoarthritis | Osteophytes | 2.05, 95% CI 1.12 to 3.74, p=0.02 |
| | Malalignment | 2.75, 95% CI 0.91 to 8.38, p=0.07 |
| | GS | 1.56, 95% CI 1.07 to 2.28, p=0.2 |
| | PD | 1.05, 95% CI 0.46 to 2.38, p=0.91 |

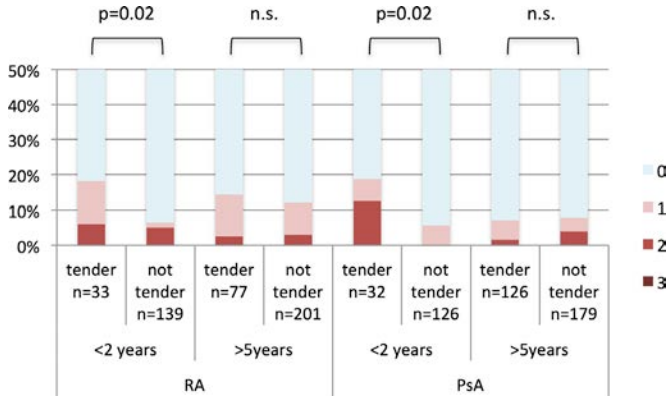


**Figure 5** Difference in power Doppler signal (0–3) between tender not swollen joints (tender) and not tender not swollen joints (not tender) in patients with rheumatoid arthritis (RA) and psoriatic arthritis (PsA) with a disease duration of less than 2 years (<2 years) and more than 5 years (>5 years). Age-adjusted and sex-adjusted logistic regression analysis was performed to determine the association of power Doppler signal on tenderness.

found in patients with PsA with a disease duration of more than 5 years (OR 0.84, 95% CI 0.46 to 1.56, p=0.59) (figure 5).

## DISCUSSION

In this study, we investigated the meaning of tenderness in RA, PsA and OA. Presence of tenderness was not associated with increased sonographic signs of synovitis in non-swollen joints in RA and PsA except for patients with a disease duration of less than 2 years. In contrast, radiographic damage had a significant association with tenderness in non-swollen joints. This suggests that tenderness might only be a sign of inflammation early in the disease course of RA and PsA, while damage has more association with tenderness in established disease.

Recently, several studies have reported poor to no association of tenderness with sonographic signs of synovitis on patient-level as well on joint-level in RA.[36 46 47] Similarly, we found no difference in PD or GS in TNS as compared with NTNS in RA. We could show the same findings in PsA, where to our knowledge this relationship has not yet been evaluated.

Another possible reason for tenderness without swelling is structural damage. When performing this study, we deliberately chose to analyse different diseases in order to see whether we can find any distinctions between the three conditions with regards to joint tenderness. We chose to include patients with inflammatory arthritides (RA and PsA) as well as those with OA which, despite certain inflammatory features, is nonetheless seen as a primarily degenerative condition. While the three diseases are indeed distinct, the key pathologic findings with regards to structural damage and indeed the radiographic methods by which we assess such damage in each of these conditions are indeed overlapping.

Osteophytes in OA were shown to be associated with pain in multiple studies[31 48–50] and secondary OA may cause pain in RA and PsA.[51] In our study, osteophytes were more common in TNS as compared with NTNS joints in OA, but not in RA or PsA. These findings suggest that secondary OA likely does not explain tenderness in patients with RA or PsA.

JSN and erosions are part of various damage scores for PsA[52 53] and RA.[42 54] JSN and erosions are seen in OA and JSN is part of a radiographic damage score for OA.[43] In addition to JSN and erosions, we wanted to assess malalignment to include more possible causes for tenderness. Malalignment or subluxation is caused by instability of the articular capsule and its ligaments as well as incongruity of articular surfaces and as is part of several radiographic damage scores in RA[54 55] and in PsA[52] and may also occur in OA[43] as well. In order to assess whether the probability of tenderness in non-swollen joints is higher in case of any joint damage, we created a binary damage score, in which a joint was regarded as damaged in case of either erosions, JSN or malalignment. Indeed, this combined damage score consisting of these three components had a significant impact on tenderness in all three diseases, also after adjustment for age and sex. Interestingly, this difference was apparent only in non-swollen joints: structural damage was not found to be more common in tender swollen joints compared with TNS joints. In RA, the multivariable regression analysis including age, sex, disease duration, erosions, JSN, osteophytes, malalignment, GS and PD resulted in exclusion of all variables except for sex and JSN, with female sex being associated with tenderness. This underlines the greater impact of radiographic damage compared with synovitis on tenderness in RA. In PsA, only malalignment remained in the analysis, again supporting the hypothesis that damage has more impact than synovitis on tenderness.

TNS joints did not have a shorter time to last observed swelling as compared with NTNS joints. This suggests that in our study, pain memory due to joint swelling within the preceding year does not explain the occurrence of tenderness without swelling.

Interestingly, we saw a significant impact of PD on tenderness in RA and PsA with a disease duration of less than 2 years. Many patients who are ultimately diagnosed with RA have a prodromal phase dominated by pain before the development of synovitis.[56] Sonographic[57 58] and MRI[59] signs of synovitis are seen before the onset of arthritis in patients with and without arthralgia. In an animal model for arthritis, histological signs of synovitis were seen before clinical arthritis.[60] Our results suggest that early in the disease course inflammation and synovitis may explain tenderness.

Another important question regarding tender joints is their predictive value for radiographic progression. Some studies, mostly on patient level, reported that as compared with swelling, tenderness is not or only poorly associated with radiographic progression in RA.[8 61 62] This underlines the findings of our study and other recent studies suggesting that tenderness without swelling may not be a sign of inflammation.[7 34]

Interestingly, in contrast to the results in RA and PsA, tender joints in OA were associated with sonographic signs of synovitis. This has been reported in several studies, although the strengths of such associations vary.[31 63] However, the association of tenderness with osteophytes and malalignment was higher compared to that with GS and PD.

Out study is not without limitations. We did not assess distal interphalangeal joints, which are among the most commonly involved joints in OA and PsA, since we aimed to perform the same assessments in all three diseases. The binary joint damage score, whereby a joint is regarded as damaged, if it exhibits any of the above-mentioned structural changes, needs to be validated in further studies. An additional limitation of our study is its cross-sectional design, which did not allow us to evaluate the predictive utility of tenderness in radiographic progression. Furthermore, we did not assess extraarticular involvement such as enthesitis or tenosynovitis as well as other potential reasons for tenderness like fibromyalgia and other chronic pain syndromes.[64 65] Furthermore, we did not assess fibromyalgia as a potential comorbidity in our study.

In conclusion, structural damage had a higher impact on tenderness in non-swollen joints in RA, PsA and OA. The results of this study suggest that an interpretation of tenderness in established inflammatory arthritides as sign of inflammation may not be appropriate. In early disease and possibly also for diagnostic purposes, tenderness may be used as a potential sign of inflammation.

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**Contributors** GI: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data, drafting the work or revising it critically for important intellectual content. MP: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. VS: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. GS: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. TD: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. MD: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. MH: drafting the work or revising it critically for important intellectual content. ML: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data. PS: drafting the work or revising it critically for important intellectual content. MZ, JSS: drafting the work or revising it critically for important intellectual content. DA: substantial contributions to the conception or design of the work, drafting the work or revising it critically for important intellectual content. PM: substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data, drafting the work or revising it critically for important intellectual content, final approval of the version published, agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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TRANSLATIONAL SCIENCE

# Tenascin-C-mediated suppression of extracellular matrix adhesion force promotes entheseal new bone formation through activation of Hippo signalling in ankylosing spondylitis

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## ABSTRACT

**Objectives** The aim of this study was to identify the role of tenascin-C (TNC) in entheseal new bone formation and to explore the underlying molecular mechanism.

**Methods** Ligament tissue samples were obtained from patients with ankylosing spondylitis (AS) during surgery. Collagen antibody-induced arthritis and DBA/1 models were established to observe entheseal new bone formation. TNC expression was determined by immunohistochemistry staining. Systemic inhibition or genetic ablation of TNC was performed in animal models. Mechanical properties of extracellular matrix (ECM) were measured by atomic force microscopy. Downstream pathway of TNC was analysed by RNA sequencing and confirmed with pharmacological modulation both in vitro and in vivo. Cellular source of TNC was analysed by single-cell RNA sequencing (scRNA-seq) and confirmed by immunofluorescence staining.

**Results** TNC was aberrantly upregulated in ligament and entheseal tissues from patients with AS and animal models. TNC inhibition significantly suppressed entheseal new bone formation. Functional assays revealed that TNC promoted new bone formation by enhancing chondrogenic differentiation during endochondral ossification. Mechanistically, TNC suppressed the adhesion force of ECM, resulting in the activation of downstream Hippo/yes-associated protein signalling, which in turn increased the expression of chondrogenic genes. scRNA-seq and immunofluorescence staining further revealed that TNC was majorly secreted by fibroblast-specific protein-1 (FSP1)+fibroblasts in the entheseal inflammatory microenvironment.

**Conclusion** Inflammation-induced aberrant expression of TNC by FSP1+fibroblasts promotes entheseal new bone formation by suppressing ECM adhesion forces and activating Hippo signalling.

## Key messages

**What is already known about this subject?**

- No targeted and effective treatments have been developed to satisfactorily prevent pathological new bone formation in ankylosing spondylitis (AS).
- Tenascin-C (TNC) is an extracellular matrix protein upregulated in multiple inflammatory conditions. TNC protein synthesis is tightly regulated with restricted distribution in adult tissues.

**What does this study add?**

- TNC is aberrantly upregulated in enthesis and ligament tissues in patients with AS and animal models.
- Genetic ablation and pharmacological inhibition of TNC suppress entheseal new bone formation in animal models.
- Inflammation-induced aberrant expression of TNC by fibroblast-specific protein-1+fibroblasts promotes entheseal new bone formation through suppression of extracellular matrix adhesion force and activation of Hippo signalling.

**How might this impact on clinical practice or future developments?**

- Suppression of aberrant expression of TNC may be a potential therapeutic strategy for prevention of pathological new bone formation in AS.

## INTRODUCTION

Axial spondyloarthritis (SpA) is a chronic inflammatory disease that mainly affects the axial skeleton and has a global prevalence of 0.32%–1.4%.[1 2] SpA includes non-radiographic SpA and radiographic axial SpA, which is also termed ankylosing spondylitis (AS).[1] In addition to inflammatory back pain, spinal ankylosis and immobilisation resulting from entheseal pathological new bone formation are typical features of AS.[3] Given that the affected population is mainly young and middle-aged men, disability caused by AS is a burden to the patients and society, resulting in considerable socioeconomic costs.[4]

Although recent investigations and medications have focused on the suppression of inflammation and pain control, treatment targeting pathological bone formation is lacking, and the prognosis of axial structural damage remains unsatisfactory.[5] The pathogenesis of entheseal pathological new bone formation that consequently leads to bony bridging is not well understood. Although some molecules that are critical for bone formation have been hypothesised in the mechanism of AS,

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including bone morphogenetic proteins,[6] Dickkopf-1[7 8] and Wnt proteins,[9] directly targeting these molecules and related pathways of general bone metabolism might be associated with excessive side effects. Therefore, more precise therapeutic targets that merely function in pathological process with less negative effect on physiological function are needed for the treatment of spinal ankylosis.

Tenascin-C (TNC) is a large molecular extracellular matrix (ECM) glycoprotein hexameric multidomain protein. Upregulation of TNC is noted in multiple inflammatory conditions, including traumatic injuries or light-damaged skin, bacterial infections and asbestos-induced damaged lungs.[10–17] Recent investigations have also reported increase of serum level of TNC in patients with rheumatic diseases,[18] including systemic lupus erythematosus, psoriatic arthritis and AS.[19] However, whether TNC plays a role in the process of entheseal pathological new bone formation is unknown.

In the current study, we found that TNC was aberrantly upregulated in ligament and entheseal tissues from patients with AS and animal models. Systemic neutralisation with specific antibody or genetic ablation of TNC significantly suppressed entheseal pathological new bone formation in animal models. Therefore, TNC might be essential for the development of pathological new bone formation. Investigation of the role of TNC in the process of pathological new bone formation and the underlying molecular mechanism might shed more light on the enigma of axial skeleton ankylosis and propose a potential therapeutic direction for the disease.

## MATERIALS AND METHODS

Additional detailed information is provided in online supplemental file.

### Patients

With ethics committee approval and patient consent, the samples (bone, ligamentum flavum, supraspinatus ligament and interspinous ligament) involved in spinal ankylosis from patients with AS and non-AS patients were collected during surgeries. Twenty-two patients (10 with AS and 12 with non-AS) were enrolled between September 2015 and June 2019. The indications of surgery for patients with AS included disabling kyphosis, loss of horizontal vision without compensation, painful spinal pseudarthrosis or Andersson lesion.[20] Non-AS patients without any systemic inflammatory condition including SpA fulfilled the indications for correction of scoliosis or spinal decompression of thoracic or lumbar stenosis.[21–23]

### Mice

DBA/1 and C57BL/6J mice were purchased from the Charles River Laboratories. The TNC knockout (KO) mouse model (C57BL/6J) was created by Cyagen Biosciences via using CRISPR/Cas-mediated genome engineering. Exon 3–5 of TNC gene (NCBI Reference Sequence: NM_011607.3; Ensembl: ENSMUSG00000028364) were selected as target site.

For spontaneous arthritis model, male DBA/1 mice (8 weeks) were mixed and caged together in groups of nine mice to induce arthritis. For antibody administration, the mice received treatment intraperitoneally once a week with TNC-neutralising antibody (5 mg/kg) (MAB2138, R&D Systems) or the equivalent volume of vehicle antibody (MAB006, R&D Systems) since the second week after caging. For Hippo pathway signalling inhibition, the mice received treatment intraperitoneally three times a week with XMU-MP-1 (2 mg/kg) (HY-100526, a reversible and selective MST1/2 inhibitor, MedChemExpress) since the second week after caging. Dimethyl sulfoxide (DMSO) was administered as a negative control.

For collagen antibody-induced arthritis (CAIA) model, wild-type (WT) and TNC KO C57BL/6J mice (male, 10 weeks) were injected intraperitoneally with Arthrogen-CIA monoclonal antibody cocktail (4 mg/20 g) (Chondrex) on day 0. Then, 100 µg lipopolysaccharide (LPS) was injected intraperitoneally into each mouse on day 3. The equivalent volume of non-specific immunoglobulin (day 0) and LPS (day 3) were used for control purposes.

At the end of each experimental time point, mice were sacrificed. Hind paw specimens were dissected and fixed with 4% paraformaldehyde for $\mu$CT and histological analyses.

### Statistical analysis

Statistical analyses were performed using SPSS V.22.0. All data obtained from experiments repeated at least three times was represented as mean±SD. Differences between two groups were analysed using two-tailed Student's t-test. Comparisons of multiple groups were analysed via one-way analysis of variance. The level of significance was set at $p<0.05$.

## RESULTS

### TNC is upregulated in ligament tissues from patients with AS and animal models

To investigate the molecular mechanism of pathological new bone formation in AS, the spinal ligament tissues were collected from patients with AS and age-matched and sex-matched controls who underwent correction surgeries (figure 1A, online supplemental figure S1A). Immunohistochemical staining showed infiltration of CD68+macrophages and expression of TNFα and IL-17A in the tissue samples from patients with AS, indicating an inflamed status (online supplemental figure S1B–D). RNA sequencing analyses showed significant enrichment of ECM-related GO terms including *Extracellular Matrix Organisation* (GO:0030198) and *Extracellular Structure Organisation* (GO:0043062) in differentially expressed genes (figure 1B). The differentially upregulated genes of these two GO terms were selected for further study (figure 1C). Gene Set Enrichment Analysis showed a high normalised enrichment score of the TNC_TARGETS gene set (figure 1D,E). Immunohistochemical staining and western blot analysis confirmed that TNC was upregulated at the entheses of spinal ligament tissues from patients with AS (figure 1F,G, online supplemental figure S1E). Two standard animal models that mimic the pathological features of entheseal pathological new bone formation of AS were established.[24–29] Entheseal pathological new bone formation was confirmed by $\mu$CT (online supplemental figure S2A–F) and histological staining (online supplemental figure S2G,H). In accordance with the findings from human tissues, TNC was also aberrantly upregulated at the entheseal site of the hind paws in these two animal models (figure 1H–K).

### Inhibition of TNC suppresses entheseal pathological new bone formation

To confirm the critical role of TNC in pathological new bone formation, a TNC-neutralising antibody was administered systemically to DBA/1 mice model 2 weeks after caging and to CAIA model 7 days after immunisation. The results showed that pathological bone formation was significantly suppressed in TNC antibody-treated group, as determined by $\mu$CT analysis, H&E staining and Safranin O Fast Green (SOFG) staining

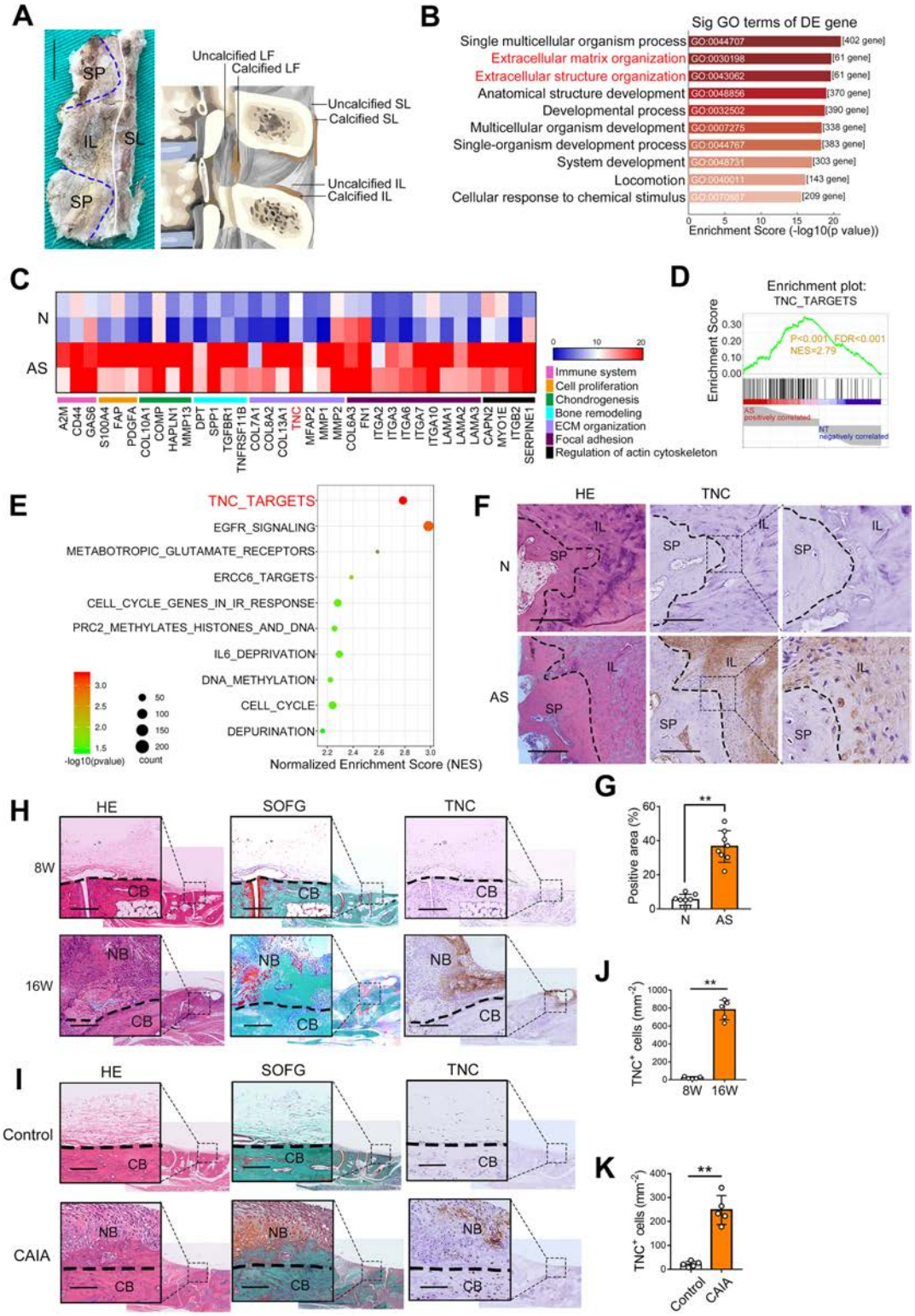


**Figure 1** TNC is upregulated in ligament tissues from patients with AS and animal models. (A) An illustration of spinal ligament tissues collection. Scale bar: 1 cm. (B) GO terms-enriched analysis of differentially expressed genes in AS entheseal tissues (top 10 significant). (C) Heat map of differentially upregulated genes from Extracellular Matrix Organization (GO:0030198) and Extracellular Structure Organization (GO:0043062). (D) Representative GSEA results for TNC_TARGETS gene set. (E) Significantly enriched signalling pathways of GSEA pathway enrichment analysis. (F,G) H&E staining, immunohistochemical analysis and quantitative analysis of TNC and in entheseal tissues from patients with AS and non-AS patients. Scale bar: 300 µm. n=8 per group. (H) H&E staining, Safranin O Fast Green (SOFG) staining and immunohistochemical analysis of TNC in hind paws of male DBA/1 model. Scale bar: 200 µm. (I) H&E staining, SOFG staining and immunohistochemical analysis of TNC in hind paws of CAIA model. Scale bar: 100 µm. (J) Quantitative analysis of TNC-positive cells ($mm^{-2}$) in (H). n=5 per group. (K) Quantitative analysis of TNC-positive cells ($mm^{-2}$) in (I). n=5 per group. Data are presented as mean±SD. **p<0.01, unpaired t-test. AS, ankylosing spondylitis; CAIA, collagen antibody-induced arthritis; CB, cortical bone; ECM, extracellular matrix; FDR, false discovery rate; GSEA, Gene Set Enrichment Analysis; IL, interspinous ligament; LF, ligamentum flavum; N, non-AS patients; NB, new bone; SL, supraspinous ligament; SP, spinous process; TNC, tenascin-C.

(figure 2A–D, online supplemental figure S3A,B). Furthermore, a CAIA model was established in TNC KO mice. The successful creation of TNC KO mice was validated before induction (online supplemental figure S4). TNC KO mice were born alive. Consistent with previous reports, they show no abnormalities physiologically.[30] No significant difference was observed in the appearance and weight of these KO mice compared with the WT mice. However, they did show some abnormal behaviours as reported, such as moving about their cages almost incessantly regardless of the dark-light cycle.[31] The results showed that the development of pathological new bone was dramatically suppressed in $TNC^{-/-}$ CAIA mice, as detected by $\mu$CT analysis, H&E staining and SOFG staining (figure 2E,F).

### TNC is critical for chondrogenesis in the process of endochondral ossification

Cartilage formation was observed in entheseal tissues from both patients with AS and animal models (online supplemental figure S5A,B), which was in accordance with previous reports indicating endochondral ossification as the major mechanism of entheseal pathological new bone formation.[32] Our results further showed that genetic ablation and pharmacological inhibition of TNC had a suppressive effect on the formation of cartilage templates in both DBA/1 model and CAIA model (figure 3A,B, online supplemental figure S5C,D), indicating that TNC was involved in the process of endochondral ossification. Immunofluorescence staining revealed that TNC was expressed around chondrocytes (online supplemental figure S5E). In a cell culture system of human mesenchymal stem cells (hBMSCs) plated on TNC-coated dishes, chondrogenic differentiation was significantly enhanced, as detected by Alcian blue staining, qPCR and western blot (online supplemental figure S5F–H). Moreover, the chondrogenic effect of TNC was suppressed by TNC antibody (figure 3C–H). To confirm the chondrogenic effect of TNC in vivo, BMSCs isolated from $TNC^{+/+}$ and $TNC^{-/-}$ mice were explanted into nude mice recipients (figure 3I). Ablation of TNC in BMSCs led to a significant reduction in chondrogenic potential (figure 3J). Collectively, these results suggest that TNC promotes chondrogenesis in the process of endochondral ossification.

### TNC promotes chondrogenesis by decreasing the matrix adhesion force

The modulation of ECM mechanical properties has been proven to regulate cell fate determination.[33–37] To determine whether the chondrogenic effect of TNC was related to this underlying mechanism, the mechanical properties of tissues from both humans and CAIA model were investigated by atomic force microscopy (figure 4A). Results showed that the adhesion force was significantly decreased in tissues from patients with AS (figure 4B,C). Similarly, the adhesion force was also significantly decreased in entheseal tissues collected from CAIA model. However, this change was much less significant in $TNC^{-/-}$ CAIA model (figure 4D,E), suggesting that TNC deposition and its chondrogenic effect occurred along with a decrease in tissue adhesion force. To confirm that TNC was involved in decreasing the matrix adhesion force, different densities of RGD peptide were plated on Matrigel matrix with or without TNC (figure 4F). TNC significantly reduced the adhesion force of the matrix with RGD (figure 4G). The chondrogenesis of mesenchymal stem cells cultured on high adhesion matrix was suppressed compared with those cultured on low adhesion matrix (figure 4H,I); in the presence of TNC, this suppressive effect was alleviated (figure 4J). These results suggest that TNC promotes chondrogenesis by modulating ECM biomechanical properties, specifically, the adhesion force.

### TNC-mediated reduction in matrix adhesion force activates Hippo/YAP signalling

To investigate the downstream signalling of the TNC-mediated changes in the ECM adhesion force, RNA sequencing of the spinal ligament tissues from patients was conducted and pathway enrichment was analysed. The results revealed a significant enrichment of genes from Hippo signalling pathway (figure 5A). Immunohistochemistry staining showed that phosphorylation of yes-associated protein (YAP), which is an indicator of Hippo signalling activation, was upregulated at the sites where TNC was highly accumulated in the spinal ligament tissues from patients with AS (figure 5B). To confirm the activating effect of TNC on Hippo/YAP signalling, cells were plated on TNC-coated dishes under chondrogenic induction. As expected, the protein levels of pYAP and pLATS1 were upregulated with TNC-coated treatment (figure 5C), and nuclear translocation of YAP was therefore decreased (figure 5D). To further investigate whether TNC activates Hippo signalling through depolymerisation of actin cytoskeleton, lysophosphatidic acid (LPA), a bioactive lipid that promotes actin stress fibre formation,[38] was applied to cells cultured under TNC-coated conditions (figure 5E). Western blot analysis showed that TNC-upregulated pYAP and pLATS1 were decreased by LPA (figure 5F), and nuclear translocation of YAP was therefore increased (online supplemental figure S6A–C). These findings indicate that TNC is involved in actin cytoskeleton depolymerisation, subsequent Hippo pathway activation and YAP degradation.

To confirm that the chondrogenic effect of TNC was dependent on Hippo/YAP pathway activation and downstream phosphorylation and degradation of YAP, YAP was overexpressed in ADTC5 cells cultured under TNC-coated conditions. As expected, YAP overexpression significantly decreased the chondrogenesis induced by TNC (figure 5G,H, online supplemental figure S6D). Consistently, XMU-MP-1, a Hippo/YAP pathway antagonist that decreases the phosphorylation and degradation of YAP, also suppressed the expression of Sox9 and the chondrogenic differentiation of ADTC5 cells (figure 5I, online supplemental figure S6E,F).

To confirm that the activation of Hippo/YAP signalling is involved in pathological new bone formation in vivo, XMU-MP-1 was systemically administered to DBA/1 model. Pathological new bone was reduced in the XMU-MP-1-treated group, and the chondrogenic process was suppressed (figure 5J–M). These results suggest that TNC-induced chondrogenesis depends on actin cytoskeleton depolymerisation-mediated YAP inactivation (online supplemental figure S6G,H).

### TNC is majorly secreted by fibroblast-specific protein-1 (FSP1)+ fibroblasts

Single-cell RNA sequencing (scRNA-seq) of entheseal tissues from CAIA models was performed to explore candidates for TNC-secreting cells. Cluster analysis using t-distributed stochastic neighbour embedding dimensionality reduction identified 10 different cell clusters (figure 6A). TNC was majorly expressed in cluster 1 (fibroblasts) (figure 6B–D). Consistent with the results of scRNA-seq, immunofluorescence staining showed that TNC was majorly co-stained with FSP1, which was a commonly used marker for fibroblasts in tissues from animal models (figure 6E) and patients with AS

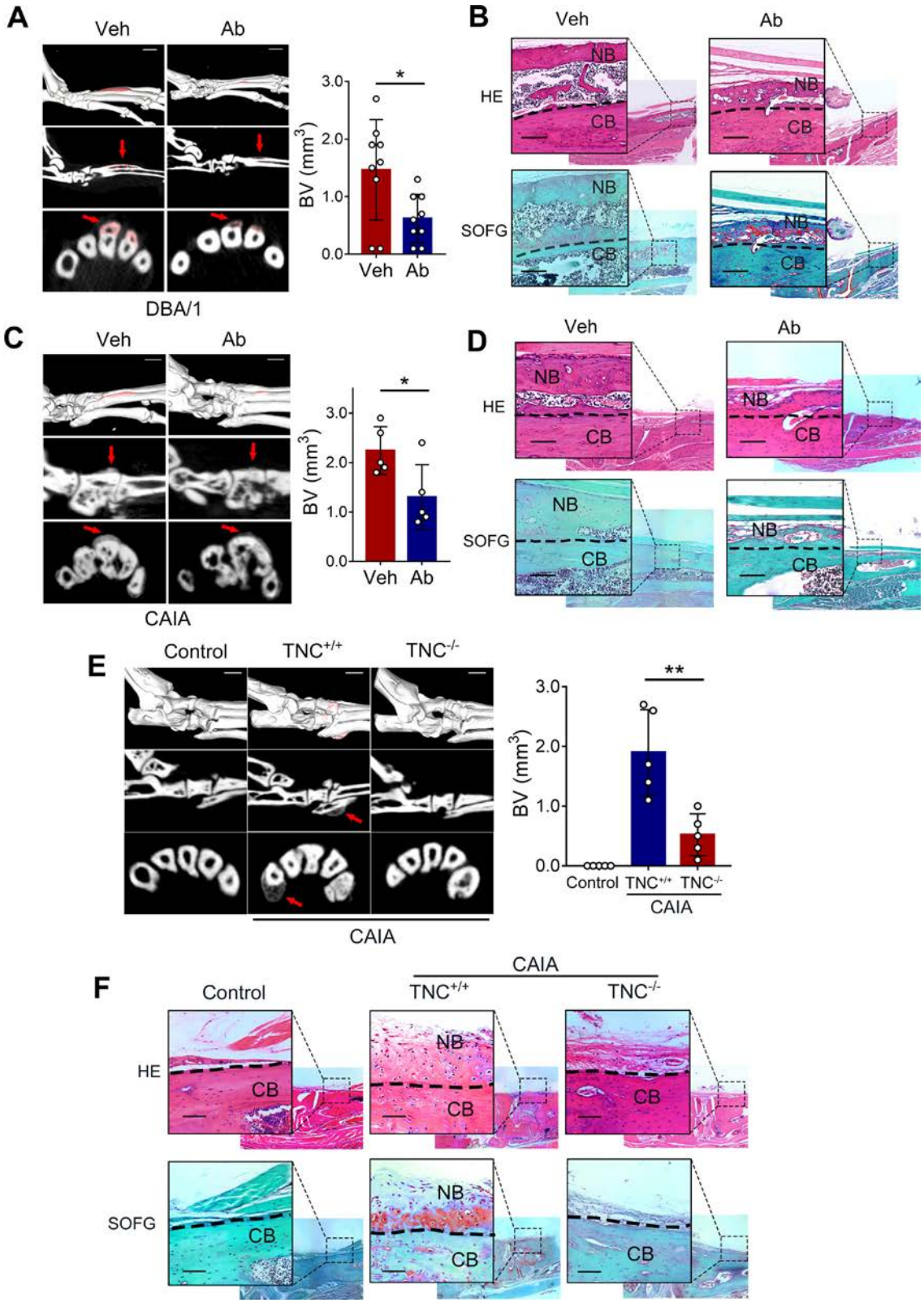


**Figure 2** Inhibition of TNC suppresses entheseal pathological new bone formation. (A) μCT images and quantitative analysis of pathological new bone formation in male DBA/1 model. Red arrows indicate the new bones. n=9 per group. Scale bar: 1 mm. (B) H&E staining and Safranin O Fast Green (SOFG) staining in hind paws of male DBA/1 model at the age of 24 w. Scale bar: 200 μm. (C) μCT images and quantitative analysis of pathological new bone formation in CAIA model. Red arrows indicate the new bone. n=5 per group. Scale bar: 1 mm. (D) H&E staining and SOFG staining in hind paws of CAIA model. Scale bar: 200 μm. (E) μCT images and quantitative analysis of hind paws of control mice and CAIA mice with and without TNC knockout. Red arrows indicate the new bone. n=5 per group. Scale bar: 1 mm. (F) H&E staining and SOFG staining in hind paws of control mice and CAIA mice with and without TNC knockout. Scale bar: 100 μm. Data are presented as mean±SD. *p<0.05, **p<0.01, determined by unpaired, two-tailed Student's t-test. Ab, antibody; BV, bone volume; CAIA, collagen antibody-induced arthritis; CB, cortical bone; NB, new bone; TNC, tenascin-C; Veh, vehicle.

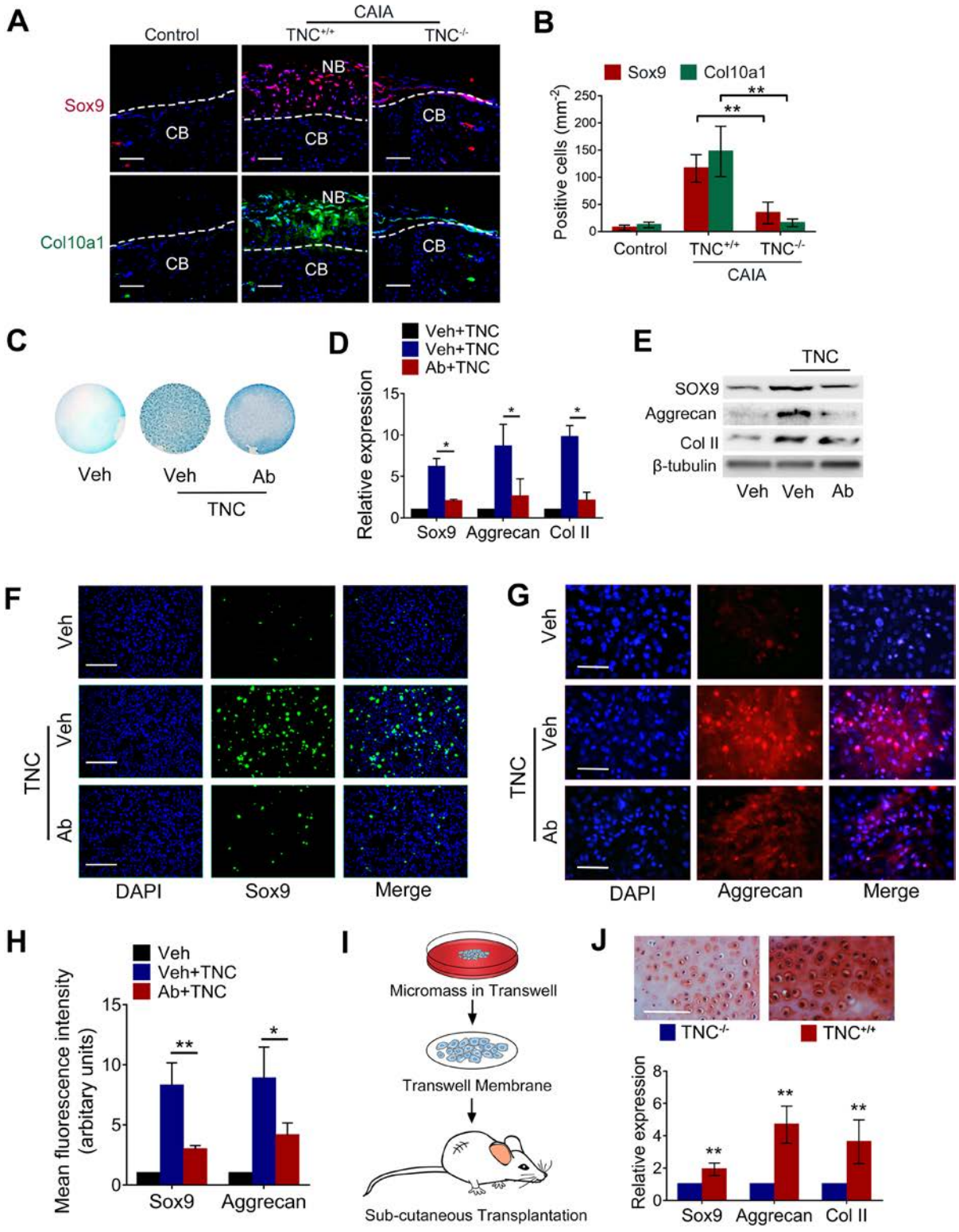


**Figure 3** TNC is critical for chondrogenesis in the process of endochondral ossification. (A) Immunofluorescence staining of Sox9 and Col10a1 in hind paws of control mice and CAIA mice with and without TNC knockout. Scale bar: 100 µm. (B) Quantitative analysis of Sox9-positive and Col10a1-positive cells ($mm^{-2}$) in (A). n=3 per group. (C) Alcian blue staining of human mesenchymal stem cells planted on TNC-coated culture dish in micromass cultures with antibody administration for 7 days. (D,E) Western blot analysis and qRT-PCR analysis of the level of Sox9, Aggrecan, Collagen II in mesenchymal stem cells planted on TNC with antibody in micromass cultures for 48 hours. n=3. (F,G) Immunofluorescence staining of Sox9 (green) and Aggrecan (red) in human mesenchymal stem cells planted on TNC-coated culture dish in micromass cultures with antibody administration for 48 hours. Scale bar: 100 µm in (F). 50 µm in (G). (H) Mean fluorescence intensity of Sox9, Aggrecan in arbitrary units of (F,G). n=3. (I) Schematic diagram illustrating the experimental setup. (J) Safranin O staining and chondrogenic markers expression levels of cartilage-like tissues isolated from mice transplanted with $TNC^{+/+}$ or $TNC^{-/-}$ MSCs. Scale bar: 50 µm. Data are presented as mean±SD. **p<0.01, *p<0.05, unpaired t-test. Ab, antibody; AS, ankylosing spondylitis; CAIA, collagen antibody-induced arthritis; CB, cortical bone; MSCs, mesenchymal stem cells; NB, new bone; TNC, tenascin-C; Veh, vehicle.

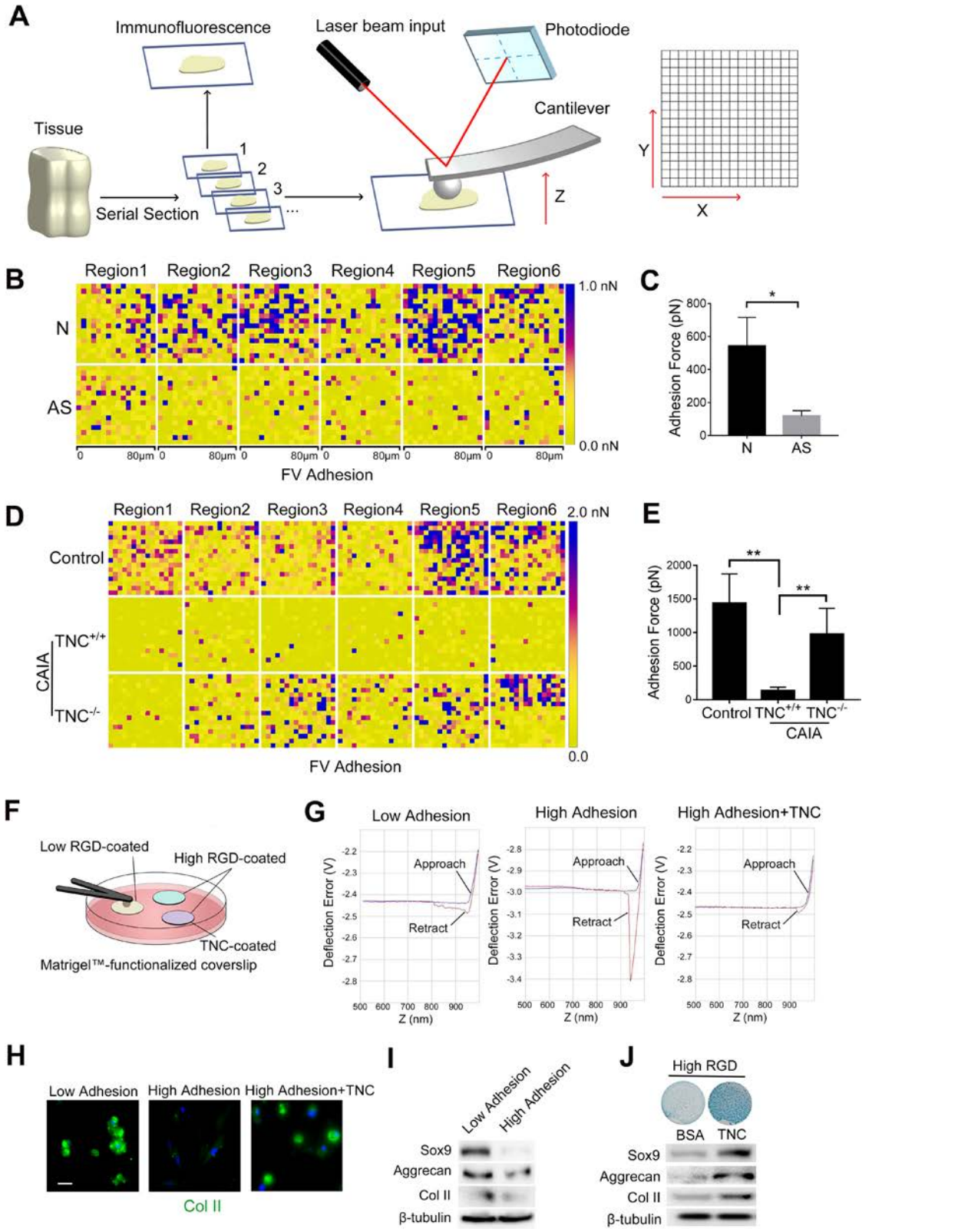


**Figure 4** TNC promotes chondrogenesis by decreasing the matrix adhesion forces. (A) Schematic diagram illustrating the experimental setup. (B) AFM maps of ECM adhesion force in human samples from patients with AS and non-AS patients. (C) Graphs illustrate the adhesion force. n=5 per group. (D) AFM maps of ECM adhesion force in CAIA mice and control mice. (E) Graphs illustrate the adhesion force. n=5 per group. (F) Schematic diagram illustrating the experimental setup. (G) Adhesion force of coated Matrigel as described in (F). (H) Immunofluorescence image of Collagen II (green) in ADTC5 cells plated on Matrigel coated with low or high density RGD for 72 hours. (I) Western blot analysis of Sox9, Aggrecan, Col II protein levels in ADTC5 cells plated on Matrigel coated with low or high density RGD for 72 hours. (J) Alcian blue staining and western blot analysis of Sox9, Aggrecan, Collagen II protein levels in human mesenchymal stem cells in micromass cultures. Data are presented as mean±SD. **p<0.01, *p<0.05, unpaired t-test. AFM, atomic force microscopy; AS, ankylosing spondylitis; BSA, bovine serum albumin; CAIA, collagen antibody-induced arthritis; ECM, extracellular matrix; FV, force volume; RGD, Arg-Gly-Asp; TNC, tenascin-C.

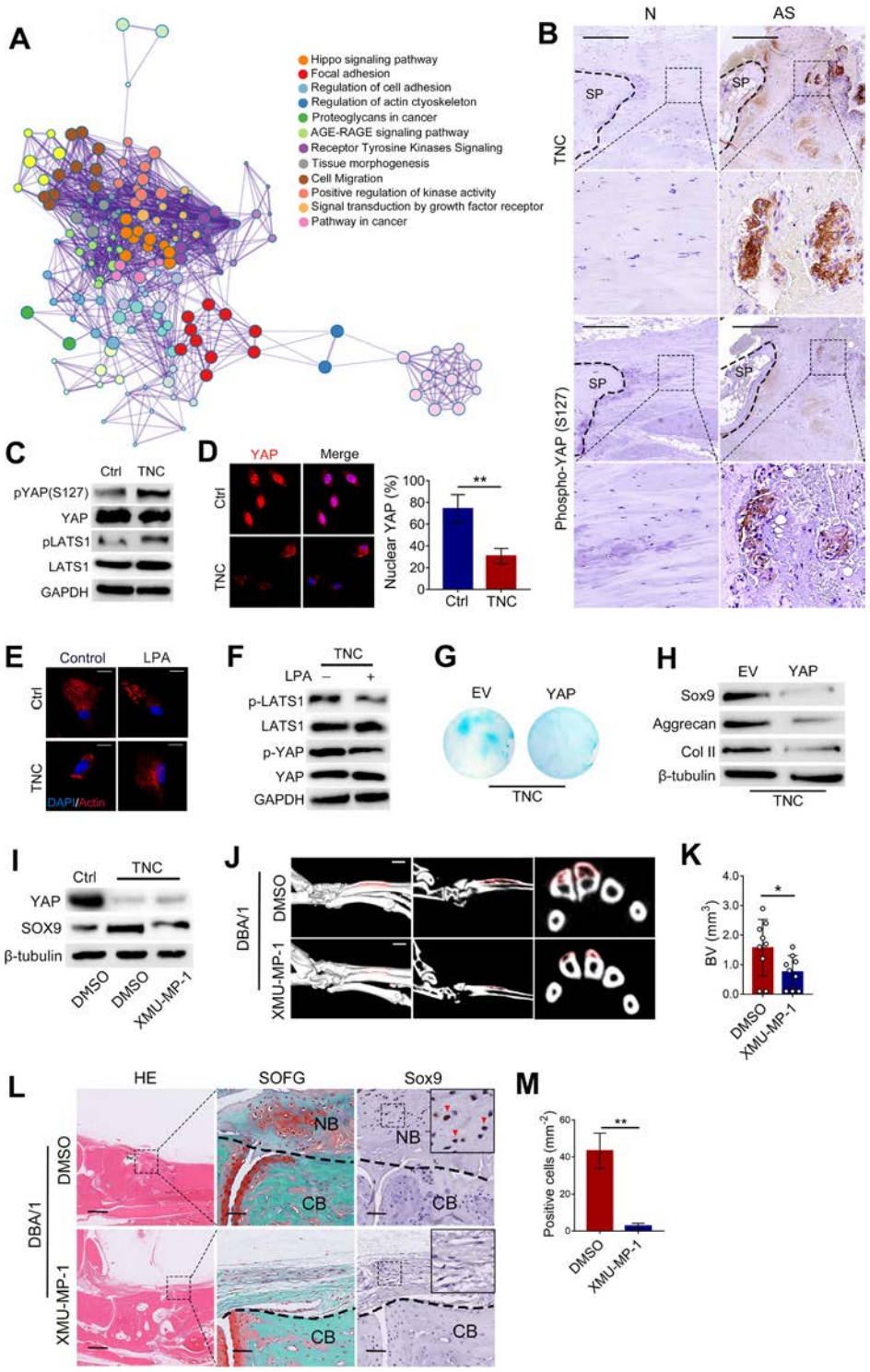


**Figure 5** TNC-mediated reduction in matrix adhesion force activates Hippo/YAP signalling. (A) Pathway enrichment analysis by Metascape of differentially expressed genes in patients with AS. (B) Immunohistochemical staining of phosphorylated YAP (S127) in entheseal tissues. Scale bar: 500 µm. (C,D) Protein levels of phosphorylated YAP (S127), total YAP, phosphorylated LATS1, total LATS1 and immunofluorescence image of YAP in ADTC5 cells plated on TNC for 6 hours. Ctrl: fibronectin 1. Scale bar: 10 µm. n=3 per group. (E,F) Immunofluorescence image of actin polymerisation (phalloidin, red) and nuclei (DAPI, blue) and protein levels of phosphorylated LATS1, total LATS1, phosphorylated YAP, total YAP in ADTC5 cells plated on TNC with or without administration of LPA for 12 hours. Scale bar: 10 µm. (G) Alcian blue staining of ADTC5 cells cultured on TNC transfected with empty vector or YAP-overexpressing vector for 7 days. (H) Protein levels of Sox9, Aggrecan and Collagen II in ADTC5 cells cultured on TNC transfected with empty vector or YAP-overexpressing vector for 48 hours. (I) Protein levels of YAP and Sox9 in ADTC5 cells plated on TNC with application of XMU-MP-1 or DMSO for 48 hours. (J,K) µCT images of new bone formation and quantitative analysis in DBA/1 mice with administration of XMU-MP-1 or DMSO for 16 weeks. Scale bar: 1 mm. n=9 per group. (L,M) H&E staining, SOFG staining, immunohistochemical analysis of Sox9 (indicated by red arrows) and quantitative analysis in DBA/1 mice with administration of XMU-MP-1 or DMSO for 8 weeks. Scale bar: 500 µm. n=5 per group. Data are presented as mean±SD. **p<0.01, *p<0.05, unpaired t-test. AS, ankylosing spondylitis; BV, bone volume; CB, cortical bone; DMSO, dimethyl sulfoxide; LPA, lysophosphatidic acid; N, non-AS patients; NB, new bone; SP, spinous process; TNC, tenascin-C; YAP, yes-associated protein.

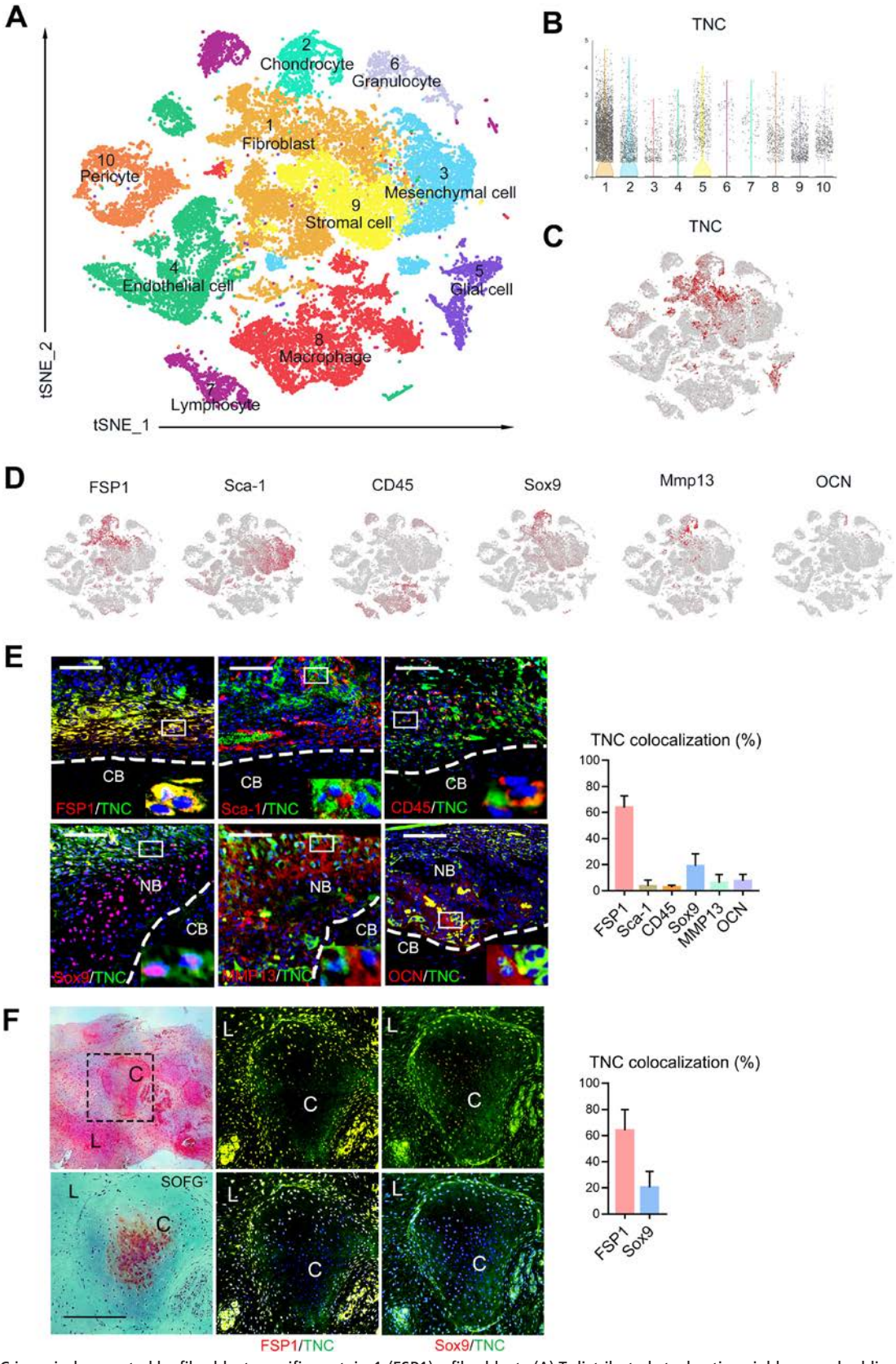


**Figure 6** TNC is majorly secreted by fibroblast-specific protein-1 (FSP1) +fibroblasts (A) T-distributed stochastic neighbour embedding (tSNE) plot of single-cell RNA sequencing from CAIA mice revealed 10 distinct cell clusters. (B) Violin plots of TNC. (C) Feature plot of TNC. (D) Feature plots displaying the single-cell gene expression of fibroblast, stem cells, inflammatory cells, chondrocytes and osteoblasts. (E) Double immunofluorescence staining in CAIA mice, including staining for FSP1, Sca-1, CD45, Sox9, MMP13, OCN and TNC. Scale bar: 100 µm. Semiquantitative analysis of TNC colocalisation. n=3. (F) H&E staining, SOFG staining and double immunofluorescence staining in spinal ligament tissues from patients with AS, including staining for FSP1, Sox9 and TNC. Scale bar: 200 µm. Semiquantitative analysis of TNC colocalisation. n=3. Data are presented as mean±SD. C, cartilage; CAIA, collagen antibody-induced arthritis; CB, cortical bone; L, ligament; NB, new bone; TNC, tenascin-C.

(figure 6F). Human fibroblasts isolated from ligament tissues were stimulated with different inflammatory cytokines related to AS pathogenesis, as identified in previous reports.[3 5] The results showed that TNC was upregulated at both the mRNA and the protein levels by TNFα, IL-17A and IL-22 (online supplemental figure S7A–C).

## DISCUSSION

Pathological new bone formation at the axial skeleton is one of the hallmark features of AS and causes spinal ankylosis, functional impairment and disability.[3] Although numerous efforts have been made to explore the pathogenesis of this disease, the mechanisms underlying pathological new bone formation are not fully understood. In the current study, we identified a secreted matrix protein, TNC, that was aberrantly upregulated in ligament and entheseal tissues from patients with AS and animal models. In addition, we found that genetic ablation and pharmacological inhibition of TNC dramatically suppressed entheseal new bone formation, indicating its essential role in this pathological process.

Various types of bone formation, including endochondral ossification, membranous ossification and chondroid metaplasia, have been described in AS, among which endochondral ossification is considered to be the most important. During this process, new bone formation occurs after the formation of a cartilage template. Chondrocytes differentiate into hypertrophic chondrocytes, which are then replaced by osteoblasts to form mature bone.[32] In the current study, we found that inhibiting TNC retarded the formation of the cartilage template, thereby suppressing subsequent pathological new bone formation. This finding was consistent with previous studies that have suggested the critical role of TNC in chondrogenesis and cartilage formation.[14–16] The findings reveal that TNC-mediated cartilage formation is essential for subsequent pathological new bone formation.

ECM constructs the basic mechanical properties of the tissue microenvironment, including stress, strain, stiffness, elasticity and adhesion.[34] It is accepted that the mechanical properties of tissues profoundly affect the differentiation process of mesenchymal stem cells, including osteogenesis, chondrogenesis and adipogenesis.[35 36] The remodelling of ECM components is a pathological feature of chronically inflamed tissues.[37 39] In this study, we found that aberrant TNC expression modified the ECM adhesion force and the subsequent mechanosignalling. TNC-mediated suppression of matrix adhesion force resulted in reduced nuclear localisation of YAP through the activation of Hippo pathway. Previous studies have shown that YAP is a negative regulator of chondrogenesis.[40 41] Deng *et al*[42] showed that the dephosphorylation and nuclear localisation of YAP inhibited chondrocyte maturation by suppressing Col10a1 expression through interaction with Runx2.[43] Similarly, Goto *et al*[44] found that dephosphorylation and nuclear localisation of YAP impaired chondrocyte proliferation and differentiation through the repression of Sox9. Consistently, we found that the dephosphorylation and nuclear localisation of YAP significantly inhibited chondrogenesis in vitro. Systemic administration of Hippo/YAP antagonist XMU-MP-1 led to a significant suppressive effect on entheseal cartilage formation and subsequent pathological new bone formation. Taken together, these results indicate that aberrant deposition of TNC in the entheseal microenvironment plays a vital role in influencing the mechanical properties of the matrix, resulting in YAP inactivation, and therefore enhancement of endochondral ossification.[45 46]

The relationship between inflammation and new bone formation in AS is still unclear.[5] However, accumulating evidence shows that inflammation is directly involved in the pathological process of new bone formation, including enhancement of osteoinductive protein production and promotion of osteoprogenitor proliferation.[47 48] In this study, we further propose that inflammation potentiates new bone formation by remodelling the ECM. The results of immunohistochemical staining showed infiltration of inflammatory cells and expression of inflammatory cytokines in the tissue samples collected from patients with AS, indicating that the regions of potential pathological new bone are inflamed, which is consistent with previous studies.[49–51] scRNA-seq analysis and immunofluorescence staining revealed that TNC was primarily secreted by FSP1+fibroblasts. FSP1, also known as S100A4, is a widely reported fibroblast marker. FSP1 is mainly expressed in fibroblasts of various organs undergoing tissue remodelling.[52 53] Fibroblasts are the majority of cells in enthesis/ligament tissues and largely proliferate on inflammation stimulation.[24] In addition, fibroblasts are also well acknowledged as secretary cells that produce various ECM proteins or cytokines to participate in the regulation of the microenvironment during multiple pathological processes.[52] In AS, a previous study showed that fibroblast-rich granulation tissue promotes new bone formation.[54] In an in vitro study, we confirmed that fibroblasts produced large amounts of TNC under the stimulation of various AS-associated inflammatory cytokines, including TNFα, IL-17A and IL-22. Taken together, it suggests that inflammation-induced aberrant TNC expression and TNC-mediated ECM remodelling contribute to the formation of an osteoinductive microenvironment and potentiate new bone formation via alteration of tissue mechanical cues. In addition, the fact that multiple inflammatory cytokines can induce TNC production explains, to a certain extent, the low pharmacological efficacy on the radiographic progression of patients with AS of antibodies that neutralise a single cytokine, such as adalimumab, ustekinumab and risankizumab.[47 48]

TNC is as an intriguingly multifunctional molecule that exhibits diverse roles in immunity, such as in the promotion of bacterial adhesion and thrombosis, in the regulation of innate and adaptive immunity, and in the control of ECM synthesis and remodelling during tissue repair.[55] Normally, TNC expression is precisely regulated. During physiologic responses to injuries or infection, it is induced at sites of inflammation and peaks once tissue rebuilding commences and down-regulates concomitant with the resolution of inflammation and tissue repair.[55] On the contrary, during abnormal wound-healing responses or pathologies associated with persistent inflammation, prolonged expression of TNC is observed. Abnormal regulation of TNC expression is found responsible for the long-lasting inflammation and pathological rebuilding in many diseases.[11 55] In the studies of rheumatoid arthritis (RA), TNC has been demonstrated as an endogenous activator of Toll-like receptor 4, which is responsible for maintaining inflammation and joint destruction.[18 56] TNC has also been shown to exhibit proinflammatory effects by activating α9 integrins in macrophages, resulting in the production of various proinflammatory molecules in the development of arthritis.[57] In addition, post-translationally citrullinated TNC achieved increased immunogenicity of the C-terminal residues, leading to the generation of autoantibodies in patients with RA.[58] Recently, serum level of TNC was reported to be elevated in patients with AS and associated with disease activity,[19] but which role that TNC plays in AS was unclear. In the current study, we found that

TNC was involved in pathological new bone formation in AS, which could be considered as a special form of abnormal tissue remodelling. However, although our results provide evidence that TNC contributed to pathological new bone formation through modulation of the biomechanical property of ECM and enhancement of chondrogenesis, given that it plays multifaceted roles in immunomodulation and inflammation, TNC might also have other critical roles in the regulation of entheseal and ligamentous microenvironment or the maintenance of chronic inflammation in AS. It will be of great interest to continue to investigate the contribution of this multifunctional molecule to the pathogenesis of AS.

There are some limitations of the current study. First, control samples from healthy individuals are extremely difficult to obtain. Nearly all of the age-matched and sex-matched controls in our research were patients suffering from adult idiopathic scoliosis. Although the sample collection location was far from the apical vertebra and spontaneous fusion zone, gene expression in these samples may still be different from that in undamaged healthy human tissue samples. Second, the AS tissue samples were collected from patients at late-stage with extensive spinal fusion. Whether the biomechanical changes of the spine contribute to the pathological process of new bone formation is unclear due to lack of available tissue samples from patients at early-stage as proper control, which requires further investigation. Third, although the AS animal models in the current study are well accepted, the triggers in rodent models may not be identical to those in human disease. For further development of therapeutic strategies, large animal models whose genetic background is more similar to that of human, will likely be required.

In summary, we demonstrated that exposure of entheseal sites to chronic inflammation causes excessive TNC deposition, which subsequently promotes chondrogenic differentiation and pathological new bone formation via suppression of ECM adhesion force and activation of the Hippo pathway. Suppression of aberrant expression of TNC may be a potential therapeutic strategy for pathological new bone formation in AS.

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**Contributors** HL conceived the ideas for experimental designs. ZihaoL and SC conducted the majority of the experiments, analysed data and prepared the manuscript. DC and ZhongpingZ conducted sample collection and performed statistical analysis. JW, ZeminL and ZhaominZ provided critical suggestions and instructions for the project and helped compose the manuscript. ZihaoL and SC provided μCT analysis. ZihaoL, SC, XL, HC and WH conducted most animal experiments and performed analysis. HL developed the concept, supervised the project and conducted data analysis.

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CLINICAL SCIENCE

# Maintaining musculoskeletal health using a behavioural therapy approach: a population-based randomised controlled trial (the MAmMOTH Study)

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## ABSTRACT

**Objective** Cognitive–behavioural therapy (CBT) has been shown to be effective in the management of chronic widespread pain (CWP); we now test whether it can *prevent* onset among adults at high risk.

**Methods** A population-based randomised controlled prevention trial, with recruitment through UK general practices. A mailed screening questionnaire identified adults at high risk of CWP. Participants received either usual care (UC) or a short course of telephone CBT (tCBT). The primary outcome was CWP onset at 12 months assessed by mailed questionnaire. There were seven secondary outcomes including quality of life (EuroQol Questionnaire-five dimensions-five levels/EQ-5D-5L) used as part of a health economic assessment.

**Results** 996 participants were randomised and included in the intention-to-treat analysis of which 825 provided primary outcome data. The median age of participants was 59 years; 59% were women. At 12 months there was no difference in the onset of CWP (tCBT: 18.0% vs UC: 17.5%; OR 1.05; 95% CI 0.75 to 1.48). Participants who received tCBT were more likely to report better quality of life (EQ-5D-5L utility score mean difference 0.024 (95% CI 0.009 to 0.040)); and had 0.023 (95% CI 0.007 to 0.039) more quality-adjusted life-years at an additional cost of £42.30 (95% CI −£451.19 to £597.90), yielding an incremental cost-effectiveness ratio of £1828. Most secondary outcomes showed significant benefit for the intervention.

**Conclusions** A short course of tCBT did not prevent onset of CWP in adults at high risk, but improved quality of life and was cost-effective. A low-cost, short-duration intervention benefits persons at risk of CWP.

**Trial registration number** ClinicalTrials.gov Registry (NCT02668003).

## Key messages

**What is already known about this subject?**

- Cognitive–behavioural therapy (CBT) has demonstrated long-term effectiveness in managing chronic widespread pain (CWP), the characteristic symptom of fibromyalgia.
- It improves patient global assessment of change and quality of life.

**What does this study add?**

- A short course of telephone CBT in persons evaluated at high risk of developing CWP does not change onset of CWP but does result in a wide range of health benefits including improved quality of life.

**How might this impact on clinical practice or future developments?**

- CBT derives benefit for a wider group of people with pain than previously established and in relation to this wider group is highly cost-effective.

## INTRODUCTION

Chronic widespread pain (CWP) is common, with an estimated population prevalence of 10.6% (95% CI 8.6% to 12.9%)[1] and is the key feature of fibromyalgia which is the second most common reason (after osteoarthritis) for referral to a rheumatologist.[2] CWP and fibromyalgia result in a substantial impact on health-related quality of life[3] even in comparison with other musculoskeletal disorders.[4]

The road to diagnosis is often tortuous and can take many years. Using general practitioner records in the UK, Hughes *et al*[5] noted that people diagnosed with fibromyalgia had higher rates of primary care visits (average 25 visits/year), prescriptions (11/year) and testing from at least 10 years prior to diagnosis, in comparison with matched persons without such a diagnosis (12 visits/year and 4.5 prescriptions/year). Current European guidelines emphasise the primary role of non-pharmacological therapies for fibromyalgia.[6] Evidence in relation to musculoskeletal pain generally, is that the longer the duration of symptoms the less likely they are to improve, including with specific interventions.[7]

A Versus Arthritis 'Research roadmap for pain' produced by scientists, clinicians and patients identified preventing future musculoskeletal pain as one of four main priorities.[8] Further recognising its importance, the International Association for the Study of Pain nominated 2020 as 'The Global Year for the Prevention of Pain'. Despite this, we are not aware of any large-scale trials which have tested approaches to the future prevention of pain.

We have previously shown, in a randomised controlled trial, short-term and long-term effectiveness of a course of cognitive–behavioural therapy delivered by telephone (tCBT) for CWP, compared



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with usual care (UC).[9 10] These results are consistent with a meta-analysis of 29 trials involving 2509 participants and comparing CBT (across all modes of delivery) with control interventions for the management of fibromyalgia, which found high-quality evidence for improving pain and reducing disability, negative mood and fatigue.[11]

We have developed, validated and refined a statistical model which identifies people at high risk for the future development of CWP.[12 13] On the basis of reporting somatic symptoms, sleep problems and aspects of illness behaviour, those classified as 'high risk' have around one in four chance of reporting CWP 1 year later. Therefore, building on the evidence for the use of tCBT in the management of CWP and the ability to identify those with risk factors for its development, we undertook a trial to test whether tCBT can reduce CWP onset among those at high risk.

## METHODS

### Study design

We conducted a randomised controlled parallel prevention trial, recruiting through a population-based sampling frame, in three health boards within the UK (National Health Service (NHS) Grampian, NHS Greater Glasgow and Clyde, and NHS Highland), the protocol for which has been previously published.[14] Recruitment was through 16 general practices.

### Participants

A short screening questionnaire, to determine eligibility for the trial, was mailed to persons aged 25 years and over registered at participating general practices in the study area. Respondents eligible for the trial were those assessed as at high risk of developing CWP, namely that they reported pain which did not satisfy the definition of CWP used in the 1990 American College of Rheumatology criteria for fibromyalgia (namely axial and contralateral body pain present for at least 3 months), and hereafter referred to as 'ACR criteria',[15] and satisfied at least two of the following: (a) a score >4 on the Illness Behaviour Subscale of the Illness Attitudes Scale,[16] (b) a score >2 on the Somatic Symptom Scale score (but excluding items on pain),[17] (c) a score >4 on the Sleep Problem Scale.[18] In order to ensure that in the event of the trial showing benefit there was a relevant clinical population to which the intervention could be applied, we added to the risk models we had developed the requirement that persons had consulted to primary care within the previous 6 months or reported consulting a doctor frequently. Respondents were not eligible to take part if they had a medical condition which would make the proposed intervention unsuitable (eg, lacked cognitive ability).

### Randomisation

Potentially eligible participants were contacted by post with information about the study, and subsequently by a study researcher by telephone to confirm their willingness to take part and provide informed consent. Participants were allocated into groups using a computer randomisation program (1:1 allocation ratio), stratified in blocks by two factors (a) the number of non-pain 'high-risk' factors they reported (two or three) since this is related to the risk of CWP onset, and (b) the general practice at which they were registered.

### Procedures

The tCBT intervention consisted of an initial assessment (45–60 min), six weekly sessions (each 30–45 min) over 6 weeks, and then booster sessions at 3 and 6 months. The intervention was delivered by therapists trained for the study and accredited by the British Association for Behaviour and Cognitive Psychotherapies. Participants were supported by a self-management manual. The therapist conducted an assessment for problem identification, and they developed with each participant a shared formulation of the current health problem. The sessions involved education about musculoskeletal pain, somatic symptoms and specific techniques such as pacing of activity, behavioural activation, diary keeping, identifying and challenging negative and unhelpful thinking patterns, and the development of a longer term management plan. Participants would record in the manuals agreed goals for the therapist and patient to work towards, and some activities to complete between sessions. Therapists delivering the intervention received a 2-day training programme conducted by the investigators. Therapists were supervised every 2 weeks (by investigators KL and PK) throughout the delivery of the intervention. The number of telephone consultations conducted was recorded, although the therapist and participant could jointly agree that no further sessions were required before all planned sessions had been completed.

The group allocated to UC received no additional intervention, reflecting the fact there is no specific intervention provided to patients currently for the prevention of CWP. There was no restriction on what this care could involve.

Follow-up questionnaires were mailed to participants at 3, 12 and 24 months after the treatment start date (for participants in the active treatment group) or dummy treatment start date (for those in UC). The dummy treatment start date for a participant randomised to UC was determined by the treatment start date of the last participant to be randomised to receive active treatment. At 3 and 12 months, participants who did not return their questionnaire were telephoned to ask them to complete and return it, while at 24 months the follow-up call also offered the option of completing a shortened version by telephone.

### Outcomes

The principal outcome time was at 12-month follow-up and the primary outcome was ACR criteria for CWP. Secondary outcomes were: Global Impression of Change, Illness Behaviour Subscale of the Illness Attitudes Scale,[16] the Somatic Symptom Scale (excluding items on pain),[17] the Sleep Problem Scale,[18] the presence of pain over the past month, Widespread Pain Index (WPI) and Symptom Severity Scale (SSS) of the 2010 (revised) criteria for fibromyalgia,[19] psychological distress measured using the General Health Questionnaire (GHQ),[20] Chalder Fatigue Scale,[21] quality of life (EuroQol Questionnaire-five dimensions-five levels/EQ-5D-5L)[22] and capability (ICEpop CAPability measure for Adults/ICECAP-A).[23] Further details of secondary outcome (including coding) are given in the online supplemental file.

### Statistical analysis

All analyses were undertaken using Stata V.15. The a priori target sample size was 946 participants, which would provide 90% power to detect a group difference of 9% (21% vs 12%) in the percentage of participants with CWP at 12-month follow-up, assuming a 5% significance level and an 80% response rate.

Where there were missing data within a scale score, we followed standard procedures (where available) as to if and how the missing values could be imputed. The analysis of the primary outcome used a binary logistic regression model with results expressed as an OR with 95% CI. Secondary outcomes were

analysed using linear, binary logistic, ordinal logistic or Poisson regression models for continuous, binary, ordinal and count variables, respectively. Model results were reported using mean differences, ORs or incidence rate ratios (IRRs) as appropriate. Except for EQ-5D-5L, mean differences less than 0 and ORs/IRRs less than 1 favour the treatment group. All models were adjusted (adj) for the number of non-pain risk factors on screening (two or three), age (years), gender, general practice (random effect) and baseline score of the outcome measure (where applicable). The primary analysis was by intention to treat—that is, participants were analysed according to randomised group regardless of the number of sessions received. Separate analyses were performed for each time point (3, 12 and 24 months). For the primary outcome, a p value less than 0.05 was regarded as statistically significant; for secondary outcomes $p<0.01$ was used. Additional sensitivity analyses were conducted for the primary outcome only and are detailed in the online supplemental file.

### Health economic analysis

Health service resource used over 24 months was assessed using responses from self-reported questionnaires. Participants were asked to recall their usage for the previous 4-week period at each follow-up. Resource use was then valued using published UK sources—NHS Reference Cost and the Personal and Social Service Research Unit for NHS primary and secondary care, and published literature for care obtained from private providers.[24] The unit costs used for the valuation of health service resource use are reported in online supplemental table S1. The intervention cost was based on the actual number and duration of telephone calls per participant ('direct time'), plus time spent on training and supervision. An allowance for indirect time spent was also included and this was based on an assumed ratio of 1:1 between time spent on participant contact and other activities conducted by therapists. Training costs were estimated using the time spent in training by trainers and trainees (tCBT therapists). A fortnightly supervision cost was estimated by assuming 30 sessions per therapist (30 min per session) were provided. Costs were expressed in 2017/2018 prices. Health utility scores were assigned based on responses to the EQ-5D-5L at each follow-up, and these were converted using the 'crosswalk' procedure to EQ-5D-3L.[25] There is currently no consensus on the preferred EQ-5D-5L tariff for use in economic evaluation, although the National Institute for Health and Care Excellence (NICE) recommends the use of the 'crosswalk' procedure (a validated mapping function) to derive health utility scores for the EQ-5D-5L from the EQ-5D-3L tariff (https://www.nice.org.uk/about/what-we-do/our-programmes/nice-guidance/technology-appraisal-guidance/eq-5d-5l accessed 20 November 2020). These utility scores were used to estimate quality-adjusted life-years (QALYs) over the 24 months using the area under the curve method.[26] Costs and QALYs incurred beyond 12 months were discounted at the rate of 3.5% per annum.

The within-trial economic analysis was conducted over 24 months from a UK NHS cost perspective. To estimate the differences in mean costs and QALYs between groups, generalised linear models with adjustment for minimisation factors, baseline cost and baseline utility score were performed. A γ family with log-link function and a Poisson family with power 0.5 link function were specified for the cost and QALY data, respectively. Missing data were addressed using multiple imputation by chained equations (MICE). Variance surrounding the incremental costs and QALYs was characterised using non-bootstrapping (500 iterations), with MICE (m=5) nested within the bootstrap loops.[27] Cost-effectiveness acceptability curves were constructed, using 500 replications of each incremental cost-effectiveness ratio (ICER) and the net monetary benefit framework, to determine the probability of the alternative interventions being considered cost-effective at different willingness to pay per QALY (£20 000–£30 000 per QALY used are commonly applied ceiling ratios in the UK). Several sensitivity analyses were performed to explore the impact on the results of uncertainty in estimates made—(1) using complete cases of costs and QALYs, (2) including private care costs, (3) using alternative tCBT costing methodology (actual trial expenses incurred by therapists and the cost of a complete tCBT course) and (4) using ICECAP tariff as the measure of effectiveness.

The trial was evaluated by the Trial Steering Committee as not requiring a Data Monitoring Committee.

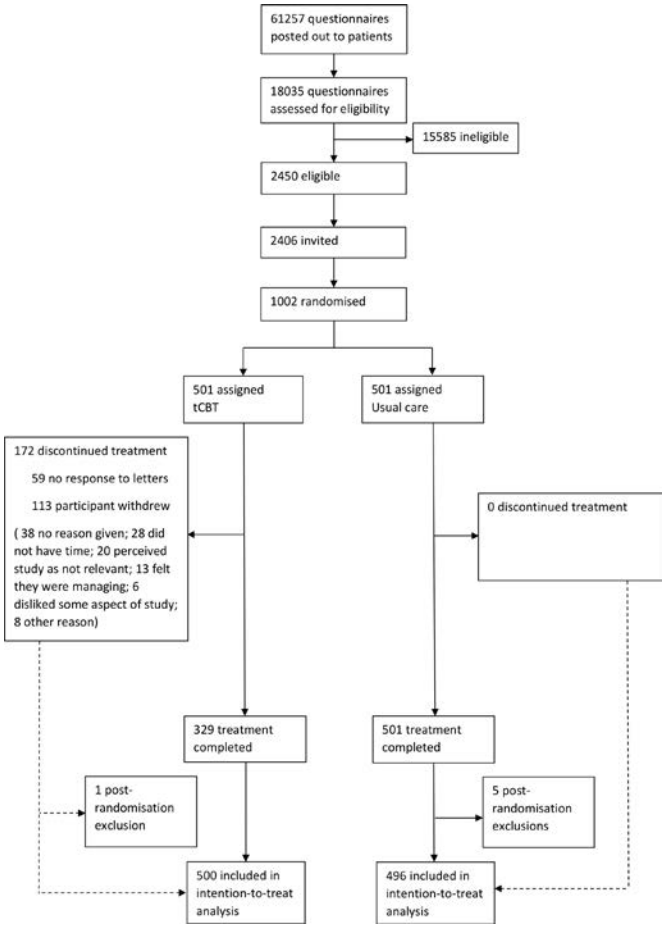


**Figure 1** Trial profile. tCBT, telephone-delivered cognitive–behavioural therapy.

## RESULTS

Of 61 257 screening questionnaires sent between 4 April 2016 and 4 November 2016 to patients registered at 16 general practices, 18 035 completed questionnaires were returned. From those returning a completed questionnaire, 2406 were identified as potentially eligible and sent invitations to take part in the trial. A total of 1002 participants were recruited to the trial and randomised, 501 to tCBT and 501 to UC, between May 2016 and March 2017. Six participants were subsequently determined to be ineligible for the trial and were excluded from analyses (see Trial profile: figure 1) leaving a final study size of 500 and 496 in the tCBT and UC arms, respectively. At the 3-month, 12-month

**Table 1** Baseline characteristics by treatment arm in the ITT population

| | Randomised groups | |
|---|---|---|
| | tCBT (n=500) | Usual care (n=496) |
| **Characteristic** | **Median (IQR)** | **Median (IQR)** |
| Age (years) | 58.8 (47.7–68.7) | 59.5 (47.9–68.9) |
| | *N (%)* | *N (%)* |
| Gender | | |
| Male | 209 (41.8) | 204 (41.0) |
| Female | 291 (58.2) | 292 (58.9) |
| Employment status | | |
| Working (full or part-time) | 277 (55.4) | 244 (49.2) |
| Unable to work because of health | 18 (3.6) | 30 (6.0) |
| Retired | 168 (33.6) | 177 (35.7) |
| Other | 37 (7.4) | 45 (9.1) |
| CWP risk profile: | | |
| Illness behaviour score >4 | | |
| No | 1 (0.2) | 2 (0.5) |
| Yes | 498 (99.6) | 494 (99.5) |
| Not known* | 1 (0.2) | 0 (0.0) |
| Somatic Symptom Scale score >2 | | |
| No | 472 (94.4) | 462 (93.1) |
| Yes | 28 (5.6) | 34 (6.9) |
| Sleep problems score >4 | | |
| No | 1 (0.2) | 2 (0.4) |
| Yes | 499 (99.8) | 493 (99.4) |
| Not known* | 0 (0.0) | 1 (0.2) |
| CWP risk profile factors present (N) | | |
| 2 | 474 (94.8) | 466 (94.0) |
| 3 | 26 (5.2) | 30 (6.0) |
| | *Median (IQR) (n)*† | *Median (IQR) (n)* |
| Psychological distress (GHQ) | 1 (0–4) (499) | 1 (0–4) (494) |
| Quality of Life (EQ-5D-5L utility score) | 0.74 (0.65–0.80) (499) | 0.74 (0.64–0.80) (496) |
| ICECAP-A | 0.91 (0.81–0.95) (495) | 0.90 (0.79–0.95) (491) |
| Fibromyalgia research criteria | | |
| WPI | 3 (1–4) (499) | 2 (1–4) (492) |
| SSS | 4 (3–6) (497) | 4 (3–5) (494) |

*Where individuals completed half or fewer items, the score was classified as not known, but individuals could still be eligible for recruitment based on their responses to other items answered.
†The number of persons for whom a scale score could be calculated.
EQ-5D-5L, EuroQol Questionnaire-five dimensions-five levels; GHQ, General Health Questionnaire; ICECAP-A, ICEpop CAPability measure for Adults; ITT, intention to treat; SSS, Symptom Severity Scale; tCBT, telephone-delivered cognitive–behavioural therapy; WPI, Widespread Pain Index.

**Table 2** Outcomes by treatment arm at 12 months (intention-to-treat analysis)

| | Randomised groups | |
|---|---|---|
| **Characteristic** | **tCBT (n=500)** | **Usual care (n=496)** |
| *Primary outcome* | *N (%)* | *N (%)* |
| Chronic widespread pain | | |
| No | 315 (82.0) | 364 (82.5) |
| Yes | 69 (18.0) | 77 (17.5) |
| *Secondary outcome* | | |
| Global impression of change | | |
| Very much better | 24 (6.5) | 15 (3.5) |
| Much better | 88 (23.7) | 59 (13.8) |
| A little better | 90 (24.3) | 84 (19.6) |
| No change | 83 (22.4) | 126 (29.4) |
| A little worse | 65 (17.5) | 119 (27.7) |
| Much worse | 18 (4.9) | 23 (5.4) |
| Very much worse | 3 (0.8) | 3 (0.7) |
| Pain reported | | |
| No | 79 (20.6) | 68 (15.4) |
| Yes | 305 (79.4) | 373 (84.6) |
| CWP risk profile | | |
| Somatic symptoms score | | |
| 0 | 210 (56.5) | 228 (52.8) |
| 1 | 103 (27.7) | 123 (28.5) |
| 2–5 | 59 (15.9) | 81 (18.8) |
| Illness behaviour score, mean (SD) (n)* | 8.21 (4.04) (371) | 8.96 (4.19) (431) |
| Sleep problems score, mean (SD) (n) | 8.20 (4.89) (373) | 9.20 (5.16) (432) |
| Psychological distress (GHQ score) | | |
| 0 | 201 (54.5) | 202 (46.8) |
| 1 | 59 (16.0) | 54 (12.5) |
| 2–5 | 68 (18.4) | 113 (26.2) |
| 6–12 | 41 (11.1) | 63 (14.6) |
| | *Mean (SD) (n)* | *Mean (SD) (n)* |
| Chalder Fatigue Score | 12.6 (4.5) (370) | 13.6 (4.4) (433) |
| | *Median (IQR) (n)* | *Median (IQR) (n)* |
| Quality of Life (EQ-5D utility score) | 0.74 (0.66–0.84) (371) | 0.74 (0.65–0.82) (435) |
| ICECAP-A | 0.91 (0.82–0.97) (368) | 0.89 (0.78–0.95) (429) |
| Fibromyalgia research criteria | | |
| WPI | 2 (1–4) (366) | 2 (1–4) (427) |
| SSS | 3 (2–5) (369) | 4 (2–5) (431) |

*The number of persons for whom a scale score could be calculated.
EQ-5D, EuroQol Questionnaire-five dimensions; GHQ, General Health Questionnaire; ICECAP-A, ICEpop CAPability measure for Adults; SSS, Symptom Severity Scale; tCBT, telephone-delivered cognitive–behavioural therapy; WPI, Widespread Pain Index.

and 24-month follow-up, there were 823, 825 and 853 respondents who provided primary outcome data, respectively. Most participants (51%) came from the lowest two quintiles of deprivation, while 18% came from the two most deprived quintiles.

Participants at the time of recruitment had a median age of 59 years (IQR 48–69), 59% were women, and 52% were working full-time or part-time (table 1). The median EQ-5D utility score was 0.74 (IQR 0.65–0.80). The vast majority satisfied only two of the non-pain criteria for eligibility, nearly always on the basis of a high score on the illness behaviour subscale of the Illness Attitudes Scale and having sleep problems. Only 6% of the study sample satisfied the somatic symptoms criterion. The tCBT and UC groups were well matched in terms of the measured health-related factors.

Results for all outcome measures at the primary time point (12 months) are shown in table 2. The corresponding results at 3 and 24 months are shown in online supplemental tables S2–S3. Table 3 provides a summary of all primary and secondary outcomes at all time points and shows adjusted and unadjusted effect sizes.

## Primary outcome

At the 12-month time point similar percentages in the tCBT and UC groups reported having CWP (tCBT: 69/384 (18.0%), UC:

**Table 3** Summary of the primary and secondary outcomes across follow-up points*

| Outcome | | Time point (months) | Analysis method (effect size) | Adjusted† effect size (95% CI) | P value | Unadjusted effect size (95% CI) | P value |
|---|---|---|---|---|---|---|---|
| *Primary outcome* | | | | | | | |
| CWP | | 3 | Logistic regression (OR) | 1.08 (0.74 to 1.58) | 0.691 | 1.07 (0.75 to 1.53) | 0.716 |
| CWP (per protocol) | | | | 1.15 (0.75 to 1.75) | 0.519 | 1.18 (0.77 to 1.66) | 0.522 |
| CWP (with multiple imputation) | | | | 1.06 (0.74 to 1.54) | 0.749 | 1.05 (0.72 to 1.53) | 0.816 |
| CWP‡ | | 12 | | 1.05 (0.75 to 1.48) | 0.771 | 1.04 (0.72 to 1.48) | 0.849 |
| CWP (per protocol) | | | | 1.11 (0.81 to 1.50) | 0.519 | 1.09 (0.74 to 1.60) | 0.673 |
| CWP (with multiple imputation) | | | | 1.04 (0.75 to 1.45) | 0.982 | 1.03 (0.74 to 1.42) | 0.964 |
| CWP | | 24 | | 0.85 (0.68 to 1.07) | 0.163 | 0.84 (0.61 to 1.18) | 0.317 |
| CWP (per protocol) | | | | 0.85 (0.64 to 1.12) | 0.241 | 0.84 (0.58 to 1.20) | 0.330 |
| CWP (with multiple imputation) | | | | 0.85 (0.66 to 1.09) | 0.220 | 0.84 (0.65 to 1.09) | 0.196 |
| CWP | | 3, 12, 24 | GEE (OR) | 1.00 (0.96 to 1.04) | 0.923 | 1.00 (0.96 to 1.04) | 0.835 |
| *Secondary outcomes* | | | | | | | |
| Global impression of change§ | | 3 | Ordinal logistic regression (OR) | 0.42 (0.32 to 0.55) | <0.001 | 0.43 (0.34 to 0.56) | <0.001 |
| | | 12 | | 0.51 (0.39 to 0.67) | <0.001 | 0.53 (0.41 to 0.68) | <0.001 |
| | | 24 | | 0.55 (0.43 to 0.70) | <0.001 | 0.58 (0.45 to 0.73) | <0.001 |
| CWP risk profile | Somatic symptoms score | 3 | Ordinal logistic regression (OR) | 0.79 (0.60 to 1.03) | 0.084 | 0.83 (0.64 to 1.08) | 0.173 |
| | | 12 | | 0.86 (0.71 to 1.04) | 0.112 | 0.85 (0.65 to 1.11) | 0.237 |
| | | 24 | | 0.81 (0.59 to 1.12) | 0.206 | 0.90 (0.67 to 1.21) | 0.498 |
| | Illness behaviour score | 3 | Linear regression (mean difference) | −0.17 (−0.58 to 0.24) | 0.385 | −0.25 (−0.79 to 0.29) | 0.360 |
| | | 12 | | −0.81 (−1.54 to −0.09) | 0.030 | −0.74 (−1.32 to −0.17) | 0.011 |
| | | 24 | | −1.25 (−2.15 to −0.35) | 0.010 | −1.20 (−1.83 to −0.58) | <0.001 |
| | Sleep problems score | 3 | Linear regression (mean difference) | −0.62 (−1.26 to 0.02) | 0.057 | −0.62 (−1.31 to 0.08) | 0.081 |
| | | 12 | | −0.95 (−1.48 to −0.42) | 0.002 | −1.00 (−1.70 to −0.30) | 0.005 |
| | | 24 | | −0.51 (−1.25 to 0.23) | 0.161 | −0.52 (−1.39 to 0.16) | 0.117 |
| Psychological distress (GHQ) | | 3 | Ordinal logistic regression (OR) | 0.55 (0.43 to 0.69) | <0.001 | 0.58 (0.45 to 0.76) | <0.001 |
| | | 12 | | 0.65 (0.50 to 0.86) | 0.002 | 0.70 (0.54 to 0.90) | 0.007 |
| | | 24 | | 0.76 (0.60 to 0.96) | 0.024 | 0.74 (0.56 to 0.98) | 0.037 |
| Chalder Fatigue Score | | 3 | Linear regression (mean difference) | −1.36 (−2.10 to −0.64) | 0.001 | −1.40 (−1.97 to −0.82) | <0.001 |
| | | 12 | | −1.02 (−1.63 to −0.42) | 0.003 | −1.03 (−1.64 to −0.42) | 0.001 |
| | | 24 | | −0.93 (−1.62 to −0.23) | 0.012 | −0.93 (−1.58 to −0.27) | 0.006 |
| Quality of Life (EQ-5D-5L utility score) | | 3 | Linear regression (mean difference) | 0.009 (−0.009 to 0.028) | 0.304 | 0.021 (−0.004 to 0.046) | 0.101 |
| | | 12 | | 0.024 (0.009 to 0.040) | 0.004 | 0.037 (0.010 to 0.064) | 0.007 |
| | | 24 | | 0.030 (0.009 to 0.050) | 0.008 | 0.040 (0.011 to 0.069) | 0.007 |
| ICECAP-A tariff | | 3 | Ordinal logistic regression (OR) | 1.14 (0.89 to 1.48) | 0.304 | 1.17 (0.86 to 1.59) | 0.323 |
| | | 12 | | 1.39 (0.94 to 2.04) | 0.096 | 1.39 (1.01 to 1.91) | 0.042 |
| | | 24 | | 0.88 (0.67 to 1.15) | 0.338 | 0.99 (0.70 to 1.41) | 0.966 |
| Fibromyalgia criteria | Widespread Pain Index | 3 | Poisson regression (IRR) | 0.98 (0.90 to 1.07) | 0.698 | 1.01 (0.93 to 1.10) | 0.771 |
| | | 12 | | 0.88 (0.80 to 0.98) | 0.018 | 0.92 (0.84 to 0.99) | 0.036 |
| | | 24 | | 0.88 (0.78 to 0.98) | 0.022 | 0.92 (0.84 to 1.00) | 0.058 |
| | Symptom Severity Scale | 3 | Linear regression (mean difference) | −0.28 (−0.52 to −0.04) | 0.026 | −0.25 (−0.57 to 0.65) | 0.118 |
| | | 12 | | −0.52 (−0.75 to −0.28) | <0.001 | −0.59 (−0.91 to −0.27) | <0.001 |
| | | 24 | | −0.29 (−0.55 to −0.02) | 0.040 | −0.28 (−0.61 to 0.05) | 0.100 |

*Analyses shaded in grey favour tCBT over usual care at prespecified significance level for secondary outcomes (p<0.01). Except for EQ-5D-5L, mean differences less than 0 and ORs less than 1 favour the treatment group.
†Adjusted analyses control for the number of risk factors (two or three), age, gender, baseline score (if applicable) and centre (random effect). Analyses are intention to treat unless otherwise stated.
‡Primary outcome.
§OR of 1 point increase in global impression of change score (worsening of health).
CWP, chronic widespread pain; EQ5D-5D-5L, EuroQol Questionnaire-five dimensions-five levels; GEE, generalised estimating equations; GHQ, General Health Questionnaire; ICECAP-A, ICEpop CAPability measure for Adults; IRR, incidence rate ratio; ;tCBT, telephone-delivered cognitive–behavioural therapy.

77/441 (17.5%); adj OR 1.05; 95% CI: 0.75 to 1.48; difference in percentages: adj 0.73, 95% CI: −4.15 to 5.61) (tables 2 and 3)). Very similar results were obtained at 3 months (17.9% vs 16.9%; adj OR: 1.08; 95% CI: 0.74 to 1.58) and 24 months (19.6% vs 22.3%; adj OR: 0.85; 95% CI: 0.68 to 1.07) (online supplemental tables S2–S3, table 3). There was no difference in the interpretation when examining unadjusted results, per protocol results or the analyses using multiple imputation (table 3). The generalised estimating equations model, incorporating data from all three time points, also showed no evidence of a difference (adj OR: 1.00; 95% CI: 0.96 to 1.04; p=0.91).

## Secondary outcomes

At 12 months, those randomised to tCBT were more likely to perceive their health to be improved (adj OR (ordinal logistic regression/OLR): 0.51, 95% CI: 0.39 to 0.67) and to report better quality of life (EQ-5D-5L utility scores) (adj mean difference

(diff): 0.024, 95% CI: 0.009 to 0.040) (tables 2 and 3). While those who received tCBT had lower illness behaviour (adj mean diff: −0.81; 95% CI: −1.54 to −0.09) and sleep problem scores (adj mean diff: −0.95; 95% CI: −1.48 to −0.42), but there was no significant difference in relation to somatic symptoms (adj OR: 0.86; 95% CI: 0.71 to 1.04). Participants randomised to tCBT had improved distress (GHQ scores) (adj OR: 0.65, 95% CI: 0.50 to 0.86) and lower levels of fatigue (Chalder Scale scores) (adj mean diff: −1.02, 95% CI: −1.63 to −0.42). There was no evidence of a difference for ICECAP-A tariffs (adj OR (OLR): 1.39, 95% CI: 0.94 to 2.04; p=0.10). In relation to the components of criteria for fibromyalgia, they had lower scores on the WPI (adj IRR: 0.88; 95% CI: 0.80 to 0.98) and SSS (adj mean diff: −0.52, 95% CI: −0.75 to −0.28). Of these receiving tCBT, 3.8% met fibromyalgia research criteria at follow-up (in comparison to 6.0% among those receiving UC).

### Outcomes across time points

Sensitivity analyses, unadjusted results and findings at 3-month and 24-month time points generally yielded similar observations as those for 12 months (table 3, online supplemental tables S1–S2). There was consistently no effect on the primary outcome. The strongest and most consistent effects were on patient global assessment of change—which showed large and consistent effects across all time points. There were also clear effects of the intervention (in comparison with UC) across all time points with respect to improvement in levels of fatigue and psychological distress. Quality of life was better in the intervention group from 12 months onwards. There was only one serious adverse event reported, it was in the intervention group but unrelated to the intervention.

### Health economic analysis

The unadjusted health service resource use and costs per participant are summarised in online supplemental table S4. Participants randomised to tCBT group had an average time of 139 min of direct contact with therapists over the 6-month t-CBT course, and the average tCBT cost was £270.19 per participant. Compared with the UC group, NHS primary and secondary care costs were lower among tCBT group, and private care costs higher. All cost-effectiveness analyses showed that tCBT was associated with an increase in health service costs and an increase in QALYs (table 4). The primary analysis generated a mean of 0.023 (95% CI 0.007 to 0.039) more QALYs per participant at an additional cost of £42.30 (95% CI −£451.19 to £597.90), yielding an ICER of £1828. Based on the results of the non-parametric bootstrap, tCBT was found to have a 91.6% chance of being the preferred strategy at a ceiling ratio of £20 000 per QALY gained (figure 2). Sensitivity analyses showed that this finding was robust to changes in study perspective, inclusion of complete cases only and different assumptions relating to delivery of the intervention in terms of tCBT staff time (online supplemental figure S1 a–d).

## DISCUSSION

A short course of tCBT among persons at high risk did not change the proportion of people developing CWP (compared with UC). Those receiving the active intervention were more likely to perceive their health as having improved and report better quality of life as well as lower levels of fatigue and psychological distress. The intervention was highly cost-effective in terms of incremental cost per QALY gained.

**Table 4** Adjusted* mean incremental costs, incremental QALYs and incremental cost-effectiveness ratio over 24 months between tCBT versus usual care

| Analysis | Mean costs, (95% CI) | | Mean QALYs, (95% CI) | | Incremental mean costs, £ (95% CI)† | Incremental mean QALYs (95% CI) | ICER (£/QALY) |
|---|---|---|---|---|---|---|---|
| | tCBT | Usual care | tCBT | Usual care | | | |
| Imputed dataset/ITT analysis (NHS perspective)‡ | 3094.68 (1775.65 to 9074.15) | 3052.38 (1735.77 to 8567.24) | 1.254 (1.238 to 1.270) | 1.231 (1.215 to 1.245) | 42.30 (−451.19 to 597.90) | 0.023 (0.007 to 0.039) | 1828 |
| SA: complete cases (NHS perspective)§ | 2684.53 (1817.69 to 5221.86) | 2454.67 (1645.66 to 4769.87) | 1.444 (1.415 to 1.471) | 1.420 (1.392 to 1.447) | 229.86 (−228.74 to 734.09) | 0.024 (−0.005 to 0.053) | 9608 |
| SA: imputed dataset (NHS+private care perspective) | 4239.22 (2135.82 to 15 332.80) | 4149.10 (2110.98 to 14 039.06) | 1.253 (1.238 to 1.270) | 1.231 (1.215 to 1.247) | 90.12 (−475.79 to 772.98) | 0.022 (0.007 to 0.039) | 4022 |
| SA: imputed dataset using actual trial expenses (NHS perspective)¶ | 3128.61 (1809.54 to 9164.04) | 3027.54 (1734.31 to 8587.83) | 1.254 (1.238 to 1.270) | 1.231 (1.215 to 1.245) | 101.07 (−373.14 to 641.98) | 0.023 (0.007 to 0.039) | 4367 |
| SA: imputed dataset using the cost of a complete tCBT course (NHS perspective)** | 3314.57 (1966.93 to 9059.99) | 2960.98 (1729.67 to 7781.19) | 1.254 (1.238 to 1.270) | 1.231 (1.215 to 1.245) | 353.59 (−80.46 to 1238.07) | 0.023 (0.007 to 0.039) | 15 280 |
| SA: imputed dataset using ICECAP (NHS perspective)†† | 4659.66 (1764.56 to 10 400.07) | 4787.56 (1815.05 to 10 632.20) | 1.288 (1.278 to 1.297) | 1.275 (1.266 to 1.284) | −127.90 (−603.19 to 545.33) | 0.013 (0.003 to 0.023)‡‡ | NA |

*Adjusted for baseline differences (age, gender, number of risk factors present, employment status, centre, baseline EQ-5D health utility score and baseline cost).
†Bootstrapped non-parametric 95% CI (2.5th/97.5th centile). Generalised linear model with γ distribution and log-link function to estimate incremental costs and generalised linear model with Poisson distribution and power 0.5 link function to estimate incremental QALYs/years of full capacity. Discounted at 3.5% per year.
‡Imputed dataset is the ITT analysis. Missing values were imputed to account for all participants included in the ITT analysis.
§593 complete cases were included (tCBT, n=297 and usual care, n=326). Complete cases are those with no missing data on cost and health utility at each time point.
¶Included the actual trial expenses per tCBT participant, £301. This was estimated using the lump-sum trial expenses incurred by therapists, including therapists' training and tCBT delivery.
**Included the cost of a complete tCBT course per participant, £443. Time spent by therapist training and supervision were included. The total time spent by the therapist was estimated by assuming that all tCBT participants attended a complete tCBT course consisting of nine sessions.
††Adjusted for baseline differences (age, gender, number of risk factors present, employment status, centre, baseline ICECAP value and baseline cost).
‡‡Incremental years of full capability.
EQ-5D, EuroQol Questionnaire-five dimensions; ICECAP, ICEpop CAPability measure for Adults; ICER, incremental cost-effectiveness ratio; ITT, intention to treat; NA, not applicable; NHS, National Health Service; QALYs, quality-adjusted life-years; SA, sensitivity analysis; tCBT, telephone-delivered cognitive–behavioural therapy.

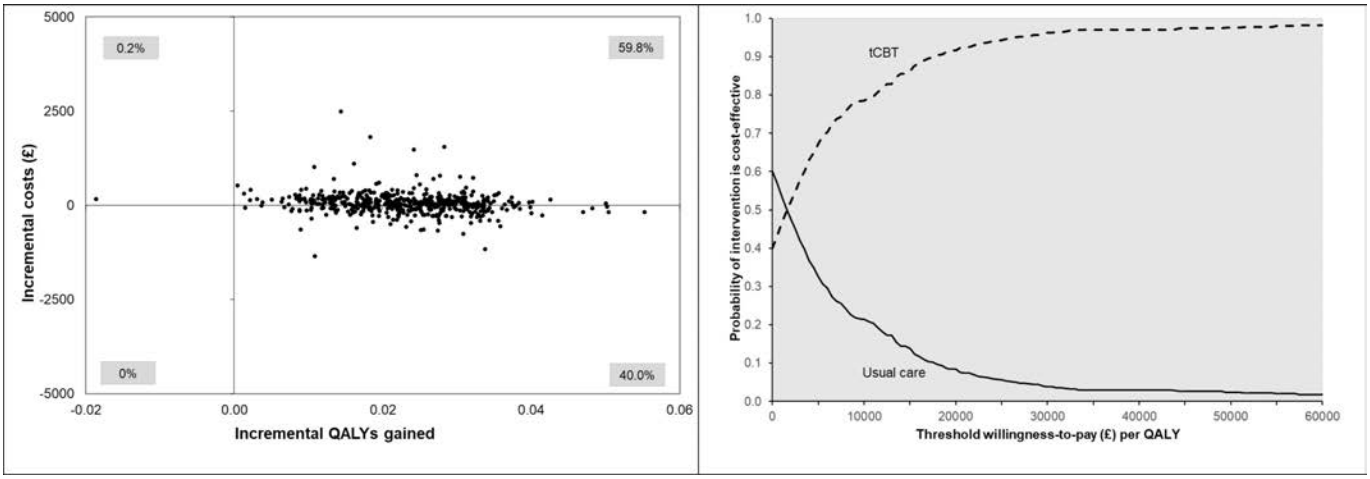


**Figure 2** Cost-Effectiveness plane and cost-effectiveness acceptability curve between groups (primary analysis using imputed dataset, NHS perspective). Cost-effectiveness planes were based on 500 bootstrap cost-effect pairs (adjusted for age, gender, number of risk factors present, employment status, centre, baseline EQ-5D health utility score and baseline cost). EQ-5D, EuroQol Questionnaire-five dimensions; NHS, National Health Service; QALY, quality-adjusted life-year; tCBT, telephone-delivered cognitive–behavioural therapy.

Undertaking a primary prevention study presents different challenges to undertaking a treatment study. Most people eligible for the trial probably would not have known what CWP is, nor that they were at high risk of its development. Thus, the intervention was described as 'maintaining musculoskeletal health' and introduced in the context of participants having reported pain and other symptoms. Although a set number of sessions for the intervention was planned, it was agreed that at any point the intervention could be stopped with mutual agreement between therapist and participant; with the intervention considered completed. Among participants, 329 (66%) were considered to be completers that is, had the assessment session and either had at least two completed treatment sessions (n=297) or had the assessment session and up to one treatment session with mutual agreement that the intervention was complete (n=32). Of those classed as 'non-completers', 97 had no assessment while 75 had an assessment and up to one treatment session.

Why did the trial clearly not change the likelihood of CWP onset while showing positive effects for a range of secondary outcomes (including quality of life)? First, it may be that CBT is not effective in relation to preventing CWP onset. We know that there is a large body of evidence that CBT (including tCBT) is effective in relation to managing CWP, and also for managing some of the symptoms which characterised people at high risk, but it may not be effective at improving the pain in CWP. Our previous trial using CBT in the management of CWP while showing large improvement in patient perception of their condition and in quality of life, did not demonstrate any benefit in terms of the Chronic Pain Grade.[10] Second, our risk model may not be the causal model. A change in hypothesised risk factors would only effect a change in outcome if the relationship was causal. This suggests that it would be beneficial to explore, among those at risk, what is the underlying causal mechanism. Altered hypothalamic–pituitary–adrenal axis function is one possible underlying causal mechanism which has been investigated.[28] Third, it is understood that there are life-course influences, specifically early life factors, on the development of CWP,[29] so it could be that intervening across the adult age range is too late to be effecting a change by means of a short-term intervention. Fourth, it may be that CWP was a poor choice as the primary outcome. There is evidence that people with CWP can move in and out of meeting criteria[30] and indeed it may be that we have identified people who commonly experience CWP but recruited them at a time when they did not meet criteria—and the interpretation would be that the intervention did not move participants off that trajectory. Recent data from a longitudinal study in Norway have shown that the transition, among people with pain, to CWP did not represent a clinically significant change in state.[31]

It is already known that CBT is effective in the management of fibromyalgia[11] and this study provides evidence that a wider range of patients may benefit in terms of quality of life. In total 54.5% of the intervention group considered their health had improved (between a little and very much) compared with 36.9% of the UC group, as well as improvements in fatigue, distress and changes in response to symptoms. The incremental cost per QALY gained of £1828 (which was robust to different assumptions modelled in various sensitivity analyses) means that this intervention is highly likely to be cost-effective at the limit, which NICE in the UK, is willing to pay. In terms of delivering behavioural therapies, it has long been recognised that there is a shortage of clinical psychologists in the UK. It is not necessary to have such persons delivering behavioural therapy to all such patients even where CBT is identified as appropriate. In this study, the intervention was delivered by therapists accredited by the British Association for Behaviour and Cognitive Psychotherapies. At a minimum this requires a Bachelor of Science degree and a 2-year course leading to a postgraduate diploma in cognitive–behaviour psychotherapies. Further there has been a considerable amount of research in terms of internet-based therapies. The potential advantage of such a self-directed approach is that it requires less input by the therapist (usually somewhere between 1 and 15 mins/week). Further, a meta-analysis of 20 studies involving 1460 participants showed that internet-delivered CBT was effective in the treatment of insomnia,[32] while a meta-analysis of 20 studies involving 1418 participants comparing face-to-face and internet-delivered CBT for psychiatric and somatic symptoms found that 'there was no evidence to conclude that they were not equivalent'.[33] Studies have also examined training members of the care team (usually nurses) to deliver behavioural therapy in terms of making any service for chronic pain sustainable, and these have been shown to be effective.[34] Thus, we need to consider different professionals and ways of delivering CBT, particularly if we widen the group

eligible to receive it, and there is no doubt that the large changes to how health services are delivered, caused by COVID-19, will only accelerate moves to the greater use of remote delivery of care.

In summary, this trial has shown that a short course of tCBT does not prevent the onset of CWP in adults assessed as being at high risk. It did however positively change most other health indicators measured, including quality of life, and was highly cost-effective. It demonstrates that a low-cost, short-duration intervention benefits a wider range of people with musculoskeletal symptoms than previously considered.

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**Contributors** GJM was CI and MB study coordinator. KL and PK were responsible for designing and overseeing the delivery of the intervention. GP was the trial statistician and designed the analysis plan, and this role was latterly taken over by NS who conducted the analysis. PM was responsible for designing the health economic analysis which was undertaken by HC. GJM drafted the manuscript with input from MB, HC, PM and NS. All authors contributed important intellectual content to trial design and execution, and commented on drafts of the manuscript.

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TRANSLATIONAL SCIENCE

# Autoantibodies targeting telomere-associated proteins in systemic sclerosis

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## ABSTRACT

**Objectives** Systemic sclerosis (SSc) is an autoimmune fibrotic disease affecting multiple tissues including the lung. A subset of patients with SSc with lung disease exhibit short telomeres in circulating lymphocytes, but the mechanisms underlying this observation are unclear.

**Methods** Sera from the Johns Hopkins and University of California, San Francisco (UCSF) Scleroderma Centers were screened for autoantibodies targeting telomerase and the shelterin proteins using immunoprecipitation and ELISA. We determined the relationship between autoantibodies targeting the shelterin protein TERF1 and telomere length in peripheral leucocytes measured by qPCR and flow cytometry and fluorescent in situ hybridisation (Flow-FISH). We also explored clinical associations of these autoantibodies.

**Results** In a subset of patients with SSc, we identified autoantibodies targeting telomerase and the shelterin proteins that were rarely present in rheumatoid arthritis, myositis and healthy controls. TERF1 autoantibodies were present in 40/442 (9.0%) patients with SSc and were associated with severe lung disease (OR 2.4, p=0.04, Fisher's exact test) and short lymphocyte telomere length. 6/6 (100%) patients with TERF1 autoantibodies in the Hopkins cohort and 14/18 (78%) patients in the UCSF cohort had a shorter telomere length in lymphocytes or leukocytes, respectively, relative to the expected age-adjusted telomere length. TERF1 autoantibodies were present in 11/152 (7.2%) patients with idiopathic pulmonary fibrosis (IPF), a fibrotic lung disease believed to be mediated by telomere dysfunction.

**Conclusions** Autoantibodies targeting telomere-associated proteins in a subset of patients with SSc are associated with short lymphocyte telomere length and lung disease. The specificity of these autoantibodies for SSc and IPF suggests that telomere dysfunction may have a distinct role in the pathogenesis of SSc and pulmonary fibrosis.

### Key messages

**What is already known about this subject?**

► A subset of patients with systemic sclerosis have markedly short telomeres in lymphocytes and a higher prevalence of interstitial lung disease.

**What does this study add?**

► The presence of autoantibodies targeting telomere-associated proteins in systemic sclerosis and their association with short telomeres provides important insights into telomere dysfunction in systemic sclerosis, and raises the possibility that some forms of telomere dysfunction can be acquired through an aberrant immune response.
► The association of telomere-associated autoantibodies with interstitial lung disease in systemic sclerosis and the presence of these autoantibodies in idiopathic pulmonary fibrosis supports a role of telomere dysregulation in pulmonary fibrosis.

**How might this impact on clinical practice or future developments?**

► These autoantibodies could serve as novel biomarkers for systemic sclerosis and specifically for systemic sclerosis lung disease.

## INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune chronic fibrosing disease of unknown aetiology that results in vasculopathy and multi-organ fibrosis. The disease is heterogeneous with a wide range of possible clinical manifestations that include skin thickening, interstitial lung disease (ILD) and Raynaud's phenomenon.[1] The majority of patients with SSc develop ILD,[2] which has some clinical similarities with the progressive lung scarring seen in idiopathic pulmonary fibrosis (IPF).[3–5] Telomere dysregulation has been observed in both SSc and IPF,[6–8] although it remains unclear if there are common mechanistic pathways underlying telomere dysfunction in these diseases.

Telomeres are repetitive nucleotide sequences that protect the ends of chromosomes from deterioration and fusions with neighbouring chromosomes. Telomeres shorten with each cell division, serving as a 'molecular clock' for cellular ageing.[9] Telomeres are elongated by telomerase containing a telomere-specific reverse transcriptase (hTERT) that adds telomere repeat sequences to the end of telomeres. hTERT is one component of the human telomerase ribonucleoprotein (RNP), which is composed of the telomerase RNA component (hTR), hTERT, and the accessory proteins DKC1, NOP10, NHP2 and GAR1.[10] Other proteins associate with the telomerase complex and act as regulators of telomerase function, including the six shelterin proteins TERF1, TERF2, POT1, TPP1, TIN2L and RAP1.[11]

Telomere dysregulation is implicated in lung disease associated with IPF and autoimmune disease including SSc.[12] Germline mutations in hTERT or hTR are present in familial clusters of

IPF, and patients with such mutations have markedly shortened telomeres.[6 13 14] The literature on telomere dysregulation in SSc is conflicting and heterogeneous, in part due to variability in assays used to measure telomere length. Several studies have identified a subgroup of patients with SSc with markedly short telomeres in lymphocytes[8 15–17] who seem to be at increased risk of ILD.[15 18] The association between germline mutations in telomere-associated genes and IPF, together with the short telomeres observed in some patients with systemic sclerosis–ILD, raises the possibility that the fibrotic lung disease observed in these two patient subgroups might be phenocopies, potentially representing the consequence of inherited and acquired defects in telomere function.

Distinct SSc clinical phenotypes have been defined by the presence of specific autoantibodies. These autoantibodies often target intracellular nuclear proteins that maintain chromosome structure and function, including proteins involved in mitosis, DNA replication and DNA repair.[19 20] Subgrouping SSc by autoantibodies has utility in predicting clinical manifestations and can provide insights into the biological mechanisms underlying this disease.[21] Since telomere lengths are relatively short in a subset of patients with systemic sclerosis, we hypothesised that this subgroup may be defined by an immune response with autoantibodies targeting the telomerase complex that is associated with a specific clinical phenotype. In this study, we identify autoantibodies targeting multiple telomere-associated proteins in a subset of patients with SSc and demonstrate an association with shortened peripheral leucocyte telomere length and fibrotic lung disease.

## METHODS

### Patient cohorts

Sera were obtained from consecutive patients who met classification criteria for SSc at the Johns Hopkins (JH) and the University of California, San Francisco (UCSF) Scleroderma Centers. These two independent cohorts have similar databases and collect identical demographic and longitudinal clinical information including pulmonary function test data and organ-specific disease severity assessed by the Medsger Disease Severity Scale (online supplemental methods 1). We also assayed sera from healthy controls and patients with myositis, rheumatoid arthritis (RA) and IPF (online supplemental methods 2).

### Patient and public involvement

Patients were recruited to participate in the longitudinal cohorts during routine clinical visits and all patients signed informed consent. Results will be disseminated through conference presentations.

### Immunoprecipitation assays for autoantibody detection

*Cell lysate immunoprecipitation:* To determine if patient sera contain autoantibodies targeting hTERT, we developed an immunoprecipitation (IP) assay using a cell lysate overexpressing telomerase. A cell line overexpressing the telomerase RNA component (hTR) and FLAG-tagged human telomerase (hTERT) was generated using a Flp-In T-Rex 293 cell line per the manufacturer's instructions (Thermo Fisher). Fifty micrograms of cell lysate was pre-cleared with protein A beads in NP40 lysis buffer (online supplemental methods 3) and immunoprecipitated with 1 µL patient serum. Immunoprecipitates were electrophoresed on SDS-PAGE gels blotted with anti-FLAG antibody (Millipore, Sigma) and visualised using enhanced chemiluminescence (ThermoFisher) in a FluorChem M chemiluminescence imager (ProteinSimple). The data were quantitated by densitometric scanning of the blots and analysed using ImageJ.[22] Each sample set was calibrated with the same positive reference IP that was run on each blot. The cut-off for a positive autoantibody was defined as the mean+4 SD of the healthy controls.

*IP using $^{35}$S-methionine-labelled proteins*: Complementary DNAs for human POT1, TPP1, TIN2L, TERF1, TERF2, RAP1, NHP2 and DKC1 (GenScript) were used to generate $^{35}$S-methioinine-labelled proteins by in vitro transcription and translation (IVTT) per the manufacturer's protocol (Promega). The radiolabeled proteins were immunoprecipitated with patient sera in lysis buffer, and the products were electrophoresed on SDS-PAGE gels and visualised by fluorography.[23]

### TERF1 ELISA

The detailed ELISA protocol is in online supplemental methods 3. ELISA plates were coated overnight at 4°C with 200 ng/well of recombinant full-length TERF1 protein (Sino Biological). Patient sera were used at 1:200 dilution and secondary antibodies were horseradish peroxidase labelled. The colour was developed using SureBlue peroxidase reagent (Seracare Life Sciences) and the absorbance was read at 450 nm. The same positive reference serum (with an optical density (OD) in the linear range) was included on every plate as a calibrator. The cut-off for autoantibody positivity was set as the mean+4 SD of 50 healthy controls. All positive sera were re-tested by ELISA alongside an uncoated well; ODs of the uncoated wells were subtracted from those obtained with TERF1-coated wells.

### Immunoblotting assays

Recombinant TERF1 protein (200 ng/lane) was electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membranes for the immunoblotting assays. Sera from patients and healthy controls were used at 1:2000 dilution for these immunoblots (see online supplemental methods 3 for details).

### Other autoantibody assays

The JH SSc sera were screened for autoantibodies targeting SSc-associated autoantibodies using the line immunoblot platform (EuroImmun: Systemic Sclerosis [Nucleoli] profile). U1RNP autoantibodies were assayed using a commercially available ELISA (Inova Diagnostics, CA). Euroimmun results were considered positive per the manufacturer's guidelines (online supplemental methods 1). Autoantibodies in the UCSF cohort were derived from clinically indicated commercial testing.

### Telomere length measurements

Two assays were used to measure telomere length: (1) a PCR-based assay measured telomere length in peripheral leucocytes from the UCSF SSc cohort as previously described[24 25] (online supplemental methods 4); (2) Flow-FISH was used to measure telomere length in banked peripheral blood mononuclear cells (PBMCs) prospectively collected from a subset of the JH SSc cohort with and without TERF1 autoantibodies. Flow-FISH was done on all samples in batch as previously described.[26 27] Telomere lengths were compared with a validated nomogram of telomere length among healthy controls.[27]

### Statistics

Fisher's exact test was used to evaluate differences in the frequency of TERF1 autoantibodies between different patient cohorts. The various demographic, clinical and serological features of systemic sclerosis, as well as differences in telomere

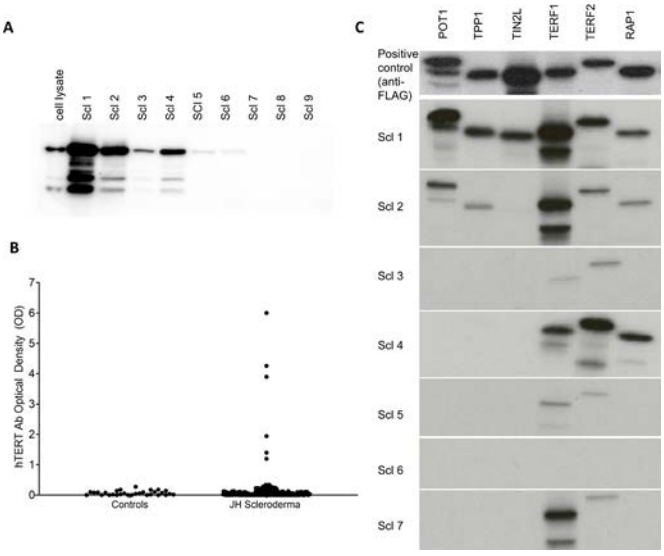


**Figure 1** Autoantibodies targeting the telomerase/shelterin complex in scleroderma. (A, B) Immunoprecipitations (IPs) were performed with patient sera (JH scleroderma cohort, n=200) using lysate made from HEK293 cells overexpressing hTERT-FLAG as input. IPs were detected by blotting with an anti-FLAG antibody. hTERT autoantibodies were found in 6/200 of the patients with scleroderma (Scl 1–6) and 0/30 healthy controls. (C) IPs were performed with patient sera using the six $^{35}$S-methionine-labelled shelterin proteins generated by in vitro transcription and translation. IPs with anti-FLAG was used for positive controls. At least one shelterin autoantibody was detected in 6/200 scleroderma patient sera. In total, 7/200 (3.5%) patient sera either immunoprecipitated hTERT or had an autoantibody targeting at least one shelterin protein.

length, were compared between the TERF1 autoantibody-negative and autoantibody-positive patients using the Wilcoxon rank-sum test or Student's t-test for continuous variables and Fisher's exact test for dichotomous variables. All statistical analyses were two-sided and were conducted using JMP V.9 (SAS Institute). A p value $<0.05$ was considered significant.

## RESULTS

### Discovery cohort: sera from a subset of patients with SSc IP telomerase and the shelterin proteins

To test whether patients with SSc have autoantibodies targeting telomerase (hTERT), we screened 200 sera from the JH SSc cohort for these autoantibodies by IP using lysate made from HEK 293 cells overexpressing FLAG-tagged hTERT and telomerase RNA (hTR).[28] The IPs were electrophoresed and hTERT was visualised by immunoblotting with anti-FLAG. Of the 200 JH SSc sera screened with this assay, 6 (3.0%) immunoprecipitated hTERT. We did not identify hTERT autoantibodies in 30 healthy control sera (figure 1A,B).

The same 200 sera from the JH SSc cohort were screened for autoantibodies targeting the six shelterin proteins (POT1, TPP1, TIN2L, TERF1, TERF2, RAP1) by IP using $^{35}$S-methionine-labelled protein generated by IVTT as input. 7/200 (3.5%) SSc sera immunoprecipitated either hTERT or one of the shelterin proteins, and 6 of these patients had multiple telomere-associated autoantibodies (figure 1C). In contrast, 0/30 healthy controls had a shelterin autoantibody. Representative images of negative controls are shown in online supplemental figure S1. None of the seven patients with telomere-associated autoantibodies had autoantibodies targeting DKC1 or NHP2.

### Validation cohort: TERF1 autoantibodies detected by ELISA in the JH and UCSF SSc cohorts

As TERF1 was the most common of the shelterin autoantibodies and overlapped with multiple other telomere-associated autoantibodies, we developed an ELISA to screen for TERF1 autoantibodies. Five out of six patients with TERF1 autoantibodies identified by IVTT IP were positive by ELISA (all except Scl 3, figure 1). In total, the ELISA detected TERF1 autoantibodies in 22/200 (11.0%) of the JH cohort. As a validation cohort, we screened 242 sera from the UCSF SSc cohort by ELISA and identified TERF1 autoantibodies in 18/242 (7.4%) patients. Table 1 includes demographic and clinical features of both cohorts. Of the 40 patients total with TERF1 autoantibodies identified by ELISA, 7/40 (18%) were positive by TERF1 IVTT IP. While the ELISA likely detected more positive sera compared with IVTT IP because of differences in TERF1 protein conformation used in the assays, we set up a third assay (immunoblotting of recombinant TERF1 protein) to better address the issue of the discrepant TERF1 autoantibody readouts. Using this, we confirmed the presence of TERF1 autoantibodies in 25/32 (78%) sera that were ELISA-positive but IP-negative using patient sera to immunoblot TERF1 protein (online supplemental figure S2). We expanded the number of healthy controls screened to 78 and found that the prevalence of TERF1 autoantibodies among patients with SSc in both cohorts (40/442 (9.0%)) was significantly higher compared with healthy controls (1/78 (1.3%)), p=0.01).

### TERF1 autoantibodies in other rheumatic diseases

To determine the specificity of TERF1 autoantibodies for systemic sclerosis, we screened 60 RA and 60 myositis sera for TERF1 autoantibodies by ELISA. The mean age of the systemic sclerosis, RA and myositis cohorts were similar, and detailed demographic and clinical information for these cohorts is in online supplemental tables S1 and S2. In each of the RA and myositis cohorts, 1/60 (1.7%) patients had a positive TERF1 autoantibody, which was similar to healthy controls (figure 2). TERF1 autoantibodies were significantly more frequent among patients with SSc (JH and UCSF combined) compared with RA or myositis (40/442 (9.0%) vs 1/60 (1.7%), p=0.05 in each case).

### TERF1 autoantibodies and telomere length in leucocytes

We next sought to determine if autoantibodies targeting the telomerase/shelterin complex are associated with abnormalities in telomere length. Telomere length was measured by qPCR in peripheral leucocytes from all UCSF patients with SSc using the same banked blood draw from which the TERF1 autoantibodies were assayed. Figure 3A shows telomere length plotted by age for all patients with and without TERF1 autoantibodies. Given that telomeres shorten with a constant linear rate in middle age,[29] we calculated each patient's expected telomere length using a linear regression model based on the relationship between age and telomere length among the TERF1 autoantibody-negative patients (expected telomere length (bp)=7028–12.62×(years of age)). The difference between the patient's telomere length and the expected telomere length was then calculated for each patient. Compared with patients without TERF1 autoantibodies, significantly more patients with TERF1 autoantibodies had a shorter telomere length than the expected age-adjusted telomere length (14/18 (78%) vs 96/224 (43%)), p=0.006). Furthermore, the difference between the patient telomere length and the expected age-adjusted telomere length was significantly more negative for patients with TERF1 autoantibodies compared with patients without TERF1 autoantibodies (median −230 (IQR −572 to

**Table 1** Demographics and disease characteristics of patients in the Johns Hopkins (JH) and University of California, San Francisco (UCSF) systemic sclerosis (SSc) cohorts

| | JH SSc cohort (n=200) | UCSF SSc cohort (n=242) | P value |
|---|---|---|---|
| Age (years), mean (SD) | 57.9 (13.4) | 54.6 (13.2) | 0.009** |
| Sex | | | |
| Female, n (%) | 171 (85) | 207 (86) | 1.0 |
| Male, n (%) | 29 (15) | 35 (14) | |
| Race | | | |
| Caucasian, n (%) | 153/198 (77) | 142/241 (59) | <0.0001*** |
| African American, n (%) | 36/198 (18) | 23/241 (10) | |
| Asian or Indian, n (%) | 9/198 (5) | 76/241 (32) | |
| SSc type | | | |
| Limited, n (%) | 127 (64) | 160 (66) | 0.62 |
| Diffuse, n (%) | 73 (36) | 82 (34) | |
| Disease duration at time of bleed | | | |
| From onset of RP, median (IQR) | 12.5 (6.6–21.4) | 11.1 (5.4–20.0) | 0.09 |
| From onset of non-RP symptom, median (IQR) | 12.1 (6.1–18.2) | 9.4 (4.3–16.3) | 0.001** |
| Autoantibody status | | | |
| Centromere, n (%) | 62/199 (31) | 60/241 (25) | 0.16 |
| U1RNP, n (%) | 17/200 (9) | 19/237 (8) | 0.86 |
| Scl70, n (%) | 44/199 (22) | 64/241 (27) | 0.32 |
| RNA polymerase III, n (%) | 39/199 (20) | 43/237 (18) | 0.81 |
| Ku, n (%) | 11/199 (6) | 82/241 (34) | 0.69 |
| No SSc-specific Ab, n (%) | 64/199 (32) | | |
| Clinical features | | | |
| History of cancer (ever), n (%) | 38/200 (19) | 39/242 (16) | 0.45 |
| Mortality, n (%) | 16/242 (7) | 6/200 (3) | 0.12 |
| Inflammatory arthritis (ever), n (%) | 40/200 (20) | 55/242 (23) | 0.56 |
| Digital ulceration or gangrene (ever), n (%) | 47/200 (24) | 100/242 (41) | <0.0001*** |
| SSc renal crisis (ever), n (%) | 5/200 (3) | 11/242 (5) | 0.31 |
| Myopathy (ever), n (%) | 43/200 (22) | 23/242 (10) | 0.0005*** |
| Max MRSS, mean (SD) | 10.9 (10.4) | 6.6 (7.2) | <0.0001*** |
| Severe muscle disease (ever), n (%) | 51/200 (26) | 18/242 (7) | <0.0001*** |
| Severe heart disease (ever), n (%) | 53/194 (27) | 52/242 (22) | 0.18 |
| Severe lung disease (ever), n (%) | 59/196 (30) | 92/242 (38) | 0.09 |
| Max RVSP (mm Hg), mean (SD) | 36.2 (11.8) | 38.4 (21.3) | 0.17 |
| Min DLCO % predicted, mean (SD) | 64.8 (20.5) | 52.3 (21.3) | <0.0001*** |
| Min FVC % predicted, mean (SD) | 74.4 (19.2) | 70.7 (22.6) | 0.07 |

200 patients were in the JH cohort and 242 in the UCSF cohort. Results are depicted as median with IQR and mean with SD. Ku autoantibody data was not available from the UCSF cohort. No SSc-specific autoantibody is defined as seronegativity for centromere, Scl70 and RNA polymerase III. Pulmonary function test and echocardiogram data were reported as the maximum (max) right ventricular systolic pressure (RVSP) and the minimum (min) diffusion capacity (DLCO) and FVC recorded in the longitudinal database. Wilcoxon rank-sum test or Student's t-test were used for continuous variables and Fisher's exact test for dichotomous variables.
*p<0.05, **p<0.01, ***p<0.001.
MRSS, modified Rodnan Skin Score.

−18) vs 53 (−272 to 304) bp, p=0.01, Wilcoxon rank sum) (figure 3B).

We next sought to confirm the association between TERF1 autoantibodies and short telomeres in the JH SSc cohort using the Flow-FISH assay, which is known to be more accurate, reproducible, sensitive and specific compared with qPCR[16] and can simultaneously differentiate telomere length in lymphocytes and granulocytes. We identified 6 patients with TERF1 autoantibodies and 10 patients without TERF1 autoantibodies who presented for routine clinical visits and agreed to donate PBMCs. ELISAs performed on serum collected concurrently were used to determine TERF1 autoantibody status. Telomere length was measured on PBMCs using Flow-FISH. The delta TL (telomere length), which is the difference between the patient's telomere length and the median telomere length for a healthy person of the same age, was significantly more negative for the TERF1 autoantibody-positive patients compared with the TERF1 autoantibody-negative patients in lymphocytes (median −1132 (IQR −1552 to −996) vs −254 (−950 to 464) bp, p=0.03, Wilcoxon rank sum). This difference was not observed in granulocytes (median −706 (IQR −1686 to 22) vs −829 (−1122 to −446) bp, p=0.8, Wilcoxon rank sum) (figure 4). The two patients with the highest titre hTERT autoantibodies both had telomere lengths below the 10th percentile in lymphocytes and granulocytes.

## Clinical and serological associations with TERF1 autoantibodies in systemic sclerosis

After identifying the existence of TERF1 autoantibodies in SSc and demonstrating an association of these autoantibodies with short telomeres in lymphocytes, we next explored associated clinical and serological features (table 2). The JH and UCSF SSc cohorts use standardised clinical definitions with harmonisation

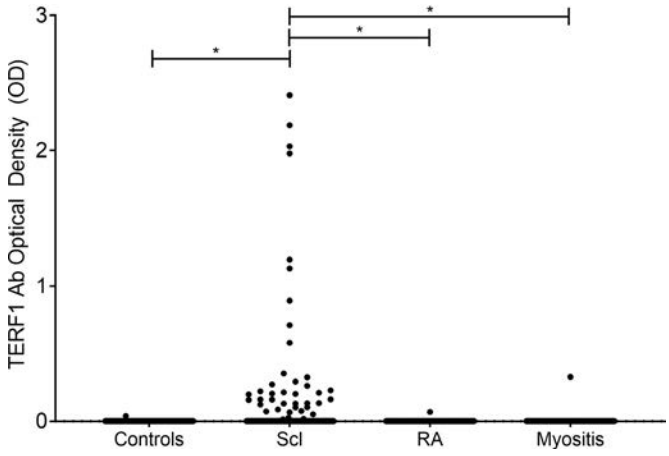


**Figure 2** TERF1 autoantibodies detected by ELISA in healthy controls (n=78), the combined JH and UCSF scleroderma cohorts (Scl, n=442), rheumatoid arthritis (n=60) and myositis (n=60). Fisher's exact test was used to compare the frequency of TERF1 autoantibodies between different cohorts. *p<0.05.

in clinical data acquisition, enabling the evaluation of clinical associations for all 40 TERF1 autoantibody-positive and 402 TERF1 autoantibody-negative patients. The length of clinical follow-up was similar between patients with and without TERF1

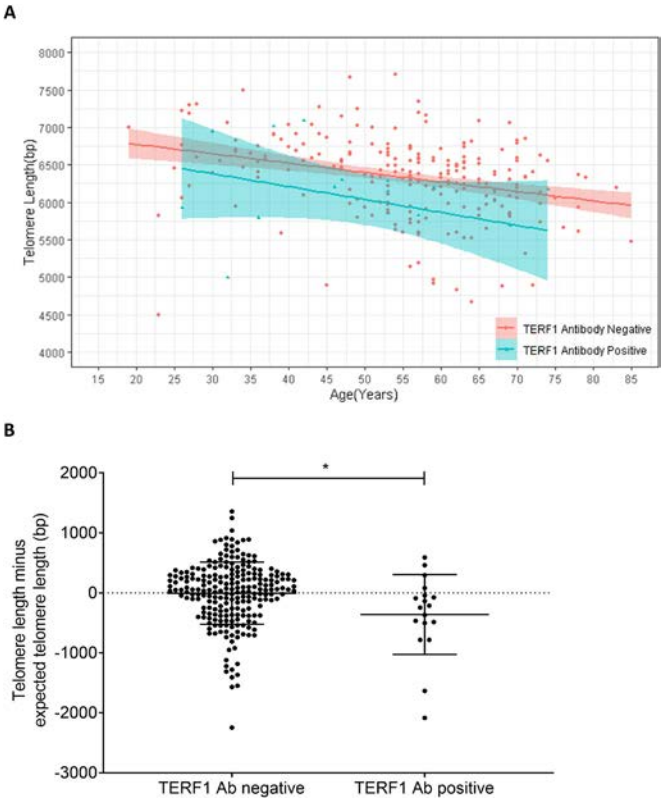


**Figure 3** Peripheral blood leucocyte telomere length measured by qPCR in 242 patients with scleroderma from the University of California, San Francisco (UCSF) Scleroderma Center. (A) Relationship between leucocyte telomere length and age for TERF1 autoantibody-positive (n=18) and autoantibody-negative (n=224) patients. (B) Patients with TERF1 autoantibodies have a significantly shorter telomere length relative to the expected age-adjusted telomere length compared with patients without TERF1 autoantibodies. Statistics were performed using Wilcoxon rank-sum test, *p<0.05.

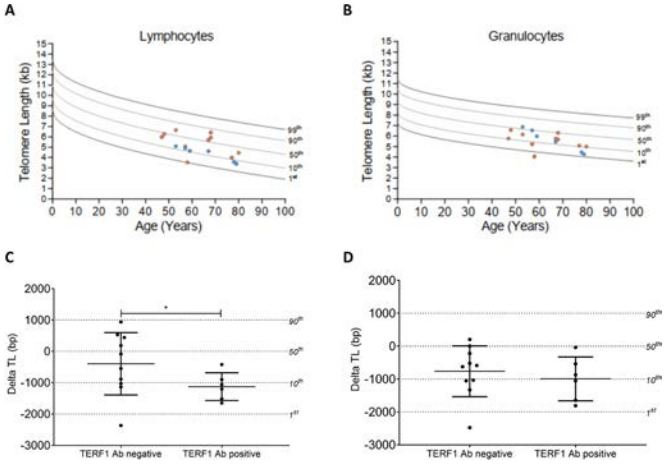


**Figure 4** Telomere length measured by Flow-FISH (flow cytometry and fluorescent in situ hybridisation) in lymphocytes and granulocytes of 6 patients with TERF1 autoantibody-positive systemic sclerosis and 10 patients with TERF1 autoantibody-negative systemic sclerosis. (A, B) Nomogram of telomere length relative to age in lymphocytes and granulocytes relative to a healthy control population depicted by percentiles. Patients with TERF1 autoantibodies are depicted in blue, and those without TERF1 autoantibodies are in red. (C, D) Patients with TERF1 autoantibodies have shorter telomere lengths in lymphocytes (C) but not granulocytes (D) compared with TERF1 autoantibody-negative patients. Delta TL is the difference between the patient telomere length and the median telomere length of a healthy control population. bp, base pairs; kb, kilobases. Statistics were performed with Wilcoxon rank-sum test, *p<0.05.

autoantibodies (6.8±6.2 vs 6.0±6.0 years, p=0.46). Patients with TERF1 autoantibodies tended to be slightly younger (52.6±13.7 vs 56.4±13.3 years, p=0.10). The presence of TERF1 autoantibodies was significantly associated with a history of severe lung disease (OR 2.4 (CI 1.2 to 4.8), p=0.04) and a lower percent predicted diffusion capacity (DLCO) within 1 year of serum collection (58.0 vs 67.9, p=0.02, Student's t-test). There was also an association with a history of severe muscle disease (OR 3.0 (CI 1.4 to 6.1), p=0.005) and inflammatory arthritis (OR 2.1 (CI 1.1 to 4.3), p=0.04). Non-white race was strongly associated with severe lung disease (OR 2.3 (CI 1.5 to 3.5), p<0.0001) and was also associated with the presence of TERF1 autoantibodies (OR 2.5 (CI 1.3 to 4.8), p=0.005). The association between TERF1 autoantibodies and severe lung disease was not statistically significant after adjusting for race (OR 1.73 (CI 0.88 to 3.4), p=0.11).

TERF1 autoantibodies were associated with U1RNP autoantibodies in the combined cohorts (OR 4.8 (CI 2.1 to 10.8), p=0.0006) and Ku autoantibodies in the JH cohort (OR 5.4 (CI 1.4 to 20.2), p=0.02) (table 2, figure 5). Ku autoantibody status was not available for the UCSF cohort. There was no association with the frequent SSc-specific autoantibodies anti-Scl-70, anti-centromere or anti-RNA polymerase III. Absence of these SSc-specific autoantibodies was observed slightly more frequently in patients with TERF1 autoantibodies compared with patients without TERF1 autoantibodies (17/40 (42%) vs 129/400 (32%), p=0.22), although this difference was not significant.

### TERF1 autoantibodies in IPF

To address whether TERF1 autoantibodies might be present in other syndromes in which telomere dysfunction and lung fibrosis are prominent, we screened 152 patients with IPF and

**Table 2** Clinical and serological characteristics among patients with TERF1 autoantibody-positive (n=40) and autoantibody-negative (n=402) systemic sclerosis (SSc) from the Johns Hopkins (JH) and University of California, San Francisco (UCSF) SSc cohorts

| | TERF1 Ab positive (n=40) | TERF1 Ab negative (n=402) | P value |
|---|---|---|---|
| Age (years), mean (SD) | 52.6 (13.7) | 56.4 (13.3) | 0.10 |
| Sex, female, n (%) | 34 (85) | 344 (86) | 1.0 |
| Race, Caucasian, n (%) | 19/40 (48) | 276/399 (69) | 0.008** |
| African American, n (%) | 10/40 (25) | 49/399 (12) | |
| Asian, n (%) | 11/40 (28) | 74/399 (19) | |
| SSc type, limited, n (%) | 24 (60) | 263 (65) | 0.49 |
| Disease duration | | | |
| From onset of RP, median (IQR) | 13.9 (7.2–22.5) | 11.6 (5.9–20.6) | 0.08 |
| From onset of non-RP symptom, median (IQR) | 12.2 (8.4–17.5) | 10.4 (4.5–17.1) | 0.08 |
| Autoantibody status | | | |
| Centromere, n (%) | 10/40 (25) | 112/400 (28) | 0.85 |
| U1RNP, n (%) | 10/40 (25) | 26/397 (7) | 0.0006*** |
| Scl70, n (%) | 11/40 (28) | 97/400 (24%) | 0.70 |
| RNA polymerase III, n (%) | 5/39 (13) | 77/397 (19%) | 0.39 |
| Ku, n (%) | 4/22 (18) | 7/177 (4) | 0.02 * |
| No SSc-specific Ab, n (%) | 17/40 (42) | 129/400 (32) | 0.22 |
| Clinical features (ever, max/min) | | | |
| History of cancer (ever), n (%) | 7/40 (18) | 70/402 (17) | 1.0 |
| Mortality, n (%) | 1/40 (3) | 21/402 (5) | 0.71 |
| Inflammatory arthritis (ever), n (%) | 14/40 (35) | 81/402 (20) | 0.04* |
| Digital ulceration or gangrene (ever), n (%) | 12/40 (30) | 140/402 (35) | 0.60 |
| SSc renal crisis (ever), n (%) | 1/40 (3) | 15/402 (4) | 1.0 |
| Myopathy (ever), n (%) | 8/40 (20) | 58/402 (14) | 0.35 |
| Max MRSS, mean (SD) | 8.0 (9.3) | 8.6 (9.0) | 0.44 |
| Severe muscle disease (ever), n (%) | 13/40 (33) | 56/402 (14) | 0.005** |
| Severe heart disease (ever), n (%) | 14/39 (36) | 91/397 (23) | 0.08 |
| Severe lung disease (ever), n (%) | 20/40 (50) | 131/398 (33) | 0.04 * |
| Max RVSP (mm Hg), mean (SD) | 39.9 (20.1) | 37.3 (17.7) | 0.46 |
| Min DLCO % predicted, mean (SD) | 53.0 (20.6) | 58.4 (21.9) | 0.13 |
| Min FVC % predicted, mean (SD) | 66.5 (20.4) | 72.9 (21.2) | 0.07 |
| PFTs within 1 year of bleed date | TERF1 Ab positive (n=34) | TERF1 Ab negative (n=354) | |
| DLCO % predicted, mean (SD) | 58.0 (22.5) | 67.9 (23.4) | 0.02 * |
| FVC % predicted, mean (SD) | 75.0 (21.3) | 80.2 (20.5) | 0.18 |

Results are depicted as median with IQR and mean with SD. No SSc-specific autoantibody is defined as seronegativity for centromere, Scl70 and RNA polymerase III. Pulmonary function test (PFT) and echocardiogram data were reported as the maximum (max) right ventricular systolic pressure (RVSP) and the minimum (min) diffusion capacity (DLCO) and FVC recorded in the longitudinal database. Wilcoxon rank-sum test or Student's t-test were used for continuous variables and Fisher's exact test for dichotomous variables.
*p<0.05, **p<0.01, ***p<0.001.
MRSS, modified Rodnan skin score.

identified TERF1 autoantibodies in 11/152 (7.2%) patients, compared with only 1/78 (1.3%) positives among healthy controls (p=0.06) (figure 6). Further details on the IPF cohort are in online supplemental table S3. The patient in the IPF cohort with the highest TERF1 autoantibody titre had a positive ANA (1:160, speckled) and subsequently developed symptoms of SSc approximately 2 years later. It was determined that this patient most likely had systemic sclerosis–ILD rather than IPF, although the TERF1 autoantibody had preceded the other clinical features of systemic sclerosis. The other patients with IPF with TERF1 autoantibodies did not have a positive ANA and have not, to our knowledge, developed any features of a systemic autoimmune disease.

## DISCUSSION

We describe a subgroup of patients with SSc with autoantibodies targeting the telomerase and shelterin complex, characterised by short telomeres in lymphocytes and the presence of lung disease. These autoantibodies are also present in a subset of patients with IPF, but are rarely detected in healthy controls, RA or myositis. While prior studies have demonstrated telomere dysregulation in SSc,[30] to our knowledge this is the first description of highly specific autoantibodies targeting the telomerase/shelterin complex in a rheumatic disease. The association of these autoantibodies with short telomeres in systemic sclerosis, and the lower prevalence of these autoantibodies in other chronic inflammatory diseases such as RA that are also known to have telomere dysfunction,[31] suggests that the mechanism of telomere dysregulation in SSc may be distinct from other inflammatory diseases. The presence of germline mutations in the essential telomerase genes in familial and sporadic IPF supports a causal role for telomere dysfunction in pulmonary fibrosis.[6 32] However, germline mutations account for only a fraction of IPF cases with short telomeres, suggesting there are other mechanisms of telomere

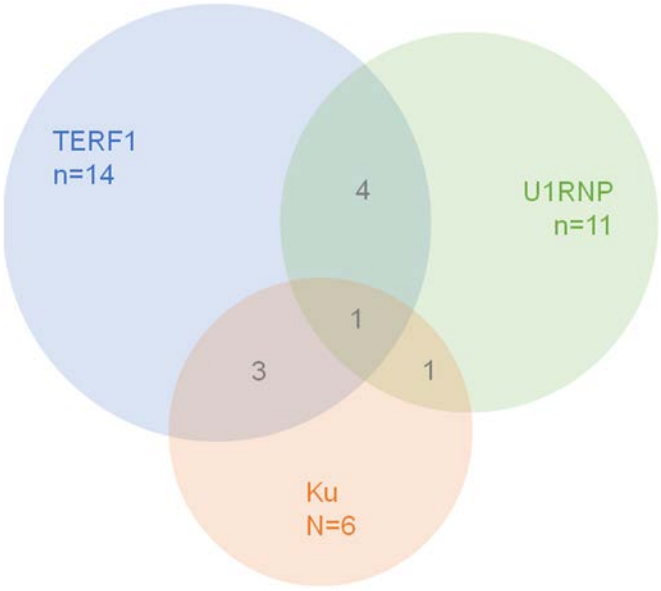


**Figure 5** Overlap among TERF1 (n=22), U1RNP (n=17) and Ku (n=11) autoantibodies in the Johns Hopkins (JH) scleroderma cohort. 40/200 (20%) of patients in the JH cohort had at least one of these autoantibodies.

dysregulation.[33] Our findings raise the possibility that an immune response directed against telomere-associated proteins may also be implicated in telomere shortening in a distinct subgroup of patients with IPF.

Although the presence of autoantibodies targeting the shelterin protein TERF1 were enriched among patients with SSc with short telomeres, the majority of patients with short telomeres did not have these autoantibodies. Therefore, there may be multiple distinct mechanisms leading to short telomeres in systemic sclerosis, and the presence of these autoantibodies may be indicative of one such mechanism. We speculate that the subset of patients with TERF1 autoantibodies have abnormal processing and presentation of telomere-associated proteins, leading to an immune response against the multimolecular telomere complex. In support of this hypothesis, patients developed autoantibodies targeting multiple components of the telomerase and shelterin complexes, suggesting loss of tolerance and epitope spreading across multiple related proteins.[34]

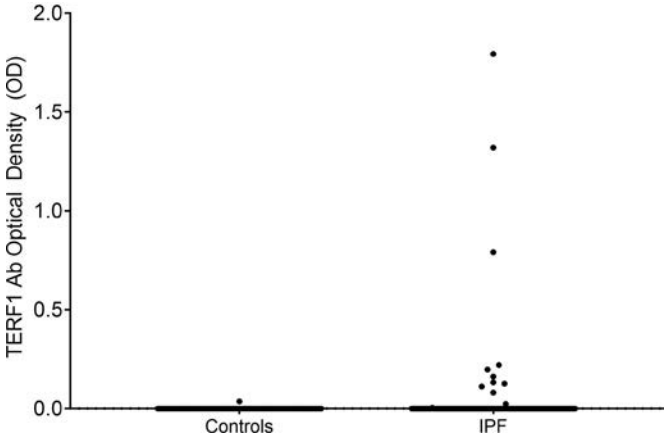


**Figure 6** TERF1 autoantibodies detected by ELISA in healthy controls (n=78) and the University of California, San Francisco (UCSF) Idiopathic Pulmonary Fibrosis (IPF) cohort (n=152).

The association between autoantibodies directed against telomere-associated proteins and short telomeres could also indicate that these autoantibodies exert a directly pathogenic effect on telomeres. We only observed telomere shortening in lymphocytes from patients with SSc with TERF1 autoantibodies. It is possible that telomeres in granulocytes might be spared from telomere shortening in the setting of a pathogenic autoantibody because of the short life-span of a granulocyte (only a few days), while lymphocytes survive and circulate for weeks to months. Additional research is needed to understand the biology underlying this highly specific association between telomere-associated autoantibodies and shortened telomeres in SSc and the significance of these autoantibodies in IPF.

The association between lung disease and TERF1 autoantibodies in SSc is consistent with previous studies which have found more severe lung disease among patients with SSc with short telomeres in lymphocytes,[15 18] and further supports a role for telomere dysfunction in the pathogenesis of SSc lung disease. The patient in the IPF cohort with the highest titre TERF1 autoantibody developed systemic symptoms consistent with SSc several years after enrolment, suggesting that some patients who meet diagnostic criteria for IPF actually have SSc lung disease. TERF1 autoantibodies may serve as an early biomarker to predict subsequent progression to a more definitive diagnosis of systemic sclerosis. This patient was notable for having a high-titre ANA unlike the other patients with IPF with TERF1 autoantibodies, suggesting that in most cases, TERF1 autoantibodies may still be an indicator of telomere dysfunction in classic IPF.

TERF1 autoantibodies were associated with anti-Ku and anti-U1RNP, specificities which are predictive of SSc overlap syndromes. However, most patients with TERF1 autoantibodies did not have Ku or U1RNP autoantibodies, indicating that TERF1 autoantibodies may provide non-redundant clinical information and could serve as another biomarker for an overlap phenotype. The protein Ku is involved in telomere capping[35 36] and it is therefore possible that Ku and TERF1 autoantibodies may both reflect underlying telomere dysfunction.

Strengths of this study are the use of two diverse and well-characterised longitudinal SSc cohorts and the use of two different telomere length assays. There was some discordance in the sensitivities of the three assays used to detect TERF1 autoantibodies, and future validation studies are needed to determine the prevalence of these autoantibodies in independent cohorts. Limitations of this study include the small sample size for the Flow-FISH assay, lack of age-matching between the cohorts and the fact that ILD was not radiographically confirmed on all patients in the SSc cohort. Future studies using RA and myositis cohorts systematically screened for ILD will enable the presence of TERF1 autoantibodies in RA–ILD or myositis–ILD to be evaluated. We had limited power for subgroup analysis, and further studies are needed to characterise the relationship between race, TERF1 autoantibodies and lung disease severity. While we did not identify an association between TERF1 autoantibodies and FVC in IPF, studies performed on larger cohorts are needed to make any definitive conclusions regarding the relationship between TERF1 autoantibodies and disease severity in IPF.

In summary, we describe a novel subgroup of patients with SSc and IPF with autoantibodies targeting the telomerase/shelterin complex that in SSc is associated with short telomeres in peripheral lymphocytes and the presence of lung disease. Telomere-associated autoantibodies may be pathogenically important in the fibrotic lung diseases with telomere dysfunction.

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**Contributors** BLA: contributed to the conception of the work and the data acquisition and interpretation, wrote manuscript. FB: contributed to data acquisition and revising the manuscript critically. PJW: contributed to data acquisition and revising the manuscript critically. COB: contributed to data acquisition and revising the manuscript critically. AAS: contributed to data acquisition and revising the manuscript critically. CG: contributed to revising the manuscript critically. LC-R: contributed to the conception of the work and the data acquisition and interpretation, revised the manuscript critically. AR: contributed to the conception of the work and the data acquisition and interpretation, revised the manuscript critically.

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**Competing interests** None declared.

**Patient consent for publication** Not required.

**Ethics approval** This study was approved by the Hopkins IRB (IRB00239503). The Institutional Review Boards at Johns Hopkins and UCSF approved the different components of this study.

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

**Data availability statement** Data are available on reasonable request. The data are stored as deidentified participant data which are available on request to BLA ( brittany.adler@jhmi.edu)



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TRANSLATIONAL SCIENCE

# Targeting human plasmacytoid dendritic cells through BDCA2 prevents skin inflammation and fibrosis in a novel xenotransplant mouse model of scleroderma

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## ABSTRACT

**Objectives** Plasmacytoid dendritic cells (pDC) have been implicated in the pathogenesis of autoimmune diseases, such as scleroderma (SSc). However, this has been derived from indirect evidence using *ex vivo* human samples or mouse pDC *in vivo*. We have developed human-specific pDC models to directly identify their role in inflammation and fibrosis, as well as attenuation of pDC function with BDCA2-targeting to determine its therapeutic application.

**Methods** RNAseq of human pDC with TLR9 agonist ODN2216 and humanised monoclonal BDCA2 antibody, CBS004. Organotypic skin rafts consisting of fibroblasts and keratinocytes were stimulated with supernatant from TLR9-stimulated pDC and with CBS004. Human pDC were xenotransplanted into Nonobese diabetic/severe combined immunodeficiency (NOD SCID) mice treated with Aldara (inflammatory model), or bleomycin (fibrotic model) with CBS004 or human IgG control. Skin punch biopsies were used to assess gene and protein expression.

**Results** RNAseq shows TLR9-induced activation of human pDC goes beyond type I interferon (IFN) secretion, which is functionally inactivated by BDCA2-targeting. Consistent with these findings, we show that BDCA2-targeting of pDC can completely suppress *in vitro* skin IFN-induced response. Most importantly, xenotransplantation of human pDC significantly increased *in vivo* skin IFN-induced response to TLR agonist and strongly enhanced fibrotic and immune response to bleomycin compared with controls. In these contexts, BDCA2-targeting suppressed human pDC-specific pathological responses.

**Conclusions** Our data indicate that human pDC play a key role in inflammation and immune-driven skin fibrosis, which can be effectively blocked by BDCA2-targeting, providing direct evidence supporting the development of attenuation of pDC function as a therapeutic application for SSc.

## Key messages

**What is already known about this subject?**

- Research to date on plasmacytoid dendritic cells (pDC) has focused only on indirect evidence using *ex vivo* human samples or mouse models, but suggests pDC involvement in interferon (IFN)-induced response, skin infiltration and fibrosis within immune-mediated inflammatory disease (IMID), such as scleroderma.

**What does this study add?**

- We devised a novel mouse model of human pDC through xenotransplantation of human pDC into immunocompromised mice, showing a significantly increased IFN-induced response to topical TLR agonist application and a strongly enhanced fibrotic and immune response to bleomycin, all of which were strongly suppressed by specific pDC BDCA2-targeting.
- We demonstrate directly that functional inactivation of human pDC through BDCA2-targeting suppresses the entire TLR9-induced transcriptome, which includes type I IFN activation and a multitude of genes that could contribute to immune-driven tissue damage.

**How might this impact on clinical practice or future developments?**

- These data offer the first direct evidence supporting the development of BDCA2-targeting as a therapeutic application for pDC-mediated skin inflammation and fibrosis.
- The development of a human pDC mouse model will allow the expansion into other IMID to confirm pDC involvement.

## INTRODUCTION

Plasmacytoid dendritic cells (pDC), specialised in the secretion of type I interferon (IFN),[1–3] activate inflammatory responses through TLR-mediated sensing of nucleic acids released from pathogens during infection or following cell death in autoimmunity.[4–8] Self-derived nucleic acids released from damaged tissues, apoptotic/necrotic cells or bound to autoantibodies, can be recognised by TLR7/8/9 and have been shown to induce pDC activation and IFN secretion.[9–15] The role of CXCL4 has been elucidated as an amplifier of TLR9-mediated pDC hyperactivation and IFN production by organising self-DNA into liquid crystalline immune complexes.[16]

TLR-induced activation of pDC triggers stable cell differentiation into three subtypes, with

PD-L1(CD274)$^{+}$CD80$^{-}$ (P1) and PD-L1$^{+}$CD80$^{+}$ (P2) subpopulations specialised in type I IFN production both in healthy volunteers (HV) and patients with autoimmune conditions.[17] pDC have been further implicated in the pathogenesis of autoimmune diseases, such as scleroderma (SSc), systemic lupus erythematosus (SLE) and psoriasis, through their ability to infiltrate the skin and secrete IFNs and proinflammatory chemokines.[13 18–22] Specifically, SSc is an immune-mediated inflammatory disease (IMID) characterised by vascular and tissue fibrosis, leading to diverse life-altering and life-threatening clinical manifestations.[23] pDC have been observed in affected skin of patients with SSc, and purified peripheral SSc pDC have been shown to spontaneously produce higher levels of type I IFN compared with HV.[24–26] Indeed, an elevated IFN gene signature in affected organs and in the blood is a common feature of severe disease in SSc,[25 27] which is present before the onset of clinical fibrosis.[28] These observations collectively support the notion that pDC activation and type I IFN play an important role in SSc pathogenesis.

BDCA2 is a type II transmembrane glycoprotein that belongs to the C-type lectin superfamily receptor that can signal to inhibit pDC type I IFN secretion.[2 29–31] BDCA2 signals through an associated transmembrane adaptor, the FcεRγ, which recruits the protein tyrosine kinase Syk, inducing protein tyrosine phosphorylation and calcium mobilisation,[32] which reduces TLR-induced activation of pDC, inhibiting type I IFN secretion and other inflammatory mediators.[30 32–34] In SLE clinical trials, BDCA2-targeting antibodies induced a significant but partial decrease in IFN response within the blood, and reduced type I IFN-induced response and immune infiltrates in skin lesions.[35 36] Recently, pDC's role in fibrosis was elucidated as elimination of mouse pDC reduced bleomycin-induced skin fibrosis,[24] further highlighting the therapeutic potential of BDCA2-targeting for SSc. However, exploring the efficacy of BDCA2-targeting during fibrosis is difficult, as BDCA2 is only expressed in primates, highlighting the need for a human-specific pDC *in vivo* model. Furthermore, there are key differences between mouse and human pDC; thus, functions determined in mouse models may not be fully transferable to human pDC.[13 37–39]

We developed human-specific models to uncover the role of pDC biology in inflammation and fibrosis, as well as attenuation of pDC function with BDCA2-targeting to determine its therapeutic application for SSc.

## RESULTS

### TLR9-induced activation of human pDC goes beyond type I IFN secretion and is hindered by BDCA2-targeting

Using RNAseq, we set out to discover the transcriptome of human pDC when stimulated with TLR9 agonist, A-class oligodeoxyribonucleotides containing CpG motifs (ODN2216/ODN), to understand the pathways that could contribute to the pathogenesis of chronic inflammation and immune-driven tissue damage seen in IMID. We performed RNAseq analysis of human pDC purified from peripheral blood mononuclear cells (PBMC) from four HV (online supplemental figure S1), as previously described.[34] Transcriptome analysis revealed 328 differentially expressed genes (DEGs ≥or ≤2 fold change, FDR≤0.05) between unstimulated (control/CTR) and ODN-stimulated pDC (ODN), with donor heterogeneity observed with ODN stimulation, suggesting pDC response donor variability (figure 1A and online supplemental table 1). Pathway analysis identified genes involved in immune response against viruses and other organisms as key enriched biological processes (figure 1B). These innate immune processes match to those previously identified in characterised inflammatory SSc skin subsets,[40] suggesting involvement of pDC activation in this specific subset. Consistent with this notion, we observed upregulation of many type I IFN-dependent pathways and IFN-related genes, such as IFN-A2, IFN-A21, IFN-B1 and CCL5, a common feature seen in SSc.[40–42] Pathway analysis also showed JAK/STAT, nuclear factor kappa B subunit 1 (NF-κB) and angiogenesis pathways to be major biological processes upregulated by ODN stimulation (figure 1B), which have been shown to be dysregulated in SSc[13 40 41] but not shown in pDC before. These data suggest that TLR stimulation of pDC can induce a multitude of genes beyond IFN, which could contribute to the pathogenesis of inflammation in SSc and other IMID. Interestingly, a recent publication showed that the majority of SSc skin samples with higher fibroblast scores had significantly increased macrophage and/or dendritic cell scores, suggesting a link between the two cell types that are important for inducing the fibroinflammatory signature.[41]

A monoclonal BDCA2 antibody (clone AC144) has previously been shown to suppress human pDC TLR-induced IFN type I secretion by interfering with the FcεRγ-Syk signalling.[19 30] To aid our understanding of human pDC in IMID, we generated mouse monoclonal antibodies (mAb) against human BDCA2 and fully humanised the lead mAb, CBS004, which had a greater affinity for BDCA2 compared with AC144 control (online supplemental figure S2A). *Ex vivo* direct competition assays showed that CBS004 and AC144 bind alternative pDC epitopes, as indicated by double staining of the pDC population gated within PBMC (LIN$^{-}$ HLA$^{+}$ CD123$^{+}$ CD304$^{+}$) (online supplemental figure S2B,C).

BDCA2-targeting using CBS004 reduced the expression of 60% of ODN-inducible DEGs ≥1.5-fold (figure 1C and online supplemental table 2). It has been recently shown that TLR-induced pDC triggers stable cell differentiation into three stable subtypes, with PD-L1(CD274)$^{+}$CD80$^{-}$ (P1) and PD-L1(CD274)$^{+}$CD80$^{+}$ (P2) subpopulations specialised in type I IFN production both in HV and patients with autoimmune conditions.[17] Consistent with these observations, ODN induced CD274 expression, which was also suppressed by BDCA2-targeting (figure 1C).[17] To validate the RNAseq findings at protein level, we measured the PD-L1 and CD80 positive subpopulations by fluorescence-activated cell sorting (FACS) (figure 1D,E). Similarly to what has been observed following viral stimulation,[17] ODN induced 62% of pDC differentiation into P1 and P2, with no differences observed between healthy and SSc samples (figure 1D,E). In this context, BDCA2-targeting caused an increase of the P3 subpopulation, which has been previously shown to produce less IFN.[17] These data were further validated by performing FACS analysis of IFN-positive pDC gated within human PBMC (online supplemental figure S2B). Functionally, ODN led to a dramatic induction of IFN positive pDC, which was reduced by 76% with BDCA2-targeting (figure 1F,G).

Beyond type I IFN signature genes, ODN-induced inflammatory interleukin (IL)-6 expression was also modulated by BDCA2-targeting. Interestingly, IL-6 and IFN secretion can synergistically activate B cells.[17 43] Among other genes of interest, serglycin (SRGN) also showed a TLR-induced and BDCA2-dependent pattern. SRGN has been shown to be secreted into the extracellular matrix and linked to promoting lymphoid cells adhesion and activation,[44 45] storage of chemokines and cytokines, as well as being able to induce epithelial–mesenchymal transition.[46 47] These data demonstrate that TLR stimulation of human pDC goes beyond IFN secretion induction and predicts a greater biological relevance of pDC activation in IMID. Our analyses show that TLR-induced pDC activation can be drastically suppressed by BDCA2-targeting.

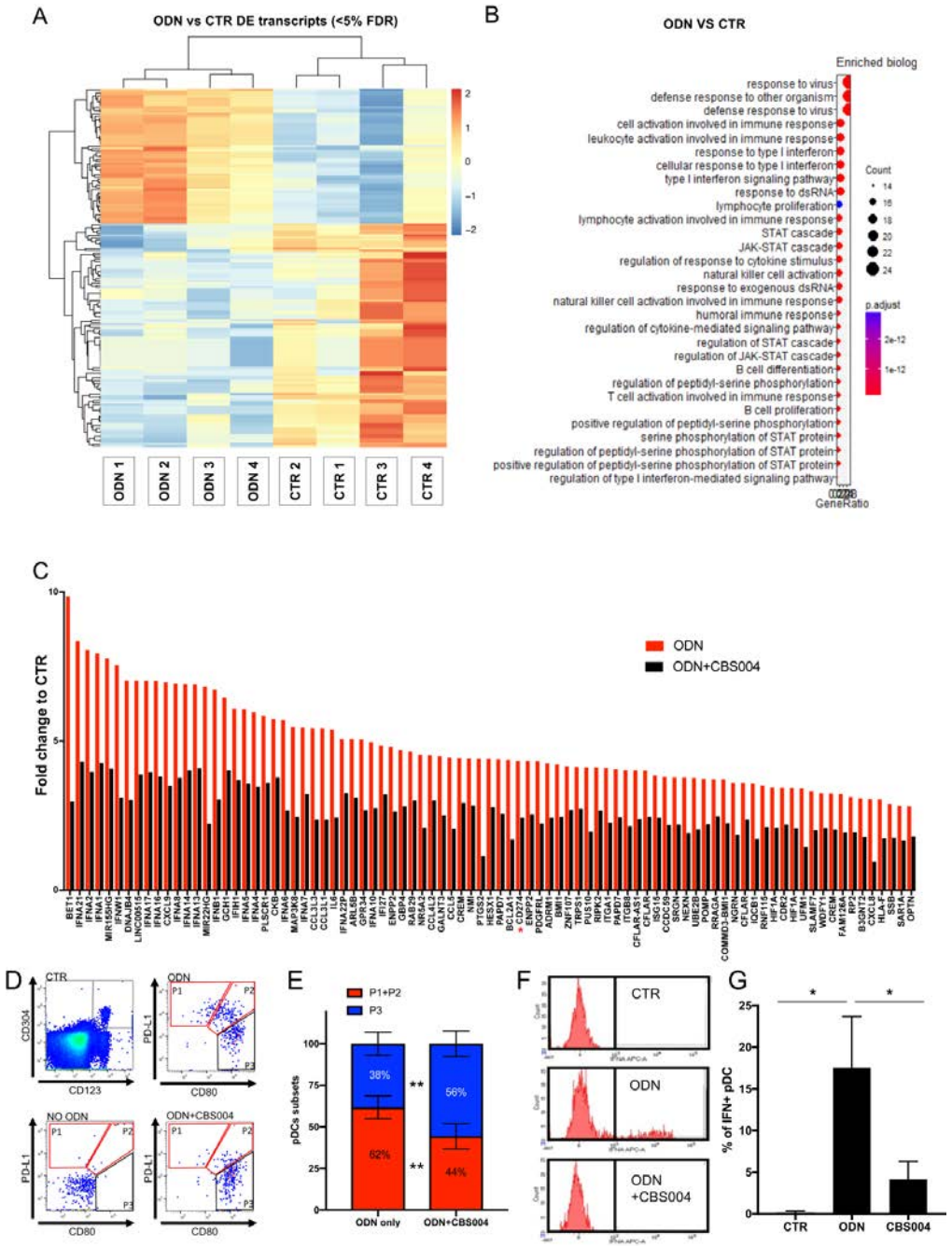


**Figure 1** TLR9-induced activation of human pDC goes beyond type I IFN secretion and is hindered by BDCA2-targeting *in vitro*. Transcriptome analysis of human pDC cultured in media alone (CTR), with 1 µM ODN2216 (ODN) or with ODN and CBS004 (10 µg/mL) (added 15 min prior to stimulation) for 16 hours (n=4). (A) Heatmap of reduced, centred normalised read counts for DE transcripts among CTR and ODN populations, <5% FDR, calculated using Benjamini-Hochberg multiple testing correction. DE transcripts≥or ≤2-fold (FDR<0.05) shown in online supplemental table 1. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showing top biological processes enriched in the set of DE transcripts between CTR and ODN pDC, and their associated p values. (C) Average fold change of DEGs for ODN and ODN+CBS004 relative to CTR (n=4). For repeat transcripts, the highest fold change was used for comparison. Red bars represent the 87 genes that were increased ≥2-fold (FDR<0.05) between CTR and ODN that are dependent on BDCA2 treatment (reduced ≥1.5-fold by CBS004) (full transcript data, online supplemental table 2). (D,E) Validation of CD274 (PD-L1) TLR-induced expression. (D) Example of subtyping FACS analysis of pDC ($CD123^+CD304^+$) in the three culture conditions (CTR, ODN and ODN+CBS004). Gating illustrates P1, P2 and P3 subtypes, with the former two previously shown to be IFN-secreting cells.[17] PBMC were cultured for 16 hours and pDC sorted as $Lin^-HLA\text{-}DR^+CD45^+CD123^+CD304^+$ (online supplemental figure S2B) and gated for PD-L1 and CD80 expression to determine P1, P2 and P3 sub-types. P1, $PD\text{-}L1^+CD80^-$; P2, $PD\text{-}L1^+CD80^+$; P3, $PD\text{-}L1^-CD80^+$.[17] (E) Quantification of sub-types based on FACS analysis of PBMC samples (healthy and SSc n=3, 4) between ODN and ODN+CBS004 culture conditions. (F,G) Validation of IFN-related gene expression. (F) Representative histogram of intracellular IFN alpha staining of pDC gated within PBMC when cultured in RPMI alone, with ODN (1 µM) and with CBS004 (10 µg/mL). (G) Percentage of IFN-positive pDC from FACS analysis from F (n=6). (E,F) Error bars represent mean±SEM, and statistical significance was evaluated using paired two-tailed t-test. *P<0.05, **P<0.01. CTR, control; DE, differentially expressed; DEG, differentially expressed gene; FACS, fluorescence-activated cell sorting; FDR, false discovery rate; IFN, interferon; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; SEM, standard error of mean; SSc, scleroderma.

### Human pDC induced IFN response in organotypic skin rafts (OSR), which is inhibited by BDCA2-targeting

Growing evidence shows pDC skin infiltration and induced IFN signature within the skin of patients with IMID, such as SSc.[24–28] Furthermore, PBMC-conditioned media are known to activate a proinflammatory response in fibroblasts.[48] To determine whether TLR9-stimulated pDC could also induce this response, and whether treatment with CBS004 may have a functional effect on target tissue cell activation, we set out to measure IFN-induced genes in an OSR model of keratinocytes and fibroblasts co-culture following exposure to pDC supernatants (figure 2A).

First, we performed IFN secretion ELISA assays on pDC purified from PBMC (online supplemental figure S1) to establish the concentration of IFN within ODN-stimulated pDC supernatants. ODN stimulation was for 16 hours to allow IFN secretion into the supernatant to accumulate, since pDC–IFN maximum production has previously been shown between 12 and 18 hours.[49 50] TLR9 induced a striking increase in IFN-α secretion. The addition of CBS004 15 min prior to ODN stimulation suppressed this by 90%, whereas human IgG (HIgG) had no significant effect (figure 2B). To ensure functional inhibition driven by BDCA2-targeting of human pDC was maintained in HV and SSc PBMC, we conducted the same experiment *ex vivo*. ODN stimulation of PBMC induced a similar but substantial increase in IFN secretion in HV and SSc samples, which again was suppressed by >98% in all samples by BDCA2-targeting (figure 2C). To determine the dose–response of CBS004 and to determine the concentration needed for maximal inhibition of IFN secretion, similar experiments were carried out on ODN-treated HV PBMC, identifying IC50 0.06 nM and IC90 0.86 nM values, with HIgG not effecting IFN production (figure 2D). The inhibitory activity of CBS004 was 17-fold higher than previously tested mAb AC144, which supports the *in vitro* data showing enhanced BDCA2 affinity and binding (online supplemental figures S3A and S2A). Importantly, BDCA2-targeting did not significantly reduce pDC viability as determined by 7AAD assay gated within HV PBMC (online supplemental figures S2B, S3B).

OSR were treated with supernatants from pDC cultured in cell media alone (CTR), ODN or ODN+CBS004 (figure 2A,B). The volume of supernatant was calculated to produce a final concentration of 6000 pg/mL of IFN in the ODN experiment, as determined via ELISA (figure 2B). Real-time quantitative reverse transcription PCR (qRT-PCR) of 78 key interferon signalling genes (ISGs) was performed on RNA collected from the keratinocyte and fibroblast collagen matrix. ODN supernatants resulted in an increase of 1.8-fold to 32-fold in 35 ISGs relative to CTR (figure 2E). Despite the limited number of models tested (n=3), eight genes showed a statistically significant upregulation, including *ISG15*, *IFITM1*, *BST2*, *IFI6*, *IFIH1*, *NMI*, *HLA-B* and *IFITM3* (induction of 3-fold to 19-fold relative to control and $p<0.05$). These data support the use of OSR to explore the effect of TLR-activated pDC in a preclinical human model. Importantly, ODN+CBS004 supernatants resulted in suppressed upregulation in all of those genes, ranging from 1.8-fold to 11-fold compared with gene expression induced by ODN (figure 2F; ANOVA, $p<0.0001$). This resulted in a transcription profile similar to CTR (figure 2G). Together these results suggest that BDCA2-targeting of pDC can suppress the IFN signature within skin cells.

### Xenotransplant of human pDC in NOD SCID mice increased skin IFN response to TLR stimulation in a BDCA2-dependent manner

To advance our understanding of the role of circulating human pDC in IFN-induced response within the skin, we developed a novel *in vivo* model. We implemented a xenotransplant transfer of purified normal human primary pDC into nonobese diabetic/severe combined immunodeficiency (NOD SCID) mice (XenoSCID) via intravenous (iv) injection followed by topical application of imiquimod-containing cream (Aldara, TLR7 agonist), with or without anti-BDCA2 (CBS004) or human IgG (HIgG) (online supplemental figure S4A). Topical imiquimod contains a TLR7 agonist that when applied to resting skin induces expression of type I IFN, primarily in macrophages.[51] Repeated application of topical imiquimod results in induction of ISG in an IFNAR1-dependent manner, the recruitment of leukocytes, skin thickening and the development of an inflammatory lesion. Xenotransplantation of human pDC into this model would allow us to determine pDC role in ISG response to imiquimod and whether it is sensitive to BDCA2-targeting. *In vitro* imiquimod-stimulation of healthy PBMC induced IFN secretion, which was BDCA2-dependent (online supplemental figure S4B). The purity of pDC isolated from healthy PBMC for *in vivo* experiments and functional responses to TLR-9 were assessed (online supplemental figures S1, S4C). FACS analysis of $CD45^+CD123^+CD304^+$cells indicated pDC skin infiltration within the Aldara+pDC condition (0.3% of total cells, figure 3A), which resulted in a functional increase in mouse skin ISG expression, as mice receiving human pDC induced a 3.2-fold increase in composite ISG score (including *Ifit1*, *Isg15*, *Mx1, Cxcl10* and *Viperin*), compared to CTR (ANOVA $P<0.05$) (figure 3B). Importantly, this is greater than Aldara treatment alone in absence of pDC, where a 1.7-fold increase in composite ISG score compared to CTR was observed (figure 3B). In agreement, IHC staining showed only a slight increase in MX1 protein expression levels between CTR and Aldara (online supplemental figure S4D). Interestingly, epidermal thickening was observed (online supplemental figure S4D), which has previously been shown be an Aldara-induced response independent of type I IFN and TLR7.[38]

In this context, we could assess the *in vivo* efficacy of BDCA2-targeting. CBS004 and HIgG control mAb were injected into XenoSCID 12 hours before pDC intravenous injection (online supplemental figure S4A). Aldara-induced pDC skin infiltration, as detected by human $CD45^+CD123^+CD304^+$ cells in the mouse treated skin, was not hindered by HIgG (0.3% of total cells) but reduced 3-fold by BDCA2-targeting (figure 3C). Most importantly, BDCA2-targeting suppressed 44% of the ISG expression observed in Aldara+pDC, which was significantly reduced compared with HIgG administration (figure 3D).

Consistent with these findings, immunohistochemistry (IHC) analysis showed a strong induction of MX1 and pSTAT1 (Tyr701) with pDC transplantation compared with NOD SCID mice, which was dramatically reduced by BDCA2-targeting and unaffected by HIgG (figure 3E,F).

### Xenotransplant of human pDC in NOD SCID mice increased the skin profibrotic response to bleomycin treatment in a BDCA2-dependent manner

Ah Kioon *et al* have shown that depletion of mouse pDC can ameliorate bleomycin-induced skin fibrosis in a mouse model of SSc.[24] While supporting the role of pDC in fibrosis in mice, the model could not directly demonstrate the role of human pDC in this setting. Thus, we developed our XenoSCID model with bleomycin-induced skin fibrosis. We supplemented every other day subcutaneous injection of bleomycin with weekly tail vein injection of human pDC for 3 weeks (online supplemental figure S5A). As anticipated in an immunocompromised mouse, bleomycin alone induced a blunted fibrotic response at 3 weeks,

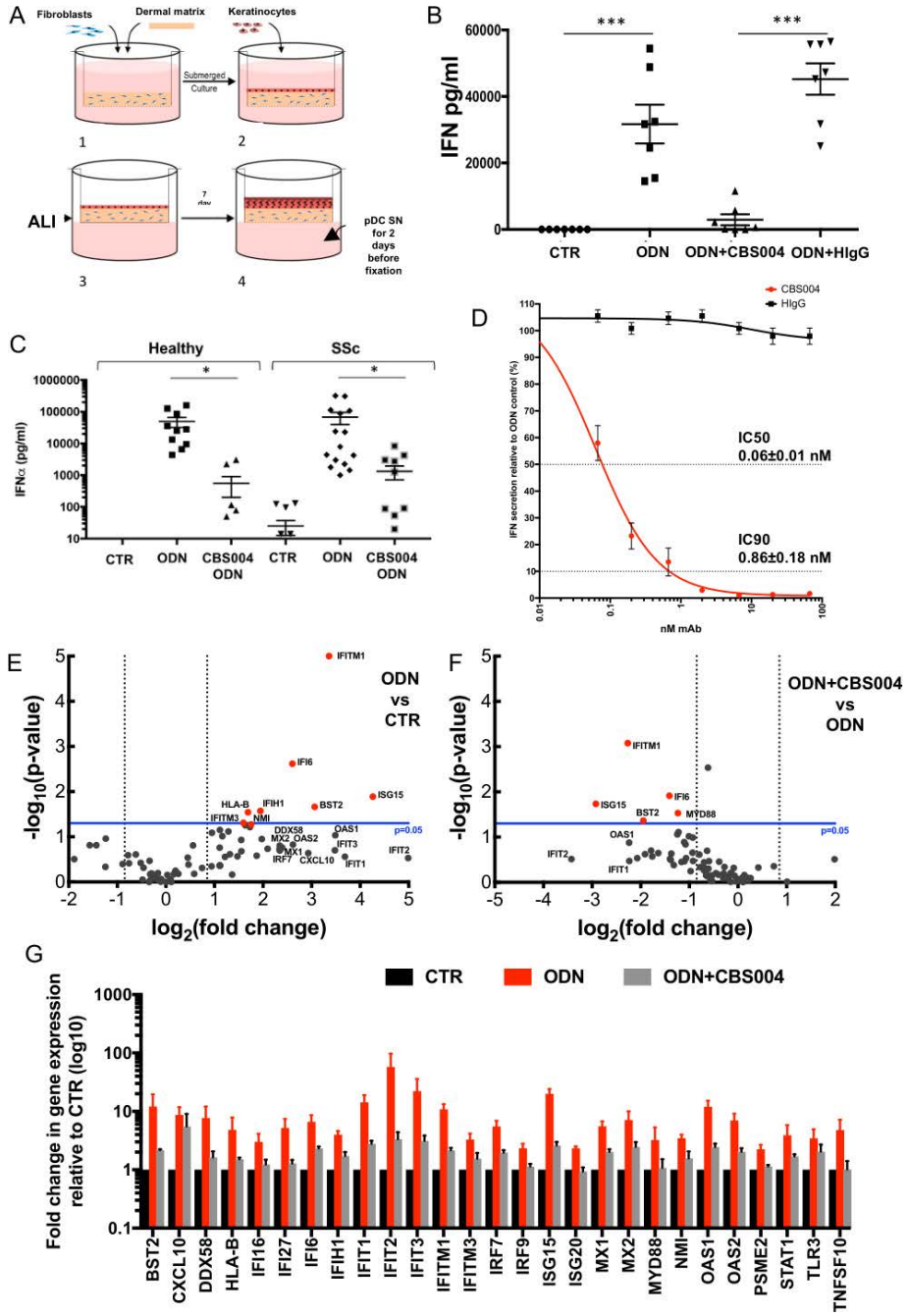


**Figure 2** Human pDC induced IFN response in OSR, which is suppressed by BDCA2-targeting. (A) Systematic outline of OSR protocol; fibroblasts are embedded into a collagen matrix and keratinocytes seeded above until confluence. OSR is brought to ALI to sustain epithelium differentiation. After 5 days, ALI media spiked with 6000 pg/mL of IFN (generated by TLR-stimulated pDC, ODN (B)) for 48 hours. CTR contains equivalent supernatant from untreated pDC (undetectable IFN) and from pDC treated with ODN+CBS004 (10 µg/mL). (B) Quantification of IFN secretion from purified HV pDC (n=7) after 16 hours of culturing in cell media alone (CTR), with ODN (1 µM) and with CBS004 or human IgG1 (10 µg/mL) (added 15 min prior to stimulation) measured by ELISA to determine volume to add to ALI. (C) CBS004 suppresses TLR-induced IFN secretion in HV and SSc PBMC. PBMC from donors were cultured in media alone (CTR), with 1 µM ODN, or with ODN and CBS004 [10 µg/mL] for 16 hours (n=15). IFN was quantified in the supernatants by ELISA. (B,C) Error bars represent mean±SEM and statistical significance was evaluated using unpaired two-tailed t-test. (D) Percentage of IFN alpha secretion, measured by ELISA, from PBMC from four donors stimulated with ODN in the presence of CBS004 or HIgG (0–66 nM) relative to ODN-stimulated pDC with no antibody (100%). Dotted lines highlight IC50s and IC90 with mean values±SEM. (E,F) RNAs harvested from 3 mm biopsies from OSR and subjected to type I IFN inducible genes superarray. Volcano plots illustrate the fold change of 79 IFN type I-related genes (black dots) between CTR and ODN (E) and between ODN and ODN+CBS004 (F) (pDC supernatant from three different donors for each condition). Grey lines represent the 1.8-fold change cut-off. The blue line represents the cut-off for statistical significance of p=0.05 calculated using Student's t-test (two-tailed distribution and equal variances between the two samples) on the triplicate 2−ΔCt values for each gene in each treatment group compared with the CTR group. (F) bar chart illustrates the IFN type I-related genes that were >1.8 fold increased in ODN relative CTR and the effect of CBS004. results are represented as means±SEM. *P<0.05, ***P<0.001. ALI, air–liquid interface; CTR, control; HIgG, human IgG; HV, healthy volunteer; IFN, interferon; OSR, organotypic skin raft; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid dendritic cell; SEM, standard error of mean; SSc, scleroderma.

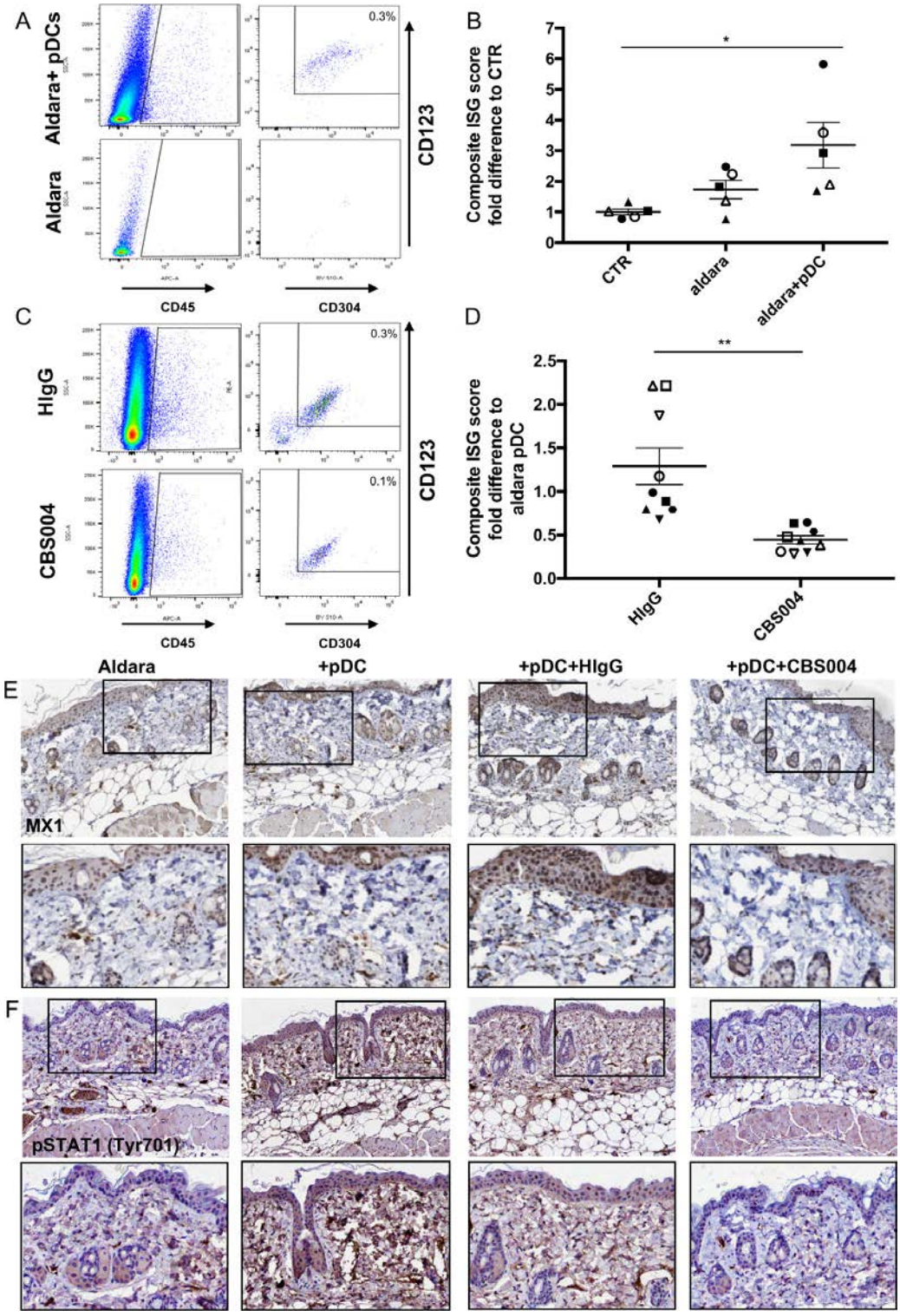


**Figure 3** XenoSCID with human pDC increased skin IFN response to TLR stimulation in a BDCA2-dependent manner. Intravenous tail injection of $2.5\times10^5$ human purified pDC and intraperitoneal injection of CBS004 mAb (5 mg/kg) or CTR human IgG to NOD SCID mice treated with topical Aldara cream application (online supplemental figure S4A, systematic diagram and timeline), with five different treatment conditions consisting of CTR (n=5), Aldara (n=5), Aldara+pDC (n=5), Aldara+pDC+CBS004 (n=9) and Aldara+pDC+HIgG (n=9). Treated skin was harvested using a 3 mm punch biopsy and processed for FACS analysis of human pDC ($CD45^+CD123^+CD304^+$) (representative analyses A and C), qRT-PCR analysis for type I IFN inducible genes (B,D), and IHC staining for MX1 and pSTAT1 (Tyr701) (E,F). (B) Composite ISG score within Aldara and Aldara+pDC conditions relative to CTR. Score shows average fold difference between relative expression of *Mx1*, *Isg15*, *Cxcl10*, *Ifit1*, *Isg15* and *Viperin* between the test condition and CTR. Different symbols represent the different mice litters/human pDC donors. Statistical significance was evaluated using analysis of variance test. (D) Illustration of the composite ISG scores for +CBS004 and +HIgG conditions relative to that of Aldara+pDC. Statistical significance was evaluated using unpaired two-tailed t-test, *P<0.05, **P<0.01. CTR, control; HIgG, human IgG; IHC, immunohistochemistry; ISG, interferon signalling gene; NOD SCID, nonobese diabetic severe combined immunodeficiency; pDC, plasmacytoid dendritic cell; qRT-PCR, real-time quantitative reverse transcription PCR; NOD SCID.

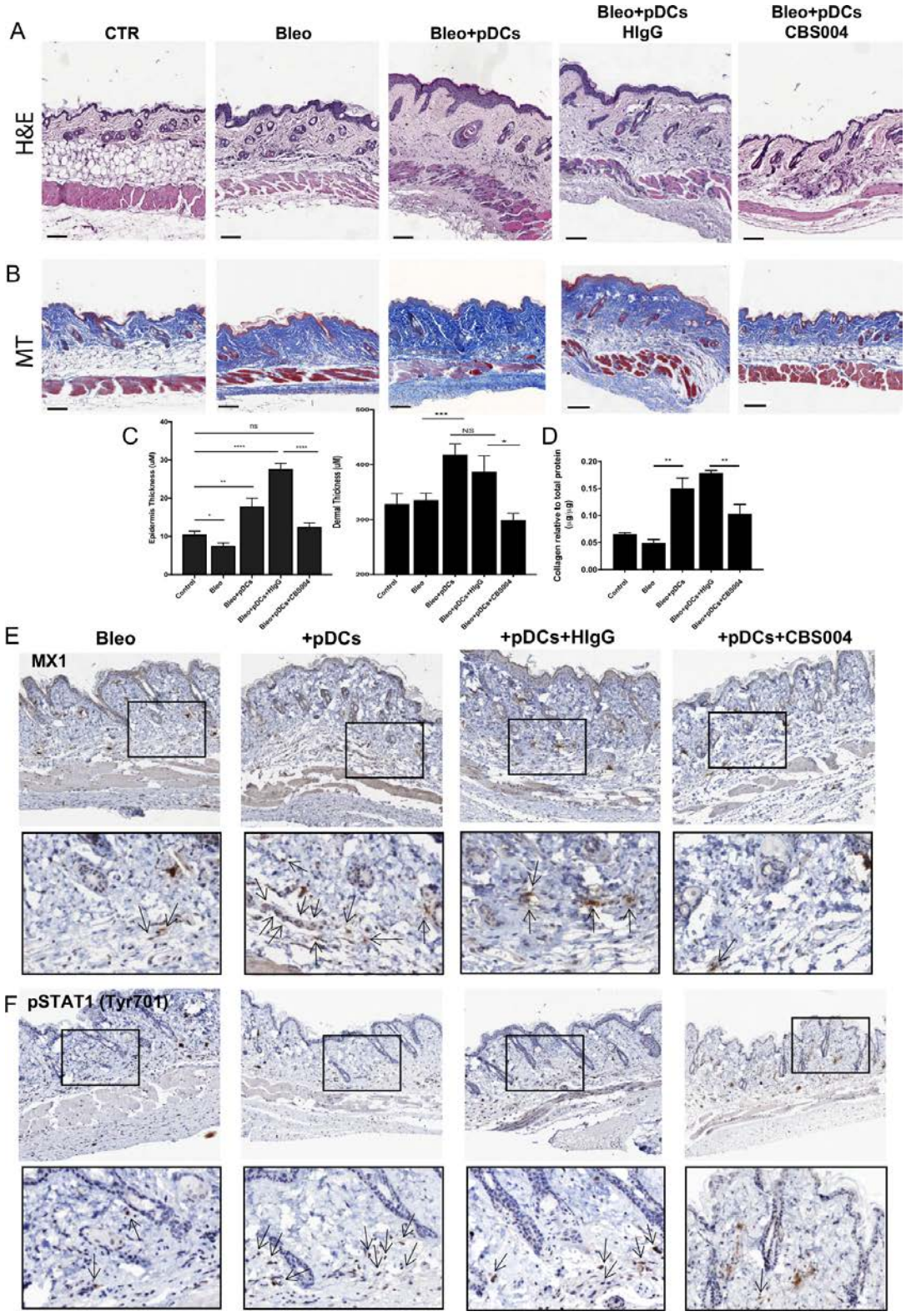


**Figure 4** XenoSCID with human pDC increased the pro-fibrotic skin response to bleomycin treatment in a BDCA2-dependent manner. Intravenous tail injection of $2.5\times10^5$ human purified pDC and intraperitoneal injection of CBS004 mAb (5 mg/kg) or CTR human IgG into NOD SCID mice treated with Bleo or PBS injections (online supplemental figure S5A); systematic diagram and timeline, with five different treatment conditions consisting of PBS/CTR, Bleo, Bleo+pDC, Bleo+pDC+CBS004 and Bleo+pDC+HIgG, each in triplicate. Treated skin was harvested using a 3 mm punch biopsy and processed for H&E (A) and MT staining (B). (C) Epidermis and dermal thickness were measured from 20 areas in each condition. (D) An additional punch biopsy was taken and used to extract protein. Total collagen content measured by Sircol™ assay and shown relative to total protein concentration. Results represented as means±SEM of triplicate experiments. Statistical significance was evaluated using paired two-tailed t-test. (E,F) IHC analysis of MX1 and pSTAT1 (Tyr701) representative images with zoomed in areas, arrows highlight positively stained cells. *P<0.05, **P<0.01, ***P<0.001. Bleo, bleomycin; CTR, control; HIgG, human IgG; IHC, immunohistochemistry; MT, Masson trichrome; NS, no significance; NOD SCID, nonobese diabetic severe combined immunodeficiency; PBS, phosphate buffered saline; pDC, plasmacytoid dendritic cell; SEM, standard error of mean.

as shown by partially retained fatty layer, as well as no significant differences observed in skin thickness and collagen content compared with control (figure 4A–D). Furthermore, no significant increase in MX1 protein expression was observed (online supplemental figure S5B,C). Injection of human pDC resulted in bleomycin-induced loss of all subdermal fat, along with increased collagen formation and a 40% increase in overall skin thickness (figure 4A–D). The fibrotic response was associated with type I IFN signalling activation as MX1 and pSTAT1 (Tyr701) protein expression was increased (figure 4E,F and online supplemental figure S5B,C).

To determine the therapeutic implications of BDCA2-targeting, bleomycin-XenoSCID were treated with intraperitoneal injection of CBS004 or HIgG (online supplemental figure S5A). pDC-induced skin fibrosis was dramatically reduced by BDCA2-targeting as demonstrated by the retention of some fatty layer tissue, similar to bleomycin-only treated mice, a 2-fold reduction in dermal and epidermal thicknesses and 1.5-fold reduction in collagen content compared with HIgG (figure 4A–D). Furthermore, reduction in MX1 and specific pSTAT1 dermal fibroblast (based on morphology) protein expression was observed compared with HIgG treatment (figure 4E,F and online supplemental figure S5B,C).

Overall, our xenotransplant models clearly show that human pDC have a crucial and direct role in skin inflammation and fibrosis and highlight pDC as a viable therapeutic target for SSc.

## DISCUSSION

In the past decade, substantial evidence has pointed to the involvement of pDC in the pathogenesis of many IMID, including SSc.[24–28] Nevertheless, research on pDC has focused only on indirect evidence using *ex vivo* human samples or mouse models. A recent key study supports that mouse pDC have a pathogenic role in fibrosis, as pDC depletion reduced bleomycin-induced IFN-I stimulated transcripts and prevented fibrosis.[24] Due to key differences between mouse and human pDC, functions determined in mouse models may not be fully transferable to human pDC.[13 37–39] In our study, we overcame these challenges by implementing novel preclinical models of human pDC function *in vitro* and *in vivo*. The development of our xenograft models has allowed for the first time to study the role of circulating human pDC within inflammation and fibrosis. Furthermore, by using CBS004, we were able to study the efficacy of specifically attenuating human pDC function by BDCA2-targeting.

Our study shows that TLR stimulation of pDC activates a gene expression profile mapping to activation of inflammation, JAK/STAT, NF-kB and angiogenesis pathways, predicting a greater biological relevance of pDC activation in IMID. A time course of ODN stimulation would be beneficial in the future to determine the transcriptome over time and to ensure key gene expression has not been overshadowed, as well as determining the effect of restimulation of previously stimulated pDC.[50] Crucially, we have shown that BDCA2-targeting is effective at blocking pDC IFN production, as well as the ODN-induced transcriptome. Furthermore, we show that BDCA2-targeting strongly suppresses the differentiation of IFN-secreting $CD274^+$ pDC[17] with a prevalent differentiation towards $CD274^-CD80^+$ pDC. Further functional studies will shed light on the effects of BDCA2-targeting on T-cell costimulation, which has been suggested to be affected by $CD274^-CD80^+$ pDC.[17]

Our preclinical organotypic model of human skin allowed us to show target cell activation by TLR-activated pDC and further supports the biological relevance of BDCA2 inhibition. Our XenoSCID model using human pDC greatly expanded this observation. By inducing local TLR-activated skin, we have shown that human pDC can migrate efficiently into Aldara-treated skin and enhance mouse IFN response, as seen in patients with SSc.[24–28] Aldara treatment alone increased a small increase in mouse IFN skin response. pDC can be found in mouse blood and lymphoid tissue of NOD SCID mice but are undetectable in skin biopsies either at rest or following imiquimod application for 24 hours,[51] indicating our observations are unlikely to be caused by host pDC. Furthermore, we clearly see that human pDC are capable of inducing IFN and fibrotic skin response when introduced into our bleomycin mouse model. While these data directly support a pro-fibrotic effect of pDC in response to bleomycin, they do not directly show that IFN is driving this effect. As shown in our *in vitro* data, pDC produce other cytokines that could plausibly drive the tissue fibrosis in this model. Nevertheless, BDAC2-targeting of pDC in situ prevented the pathogenic responses to proinflammatory and pro-fibrotic stimuli, identifying specific pDC targeting to be a viable therapeutic application for SSc. Our data are supported by similar observations seen when mouse pDC were depleted in a bleomycin-induced fibrotic model of SSc and when specific BDCA2-targeting of resident human pDC in a xenograft Psoriasis model prevented progression into psoriatic skin.[19 24 52]

The development of our XenoSCID model is a novel tool that can be used to study the biology of human pDC in mice and can be applied in the research of other IMID affecting the skin, such as psoriasis or SLE. A limitation of this approach is the lack of adaptive immune response in these animals. Therefore, the consequence of pDC inhibition in a competent immune system remains unknown. Nevertheless, the studies from Rowland *et al* showed that in a mouse model of SLE, elimination of pDC strongly impaired expansion and activation of T and B cells.[21] In this context, xenotransplant models of human PBMC with and without pDC depletion would be extremely informative although falling beyond the scope of this study.

Together, our data indicate that human pDC, and their cytokine production, are a key cell type in the pathogenesis of SSc. As shown in our *in vitro* and *in vivo* models, BDCA2-targeting of human pDC can reduce ISG response and inflammation, as well as prevent fibrosis. For effective therapeutic application, stratification of patients for those with pDC infiltration and higher IFN score should aid responsiveness to BDCA2 suppression of fibrosis.

## MATERIAL AND METHODS

Detailed description of experimental methods is available in online supplemental file 1.

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**Contributors** FDG, CSM, YME-S, SH and RLR designed the study. RLR, CC, GM and YME-S performed the experiments and analysed the data, with additional help from CWW, IG and AC. RNAseq analysis was performed by IMC and AA. JHWD gave conceptual advice and helped with the data interpretation and manuscript draft. RLR and FDG wrote the manuscript draft. All authors contributed to the draft review.

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**Patient consent for publication** Not required.

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EPIDEMIOLOGICAL SCIENCE

# Factors associated with COVID-19-related death in people with rheumatic diseases: results from the COVID-19 Global Rheumatology Alliance physician-reported registry

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## ABSTRACT

**Objectives** To determine factors associated with COVID-19-related death in people with rheumatic diseases.

**Methods** Physician-reported registry of adults with rheumatic disease and confirmed or presumptive COVID-19 (from 24 March to 1 July 2020). The primary outcome was COVID-19-related death. Age, sex, smoking status, comorbidities, rheumatic disease diagnosis, disease activity and medications were included as covariates in multivariable logistic regression models. Analyses were further stratified according to rheumatic disease category.

**Results** Of 3729 patients (mean age 57 years, 68% female), 390 (10.5%) died. Independent factors associated with COVID-19-related death were age (66–75 years: OR 3.00, 95% CI 2.13 to 4.22; >75 years: 6.18, 4.47 to 8.53; both vs ≤65 years), male sex (1.46, 1.11 to 1.91), hypertension combined with cardiovascular disease (1.89, 1.31 to 2.73), chronic lung disease (1.68, 1.26 to 2.25) and prednisolone-equivalent dosage >10 mg/day (1.69, 1.18 to 2.41; vs no glucocorticoid intake). Moderate/high disease activity (vs remission/low disease activity) was associated with higher odds of death (1.87, 1.27 to 2.77). Rituximab (4.04, 2.32 to 7.03), sulfasalazine (3.60, 1.66 to 7.78), immunosuppressants (azathioprine, cyclophosphamide, ciclosporin, mycophenolate or tacrolimus: 2.22, 1.43 to 3.46) and not receiving any disease-modifying anti-rheumatic drug (DMARD) (2.11, 1.48 to 3.01) were associated with higher odds of death, compared with methotrexate monotherapy. Other synthetic/biological DMARDs were not associated with COVID-19-related death.

**Conclusion** Among people with rheumatic disease, COVID-19-related death was associated with known general factors (older age, male sex and specific comorbidities) and disease-specific factors (disease activity and specific medications). The association with moderate/high disease activity highlights the importance

## Key messages

**What is already known about this subject?**

- To date, most available data on outcomes for people with rheumatic diseases infected with SARS-CoV-2 come from single centre or single country case series or from one large international registry; the COVID-19 Global Rheumatology Alliance (GRA) physician registry.
- The first GRA publication identified factors associated with higher odds of COVID-19 hospitalisation, including older age, presence of comorbidities and higher dosages of glucocorticoids (≥10 mg/day of prednisolone equivalent).
- Clinical outcome information on patients with COVID-19 who have rheumatic disease therefore remains limited, particularly with regard to factors associated with COVID-19-related death.

**What does this study add?**

- In this analysis of 3729 patients with rheumatic diseases, older age, male sex, and cardiovascular and chronic lung disease were associated with COVID-19-related death.
- Disease-specific factors, namely, moderate/high disease activity and certain medications (rituximab, sulfasalazine and immunosuppressants (as opposed to immunomodulators like disease-modifying anti-rheumatic drugs (DMARDs)) were also associated with COVID-19-related death.

## Key messages

**How might this impact on clinical practice or future developments?**

- There is differential risk of COVID-19-related death according to disease activity and treatments in patients with rheumatic disease, highlighting the need for adequate disease control with DMARDs, preferably without increasing the glucocorticoid dosage.

of adequate disease control with DMARDs, preferably without increasing glucocorticoid dosages. Caution may be required with rituximab, sulfasalazine and some immunosuppressants.

## INTRODUCTION

There is a lack of robust data to inform our understanding of outcomes following SARS-CoV-2 infection in patients with inflammatory rheumatic diseases, leading to uncertainties regarding chronic disease management, especially for those taking immunosuppressant or immunomodulatory drugs.[1–3]

Whether people with rheumatic diseases belong to a vulnerable, higher risk population for SARS-CoV-2 infection and have poorer outcomes is unclear.[1–8] In general, this population seems to have similar or only slightly poorer outcomes compared with those without rheumatic disease.[7–9] However, important confounding disease-related factors, such as disease activity or treatments, have previously not been addressed.

Medications commonly used to treat rheumatic diseases have been used or are being tested for the prevention and/or treatment of COVID-19 and its complications,[10] raising questions about the impact of these treatments on the outcomes of SARS-CoV-2 infection. Continuation of immunomodulatory or immunosuppressive therapy is essential for controlling rheumatic disease activity, avoiding disease progression and preventing joint or organ-damage related to sustained inflammation. Withdrawal of effective treatments should be based on sound evidence, even during a pandemic.

To generate more granular data relevant to rheumatic diseases, a global network of rheumatologists, data scientists and patients developed a COVID-19 physician-reported case registry in March 2020.[11 12] Analysis of the first 600 patients revealed that older age and comorbidities were associated with hospitalisation,[13] similar to results in the general population.[8 14] More robust data on the risk of poor outcomes, in particular risk of death, are required.

The aim of this study was to investigate factors associated with COVID-19-related death in patients with rheumatic diseases and to analyse these associations by disease group.

## METHODS

### Data source

The COVID-19 Global Rheumatology Alliance (C19-GRA) physician-reported registry is an observational registry launched on 24 March 2020. Data are entered voluntarily by rheumatologists or under supervision of rheumatologists; patients are eligible for inclusion if they have a pre-existing rheumatic disease and a COVID-19 diagnosis. Data are entered either directly into the global or European data entry systems or transferred from national registries (France, Germany, Italy, Portugal and Sweden).

We used data collected on or before 1 July 2020. Further details of this registry have been described elsewhere.[11–13] Countries were assigned to the six WHO regions (www.who.int); the 'Americas' was further divided into north and south. Given the registry collects anonymous data, the UK Health Research Authority and the University of California San Francisco Institutional Review Board considered it exempt from patient consent.

### Patient stratification into diagnostic groups

Rheumatic diseases differ regarding the disease-modifying antirheumatic drugs (DMARDs) approved for their treatment. To minimise the impact of this heterogeneity on the associations of interest, in addition to the main analysis with all patients, diagnostic categories were defined (figure 1) and stratified analyses were undertaken for patients with (1) inflammatory joint diseases (IJD), (2) rheumatoid arthritis (a subset of the IJD subgroup) and (3) connective tissue diseases (CTD)/vasculitis.

### COVID-19 reporting and outcome

Both confirmed and presumptive cases of COVID-19 were reported. The method of COVID-19 diagnosis was specified: PCR, CT scan, metagenomic testing, laboratory assays or based on symptoms only.

For analysis, patients were subsequently categorised into (1) *confirmed* or high likelihood of COVID-19 (chest imaging (CT or chest X-ray) showing bilateral infiltrates and/or symptoms after close contact with a known laboratory-confirmed COVID-19 positive patient) or (2) *presumptive* cases based on symptoms alone.

The primary outcome was COVID-19-related death.

### Treatment prior to COVID-19

Antirheumatic medications used prior to COVID-19 diagnosis were categorised into groups shown in figure 1. Immunomodulatory drugs (conventional synthetic (cs)/biological (b)/targeted synthetic (ts) DMARDs) were distinguished from immunosuppressive drugs (azathioprine, cyclophosphamide, ciclosporin, mycophenolate mofetil/mycophenolic acid, tacrolimus) as recommended by Isaacs and Burmester[15]; glucocorticoids are also immunosuppressive but they were examined separately and categorised by prednisolone-equivalent dosage (1–10 mg/day and >10 mg/day). Methotrexate monotherapy was adopted as the medication reference group; methotrexate is the anchor drug in multiple rheumatic diseases[16] and it represents the largest medication category in the registry.

### Statistical analyses

Descriptive tables were produced for the whole cohort and then by diagnostic group, country (for the six countries with the highest number of cases: France, Germany, Italy, Spain, UK and USA) and medication. Independent associations between demographic and disease features and COVID-19-related death were estimated using multivariable logistic regression and reported as OR and 95% CI. Covariates included in the model were age, sex, key comorbidities (hypertension alone or cardiovascular disease (CVD) alone, hypertension combined with CVD, chronic lung disease, chronic kidney disease (CKD) and diabetes), smoking status (ever vs never), rheumatic disease diagnostic group, disease activity as per the physician's global assessment (severe/high or moderate disease activity vs minimal/low disease activity or remission), rheumatic disease treatment prior to COVID-19 diagnosis and prednisolone-equivalent glucocorticoid use.

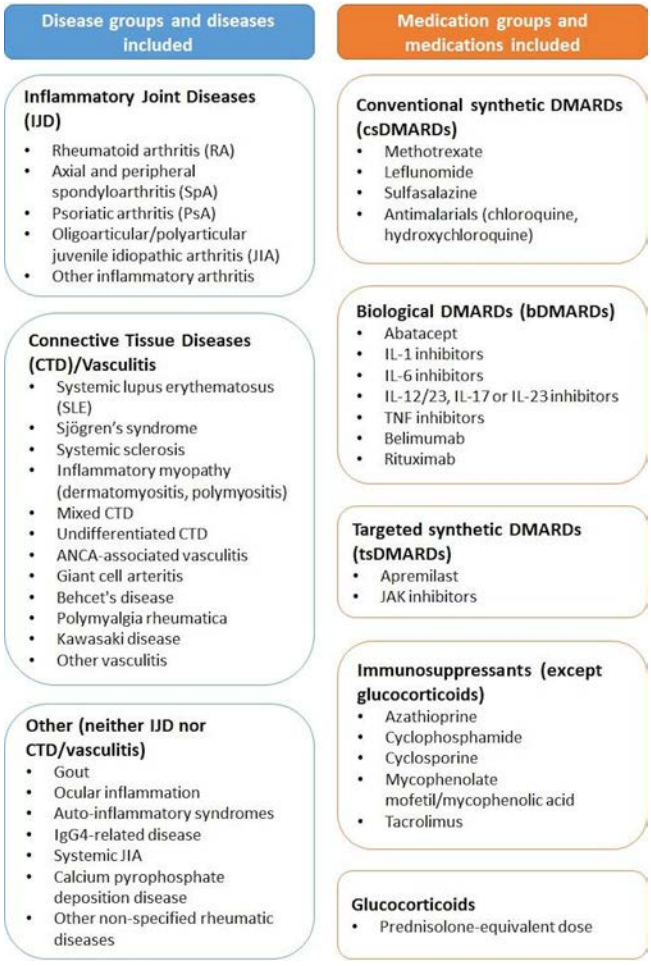


**Figure 1** Disease and medication groups. ANCA, anti-neutrophil cytoplasm antibodies; DMARD, disease-modifying antirheumatic drugs; IgG, immunoglobulin; IL, interleukin; JAK, Janus kinase; TNF, tumour necrosis factor.

All patients with confirmed or presumptive COVID-19 were included in the main analyses. Patients with missing primary outcome (N=82) or missing values for age, sex and DMARD (N=19) were excluded from analysis. Missing values for comorbidities, smoking status, glucocorticoid therapy and disease activity were derived by multiple imputation using full conditional specification.[17] Results of the logistic regression analyses for 10 imputed datasets were pooled by Rubin's rules. As disease activity was missing for all French patients, country-level life expectancy was used in the imputation model to explain potential structural differences in disease activity between countries not accounted for in the patient-level data (data from 2018, source: http://hdr.undp.org/).

To account for pronounced heterogeneity between participating countries regarding both healthcare systems and infection dynamics, countries were implicitly considered as data clusters in the regression analysis by assuming that the data arose from a cluster sample design; this was done by applying a Taylor series linearisation in the variance estimation.[18]

For patients listed as having more than one rheumatic disease or being treated with more than one of the medications of interest, we created a hierarchy based on clinical expertise to categorise patients. This process creates disjoint categories, allowing a clear reference group for interpretation of the regression models and avoiding collinearities. Patients with more than one of the following diseases were grouped according to the following hierarchy: systemic lupus erythematosus (SLE)>vasculitis>other CTD>RA>psoriatic arthritis (PsA)>(other) spondyloarthritis (SpA)>other IJD>other non-IJD/non-CTD rheumatic disease. Patients receiving multiple csDMARDs or immunosuppressants (except glucocorticoids) were grouped according to the following hierarchy: immunosuppressants>sulfasalazine>antimalarials>leflunomide>methotrexate. Patients receiving a b/tsDMARD were considered solely in the b/tsDMARD group. Patients treated with more than one b/tsDMARD (N=4), patients receiving IL-1 inhibitors (N=20) and patients receiving DMARDs atypical for their disease subgroup (N=48) were excluded from analysis due to very low numbers (figure 2). Patients were excluded from a particular analysis if the medication they received provided ≤20 patients for that analysis or if there were no deaths reported for that specific medication.

The following sensitivity analyses were performed to examine the robustness of our findings to procedures for handling missing data: (1) excluding patients from France (no disease activity data available); (2) complete case analysis. Further sensitivity analyses were conducted to assess the stability of the results: (1) limited to patients with confirmed or highly likely COVID-19; (2) using the alternative outcome 'death or invasive ventilation'; (3) using a reduced number of covariates to assess the risk of overfitting; (4) analysis explicitly controlling for country, using data from the top six reporting countries; (5) analysis stratified for several binary key variables (age >65 or not, sex, ever smoked vs not, high/moderate/severe disease activity vs remission/low disease activity, CVD, chronic lung disease, glucocorticoid use) to assess the possibility of interactions.

Data were considered statistically significant for p values <0.05. All analyses were conducted in SAS (V.9.4) and R (V.3.6.3).

## RESULTS

As of 1 July 2020, 3830 patients were in the registry, of whom 3729 had no missing values for death, age, sex and DMARD therapy (table 1, results for all patients; online supplemental table 1, results stratified by diagnostic subgroup; online supplemental table 2, results stratified by country; online supplemental table 3, results stratified by medication of interest).

### Patient characteristics and outcomes of COVID-19

Mean age was 57 (15.7) years and most patients were ≤65 years (2586/3729, 69.3%) and female (2534/3729, 68%). The most common disease was RA (1394/3729, 37.4%), followed by CTDs other than SLE (533/3729, 14.3%), SLE (391/3729, 10.5%), PsA (440/3729, 11.8%) and other SpA (431/3729, 11.6%).

Patients were primarily from Europe (2315/3729, 62.1%) or North America (1105/3729, 29.6%). Nearly half (1309/2758, 47.5%) had minimal or low disease activity and one-third (893/2758, 32.4%) were in remission before COVID-19. One-quarter of all patients (776/3164, 24.5%) were ever smokers.

Most patients had a laboratory-confirmed diagnosis of COVID-19 (2897/3729, 77.7%); 2.4% (91/3729) had a high likelihood of infection based on imaging or confirmed COVID-19 contacts.

Death occurred in 10.5% (390/3729) of patients; 68.7% (268/390) of those who died were >65 years. Nearly half of all patients (1739/3546; 49.0%) were hospitalised. Invasive ventilation was reported in 6.2% (187/2995) of patients, but in 40.8% (120/294) of those who died.

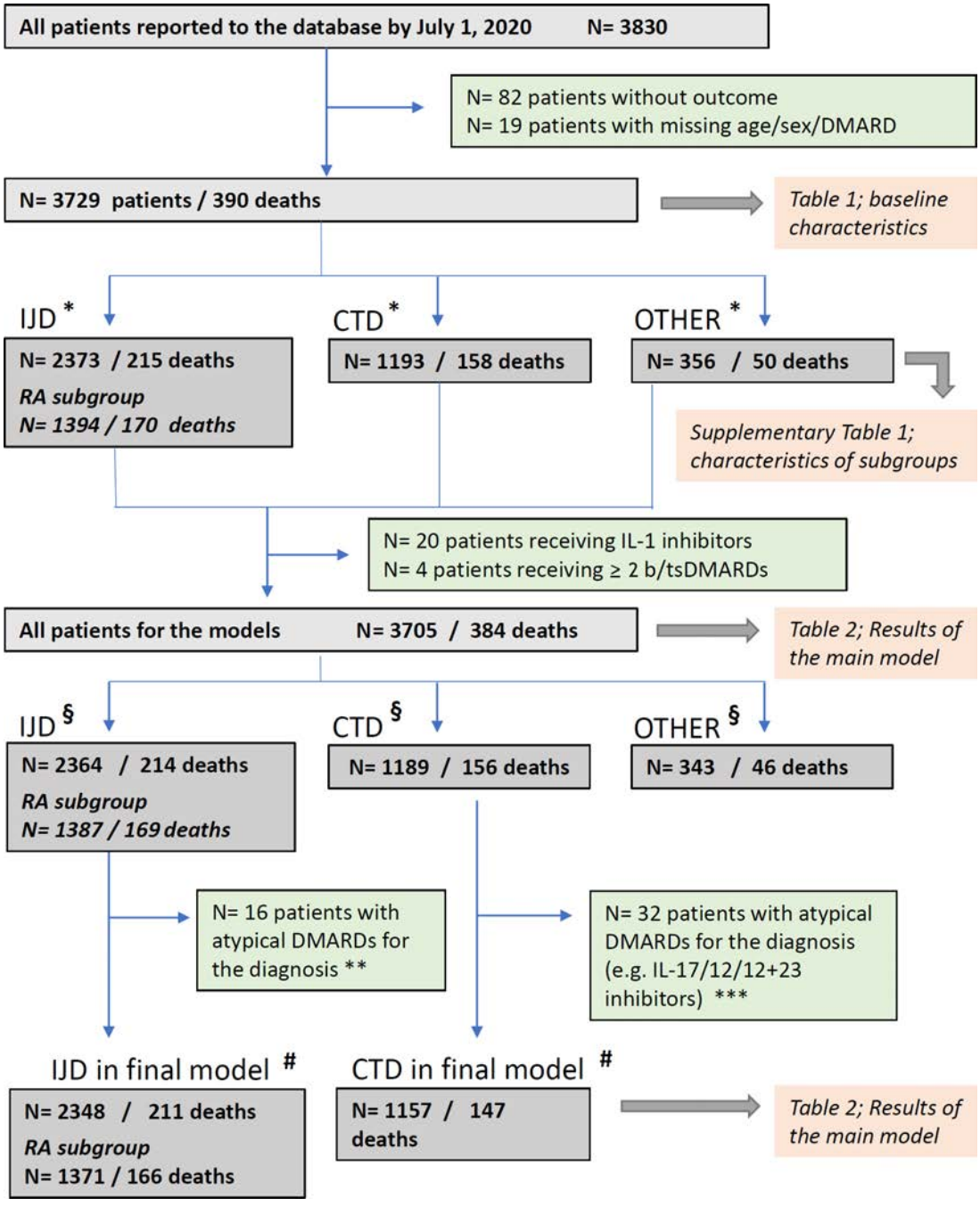


**Figure 2** Patient flowchart. Some patients had diagnoses in multiple groups; as a result, the sum of patients in each group is greater than the total number of patients. (*) Patients belonging to more than one diagnosic group: IJD and CTD: N=78 (10 deaths); IJD and other: N=70 (12 deaths); CTD and other: N=50 (13 deaths); IJD and CTD and other: N=5 (2 deaths). (§) Patients belonging to more than one diagnosic group: IJD and CTD: N=77 (10 deaths); IJD and other: N=70 (12 deaths); CTD and other: N=49 (12 deaths); IJD and CTD and other: N=5 (2 deaths). (#) Patients belonging to more than one diagnosic group: IJD and CTD: N=59 (7 deaths). (**) Non-typical DMARDs for IJD and RA: immunosuppressants and belimumab; non-typical DMARDs for RA: IL-17/IL-23/IL-12+23 inhibitors. (***) Non-typical DMARDs for CTD: abatacept, IL-17/IL-23/IL-12+23 inhibitors, sulfasalazine, leflunomide and tsDMARDs. b/tsDMARDs, biological/targeted synthetic disease-modifying antirheumatic drugs; CTD, connective tissue disease/vasculitis; DMARDs, disease-modifying anti-rheumatic drugs; IJD, inflammatory joint disease; IL, interleukin; RA, rheumatoid arthritis.

### Comorbidities

Most patients (2582/3700, 69.8%) had at least one comorbidity, and 20.5% (760/3700) had more than three. The most frequent were hypertension (1307/3700, 35.3%), chronic lung disease (719/3700, 19.4%), obesity (BMI ≥30; 597/3700, 16.1%), diabetes (505/3700, 13.6%), other CVD (442/3700, 11.9%) and CKD (258/3700, 7.0%). Among deceased patients, the proportion of those with comorbidities was higher, with 42.7% (165/386) having ≥3 comorbidities, namely, 54.9% (212/386) with hypertension, 35.8% (138/386) with chronic lung disease, 24.6% (95/386) with diabetes, 32.1% (124/386) with other CVD and 19.9% (77/386) with CKD.

### Treatments

At the time of COVID-19 diagnosis, 40.6% (1514/3729) of patients were treated only with csDMARDs, immunosuppressants or combinations of these; 35.7% (1331/3729) received

**Table 1** Patient demographic and clinical characteristics

| Parameter | Not deceased | Deceased | Total |
|---|---|---|---|
| **N** | **3339** | **390** | **3729** |
| **General** | | | |
| Age (years) | 55.5 (15.2) | 69.7 (14.6) | 57.0 (15.7) |
| ≤30 | 197 (5.9) | 9 (2.3) | 206 (5.5) |
| 31–50 | 1012 (30.3) | 31 (7.9) | 1043 (28) |
| 51–65 | 1255 (37.6) | 82 (21) | 1337 (35.9) |
| 66–75 | 536 (16.1) | 109 (27.9) | 645 (17.3) |
| >75 | 339 (10.2) | 159 (40.8) | 498 (13.4) |
| Male sex | 1031 (30.9) | 164 (42.1) | 1195 (32) |
| Ever smoker | 664 (23.3) (*N=2854*) (*Missing=485*) | 112 (36.1) (*N=310*) (*Missing=80*) | 776 (24.5) (*N=3164*) (*Missing=565*) |
| Regions | | | |
| African region | 14 (0.4) | 2 (0.5) | 16 (0.4) |
| Eastern Mediterranean region | 83 (2.5) | 11 (2.8) | 94 (2.5) |
| European region | 2040 (61.1) | 275 (70.5) | 2315 (62.1) |
| North American region | 1024 (30.7) | 81 (20.8) | 1105 (29.6) |
| South American region | 112 (3.4) | 10 (2.6) | 122 (3.3) |
| South-East Asian region | 11 (0.3) | 0 | 11 (0.3) |
| Western Pacific region | 55 (1.6) | 11 (2.8) | 66 (1.8) |
| **Inflammatory joint diseases** | | | |
| Rheumatoid arthritis | 1224 (36.7) | 170 (43.6) | 1394 (37.4) |
| Spondyloarthritis | 416 (12.5) | 15 (3.8) | 431 (11.6) |
| Psoriatic arthritis | 420 (12.6) | 20 (5.1) | 440 (11.8) |
| Juvenile idiopathic arthritis (poly, oligo, not systemic) | 21 (0.6) | 4 (1) | 25 (0.7) |
| Other inflammatory arthritis | 90 (2.7) | 8 (2.1) | 98 (2.6) |
| Total Inflammatory joint diseases | 2158 (64.6) | 215 (55.1) | 2373 (63.6) |
| **Connective tissue diseases/Vasculitis** | | | |
| Systemic lupus erythematosus | 355 (10.6) | 36 (9.2) | 391 (10.5) |
| Connective tissue diseases (other than SLE) | 473 (14.2) | 60 (15.4) | 533 (14.3) |
| Vasculitis | 258 (7.7) | 68 (17.4) | 326 (8.7) |
| Total CTD | 1035 (31) | 158 (40.5) | 1193 (32.0) |
| **Other RMDs** | | | |
| Total | 306 (9.2) | 50 (12.8) | 356 (9.5) |
| **Disease activity** | *N=2464* (*Missing=875*) | *N=294* (*Missing=96*) | *N=2758* (*Missing=971*) |
| Remission | 799 (32.4) | 94 (32) | 893 (32.4) |
| Minimal/low disease activity | 1202 (48.8) | 107 (36.4) | 1309 (47.5) |
| Moderate disease activity | 388 (15.7) | 60 (20.4) | 448 (16.2) |
| Severe/high disease activity | 75 (3) | 33 (11.2) | 108 (3.9) |
| **Other outcomes** | | | |
| Hospitalised | 1368 (43.3) (*N=3162*) (*Missing=177*) | 371 (96.6) (*N=384*) (*Missing=6*) | 1739 (49) (*N=3546*) (*Missing=183*) |
| Invasive ventilation | 67 (2.5) (*N=2701*) (*Missing=638*) | 120 (40.8) (*N=294*) (*Missing=96*) | 187 (6.2) (*N=2995*) (*Missing=734*) |
| **Comorbidities** | *N=3314* (*Missing=25*) | *N=386* (*Missing=4*) | *N=3700* (*Missing=29*) |
| Hypertension | 1095 (33) | 212 (54.9) | 1307 (35.3) |
| Cardiovascular disease | 318 (9.6) | 124 (32.1) | 442 (11.9) |
| Cerebrovascular disease | 89 (2.7) | 20 (5.2) | 109 (2.9) |
| Chronic lung disease | 581 (17.5) | 138 (35.8) | 719 (19.4) |
| Chronic kidney disease | 181 (5.5) | 77 (19.9) | 258 (7) |
| Obesity (BMI ≥30) | 539 (16.3) | 58 (15) | 597 (16.1) |
| Morbid obesity (BMI ≥40) | 106 (3.2) | 16 (4.1) | 122 (3.3) |
| Diabetes | 410 (12.4) | 95 (24.6) | 505 (13.6) |
| Cancer | 165 (5) | 49 (12.7) | 214 (5.8) |
| Other comorbidities | 771 (23.3) | 126 (32.6) | 897 (24.2) |
| Number of comorbities | 1.3 (1.3) | 2.5 (1.6) | 1.4 (1.3) |
| No comorbidity | 1090 (32.9) | 28 (7.3) | 1118 (30.2) |
| One comorbidity | 1032 (31.1) | 83 (21.5) | 1115 (30.1) |
| Two comorbidities | 597 (18) | 110 (28.5) | 707 (19.1) |
| ≥3 comorbidites | 595 (18) | 165 (42.7) | 760 (20.5) |
| **DMARD therapies** | | | |
| csDMARDs monotherapy | 592 (17.7) | 59 (15.1) | 651 (17.5) |
| csDMARDs combination therapy | 692 (20.7) | 61 (15.6) | 753 (20.2) |
| Methotrexate monotherapy | 531 (15.9) | 47 (12.1) | 578 (15.5) |
| Methotrexate combination therapy | 607 (18.2) | 52 (13.3) | 659 (17.7) |
| Leflunomide monotherapy | 61 (1.8) | 12 (3.1) | 73 (2) |
| Leflunomide combination therapy | 120 (3.6) | 10 (2.6) | 130 (3.5) |
| Sulfasalazine monotherapy | 51 (1.5) | 16 (4.1) | 67 (1.8) |
| Sulfasalazine combination therapy | 129 (3.9) | 26 (6.7) | 155 (4.2) |
| Antimalarial monotherapy | 287 (8.6) | 17 (4.4) | 304 (8.2) |
| Antimalarial combination therapy | 322 (9.6) | 39 (10) | 361 (9.7) |
| Immunosuppressants monotherapy | 149 (4.5) | 26 (6.7) | 175 (4.7) |
| Immunosuppressants combination therapy | 147 (4.4) | 21 (5.4) | 168 (4.5) |
| Mycophenolate mofetil monotherapy | 68 (2) | 14 (3.6) | 82 (2.2) |
| Mycophenolate mofetil combination therapy | 81 (2.4) | 15 (3.8) | 96 (2.6) |
| Azathioprine monotherapy | 63 (1.9) | 7 (1.8) | 70 (1.9) |
| Azathioprine combination therapy | 51 (1.5) | 3 (0.8) | 54 (1.4) |
| Cyclophosphamide monotherapy | 10 (0.3) | 3 (0.8) | 13 (0.3) |
| Cyclophosphamide combination therapy | 5 (0.1) | 5 (1.3) | 10 (0.3) |
| Tacrolimus monotherapy | 5 (0.1) | 2 (0.5) | 7 (0.2) |
| Tacrolimus combination therapy | 11 (0.3) | 0 | 11 (0.3) |
| Ciclosporin monotherapy | 3 (0.1) | 0 | 3 (0.1) |
| Ciclosporin combination therapy | 11 (0.3) | 1 (0.3) | 12 (0.3) |
| bDMARDs monotherapy | 675 (20.2) | 48 (12.3) | 723 (19.4) |
| bDMARDs combination therapy | 562 (16.8) | 46 (11.8) | 608 (16.3) |
| TNF inhibitors monotherapy | 434 (13) | 13 (3.3) | 447 (12) |
| TNF inhibitors combination therapy | 340 (10.2) | 17 (4.4) | 357 (9.6) |
| Abatacept monotherapy | 28 (0.8) | 4 (1) | 32 (0.9) |
| Abatacept combination therapy | 46 (1.4) | 5 (1.3) | 51 (1.4) |
| B-cell-targeted bDMARDs monotherapy | 71 (2.1) | 25 (6.4) | 96 (2.6) |
| B-cell-targeted bDMARDs combination therapy | 106 (3.2) | 18 (4.6) | 124 (3.3) |
| Rituximab monotherapy | 66 (2) | 25 (6.4) | 91 (2.4) |
| Rituximab combination therapy | 85 (2.5) | 17 (4.4) | 102 (2.7) |
| Belimumab monotherapy | 5 (0.1) | 0 | 5 (0.1) |
| Belimumab combination therapy | 22 (0.7) | 1 (0.3) | 23 (0.6) |
| IL-6 inhibitors monotherapy | 51 (1.5) | 3 (0.8) | 54 (1.4) |
| IL-6 inhibitors combination therapy | 34 (1) | 2 (0.5) | 36 (1) |

Continued

**Table 1** Continued

| Parameter | Not deceased | Deceased | Total |
|---|---|---|---|
| IL-1 inhibitors monotherapy | 10 (0.3) | 2 (0.5) | 12 (0.3) |
| IL-1 inhibitors combination therapy | 4 (0.1) | 4 (1) | 8 (0.2) |
| IL-17, IL-23, IL-12/23 inhibitors monotherapy | 79 (2.4) | 1 (0.3) | 80 (2.1) |
| IL-17, IL-23, IL-12/23 inhibitors combination therapy | 36 (1.1) | 0 | 36 (1) |
| tsDMARDs monotherapy | 61 (1.8) | 5 (1.3) | 66 (1.8) |
| tsDMARDs (*) combination therapy | 71 (2.1) | 10 (2.6) | 81 (2.2) |
| JAK inhibitors monotherapy | 54 (1.6) | 4 (1) | 58 (1.6) |
| JAK inhibitors combination therapy | 67 (2) | 9 (2.3) | 76 (2) |
| Apremilast monotherapy | 7 (0.2) | 1 (0.3) | 8 (0.2) |
| Apremilast combination therapy | 3 (0.1) | 1 (0.3) | 4 (0.1) |
| No DMARD therapies | 615 (18.4) | 124 (31.8) | 739 (19.8) |
| **Further therapies** | | | |
| Glucocorticoids (#) | 1056 (32) (*N=3302*) (*Missing=37*) | 217 (57.1) (*N=380*) (*Missing=10*) | 1273 (34.6) (*N=3682*) (*Missing=47*) |
| Glucocorticoids 1–10 mg/day | 833 (25.6) (*N=3254*) (*Missing=85*) | 150 (41.3) (*N=363*) (*Missing=27*) | 983 (27.2) (*N=3617*) (*Missing=112*) |
| Glucocorticoids>10 mg/day | 171 (5.3) (*N=3254*) (*Missing=85*) | 49 (13.5) (*N=363*) (*Missing=27*) | 220 (6.1) (*N=3617*) (*Missing=112*) |
| NSAIDs | 600 (19.3) (*N=3103*) (*Missing=236*) | 38 (11.0) (*N=345*) (*Missing=45*) | 638 (18.5) (*N=3448*) (*Missing=281*) |

Data are N (column %) for categorical variables or mean (SD) for continuous variables. The table includes all patients with a non-missing outcome and non-missing values for age, sex and disease-modifying anti-rheumatic drugs (DMARDs) (101 patients excluded). Data refer to patients with non-missing values for the respective variable; total N for patients with non-missing values is given in parentheses for variables with missing values; the total number of missing values is also given in parenthesis, for the applicable variables. (*) Includes one patient on a study medication (Lenabasum). (#) Includes patients with a missing glucocorticoid dosage.
bDMARD, biological disease-modifying antirheumatic drug; BMI, body mass index; csDMARD, conventional synthetic disease-modifying antirheumatic drug; CTD, connective tissue diseases; DMARD, disease-modifying antirheumatic drug; IL, interleukin; JAK, Janus kinase; JIA, juvenile idiopathic arthritis; N, number; NSAID, non-steroidal anti-inflammatory drugs; SLE, systemic lupus erythematosus; TNF, tumour necrosis factor; tsDMARD, targeted synthetic disease-modifying antirheumatic drug.

bDMARDs and 3.9% (147/3729) received tsDMARDs. One-fifth (739/3729, 19.8%) were not receiving any DMARD/immunosuppressive treatment (except glucocorticoids), and this proportion was higher among deceased patients (124/390, 31.8%).

Among the patients not receiving any DMARD/immunosuppressive treatment, 39.8% (290/729) received glucocorticoids, 9.8% (70/712) with a prednisolone-equivalent dosage of >10 mg/day; the most frequent diagnostic categories being other non-specified rheumatic diseases (173/739, 23.4%), vasculitis (161/739, 21.8%), CTD other than SLE (156/739, 21.1%) and RA (110/739, 14.9%).

### Country-specific differences

The majority of cases (2993/3729, 80.3%) were reported from six countries with considerable differences in reported percentages of death (online supplemental table 2). Overall, 10.5% (390/3729) of patients died, with highest proportions in the UK (91/435, 20.9%) and Italy (53/315, 16.8%). Death was reported in lower proportions in the USA (70/1005, 7.0%), Germany (15/198, 7.6%), France (62/793, 7.8%) and Spain (21/247, 8.5%). Other major differences between the countries were the distribution of rheumatic diseases and the distribution and frequency of comorbidities.

### Factors associated with death

In multivariable analyses (table 2, figure 3), patients between 66 and 75 years of age were more likely to have died (OR 3.00, 95% CI 2.13 to 4.22) than those ≤65 years. The association was even more pronounced in patients over 75 years (6.18, 4.47 to 8.53; vs ≤65 years). Male sex was also associated with higher odds of death (1.46, 1.11 to 1.91). Current or former smoking was only associated with death in the RA subgroup (1.45, 1.02 to 2.04).

Other factors associated with death included chronic lung disease (1.68, 1.26 to 2.25) and CVD combined with hypertension (1.89, 1.31 to 2.73), whereas hypertension or CVD alone did not show a significant association. CKD was significantly associated with death in patients with CTD or vasculitis (2.30, 1.37 to 3.88) but not in other disease subgroups.

Across all diagnostic groups, treatments with leflunomide, antimalarials, TNF inhibitors, abatacept, belimumab, IL-6 inhibitors, IL-17/IL-23/IL-12+23 inhibitors and tsDMARDs were not associated with death, as compared with methotrexate monotherapy. In the overall model, not receiving DMARD treatment was associated with death (2.11, 1.48 to 3.01) compared with methotrexate monotherapy. This was also seen in the IJD, RA and CTD subgroups.

Compared with methotrexate monotherapy, treatments associated with a higher odds of death were rituximab (4.04, 2.32 to 7.03, in the overall model; 5.42, 2.77 to 10.61, in the IJD subgroup; 4.99, 2.43 to 10.26, in the RA subgroup; 3.72, 1.21 to 11.48, in the CTD/vasculitis subgroup), sulfasalazine (3.60, 1.66 to 7.78, in the overall model and consistent across all subgroups) and immunosuppressants (azathioprine, cyclophosphamide, ciclosporin, mycophenolate or tacrolimus: 2.22, 1.43 to 3.46, in the overall model; 2.44, 1.06 to 5.65, in the CTD/vasculitis subgroup; not applicable to other subgroups).

An additional analysis indicated that the association of sulfasalazine with an increased odds for death was mainly driven by the larger group of sulfasalazine monotherapy and persisted even when sulfasalazine combination treatment (plus either antimalarials, leflunomide or methotrexate) was considered separately (data not shown).

Treatment with higher dosages of glucocorticoids (>10 mg/day prednisolone-equivalent dose vs no use) was also found to be associated with death (1.69, 1.18 to 2.41), particularly in the CTD/vasculitis subgroup (1.93, 1.11 to 3.36).

Higher disease activity at COVID-19 diagnosis was consistently associated with death across all disease groups. Patients with high/moderate/severe disease activity had higher odds of death (1.87, 1.27 to 2.77) than patients with low disease activity or in remission (overall model and consistent across all subgroups).

### Sensitivity analyses

Results were largely consistent in our sensitivity analyses (online supplemental tables 4–9). In the complete case analysis (online supplemental table 5), the association between sulfasalazine and death was no longer statistically significant. In stratified analyses (online supplemental tables 10–16), sulfasalazine use was not associated with death among patients that never smoked, with the OR among ever smokers being almost threefold than among non-smokers (online supplemental table 12).

## DISCUSSION

With global cooperation, the C19-GRA physician-reported registry is the largest collection to date of patients with rheumatic

**Table 2** Multivariable logistic regression analysis of factors associated with COVID-19-related death in patients with rheumatic diseases (all patients)

| | All | | | Patients with inflammatory joint diseases (IJDs) | | | Only patients with rheumatoid arthritis | | | Patients with connective tissue diseases (CTDs) or vasculitis | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 384/3705 (10.4%) | | | 211/2348 (9.0%) | | | 166/1371 (12.1%) | | | 147/1157 (12.7%) | | |
| N deaths/patients (%) | N deaths/patients | OR | 95% CI | N deaths/patients | OR | 95% CI | N deaths/patients | OR | 95% CI | N deaths/patients | OR | 95% CI |
| Age, years | | | | | | | | | | | | |
| Age≤65 | 118/2565 | 1 | Reference | 55/1657 | 1 | Reference | 40/840 | 1 | Reference | 56/779 | 1 | Reference |
| 65 years<Age≤75 | 109/644 | **3** | **2.13 to 4.22** | 71/426 | **3.63** | **2.55 to 5.15** | 55/314 | **3.10** | **1.68 to 5.72** | 33/187 | **2.29** | **1.34 to 3.93** |
| Age>75 | 157/496 | **6.18** | **4.47 to 8.53** | 85/265 | **8.21** | **5.54 to 12.18** | 71/217 | **7.30** | **4.42 to 12.06** | 58/191 | **4.08** | **2.27 to 7.36** |
| Male sex (vs female) | 161/1188 | **1.46** | **1.11 to 1.91** | 82/788 | 1.31 | 0.95 to 1.8 | 55/345 | 1.17 | 0.78 to 1.76 | 63/296 | 1.66 | 0.96 to 2.86 |
| Ever smoked (vs never) | 140/922 | 1.21 | 0.94 to 1.57 | 84/607 | 1.26 | 0.93 to 1.72 | 71/385 | **1.45** | **1.02 to 2.04** | 42/248 | 1.11 | 0.67 to 1.86 |
| Comorbidities | | | | | | | | | | | | |
| Hypertension alone or CVD alone | 155/1150 | 1.19 | 0.89 to 1.59 | 79/690 | 1.04 | 0.74 to 1.46 | 66/454 | 1.11 | 0.74 to 1.67 | 69/406 | **1.56** | **1.06 to 2.29** |
| Hypertension and CVD | 89/301 | **1.89** | **1.31 to 2.73** | 53/168 | **2.29** | **1.25 to 4.23** | 38/118 | **2.03** | **1.03 to 3.97** | 28/106 | 1.57 | 0.78 to 3.16 |
| Chronic lung disease | 136/721 | **1.68** | **1.26 to 2.25** | 76/406 | **1.52** | **1.04 to 2.21** | 63/293 | 1.44 | 0.99 to 2.09 | 54/285 | **2.05** | **1.47 to 2.85** |
| Chronic kidney disease | 76/259 | 1.67 | 0.99 to 2.8 | 27/111 | 1.09 | 0.54 to 2.21 | 21/83 | 1.01 | 0.46 to 2.24 | 41/124 | **2.30** | **1.37 to 3.88** |
| Diabetes mellitus | 96/508 | 1.38 | 0.88 to 2.17 | 55/313 | 1.31 | 0.95 to 1.79 | 39/213 | 1.08 | 0.72 to 1.61 | 32/154 | 1.39 | 0.64 to 3 |
| Rheumatic disease | | | | | | | | | | | | |
| Rheumatoid arthritis | 160/1326 | 1 | Reference | 166/1373 | 1 | Reference | | n.a. | | | n.a. | |
| Systemic lupus erythematosus | 36/391 | 1.2 | 0.70 to 2.04 | | n.a. | | | n.a. | | 32/378 | 1 | Reference |
| Vasculitis | 67/325 | 0.8 | 0.60 to 1.08 | | n.a. | | | n.a. | | 64/318 | 0.81 | 0.49 to 1.33 |
| Other connective tissue diseases | 53/473 | **0.75** | **0.58 to 0.97** | | n.a. | | | n.a. | | 51/461 | 0.78 | 0.39 to 1.54 |
| Psoriasis arthritis | 19/429 | 0.75 | 0.53 to 1.07 | 19/437 | 0.82 | 0.55 to 1.22 | | n.a. | | | n.a. | |
| Spondyloarthritis | 15/423 | 0.72 | 0.34 to 1.54 | 15/424 | 0.82 | 0.4 to 1.69 | | n.a. | | | n.a. | |
| Other inflammatory arthritis or non-systemic JIA | 10/109 | 0.79 | 0.46 to 1.34 | 11/114 | 0.76 | 0.43 to 1.36 | | n.a. | | | n.a. | |
| Other rheumatic diseases (not IJDs/CTDs/vasculitis) | 24/229 | **0.51** | **0.35 to 0.73** | | n.a. | | | n.a. | | | n.a. | |
| High/moderate/severe disease activity (DA) vs remission/low DA | 109/722 | **1.87** | **1.27 to 2.77** | 54/453 | **1.6** | **1.13 to 2.26** | 44/274 | **1.60** | **1.03 to 2.47** | 51/230 | **2.45** | **1.49 to 4.02** |
| Medication | | | | | | | | | | | | |
| Methotrexate | 47/595 | 1 | Reference | 41/487 | 1 | Reference | 34/354 | 1 | Reference | 6/94 | 1 | Reference |
| No DMARD therapy | 124/739 | **2.11** | **1.48 to 3.01** | 38/239 | **2.08** | **1.38 to 3.14** | 25/110 | **2.12** | **1.34 to 3.37** | 67/353 | **3.18** | **1.61 to 6.27** |
| Leflunomide | 12/90 | 1.56 | 0.9 to 2.7 | 10/83 | 1.37 | 0.69 to 2.73 | 9/68 | 1.43 | 0.71 to 2.86 | | n.a. | |
| Antimalarials | 27/426 | 0.99 | 0.66 to 1.48 | 17/167 | 1.14 | 0.65 to 2 | 17/141 | 1.24 | 0.7 to 2.19 | 11/271 | 1.38 | 0.48 to 4.02 |
| Sulfasalazine | 33/144 | **3.6** | **1.66 to 7.78** | 31/137 | **3.40** | **1.46 to 7.93** | 21/85 | **2.62** | **1.21 to 5.68** | | n.a. | |
| Immunosuppressants | 38/276 | **2.22** | **1.43 to 3.46** | | n.a. | | | n.a. | | 32/247 | **2.44** | **1.06 to 5.65** |
| TNF inhibitors | 30/803 | 0.85 | 0.52 to 1.36 | 26/764 | 0.77 | 0.42 to 1.41 | 16/292 | 0.82 | 0.39 to 1.76 | 4/39 | 2.00 | 0.36 to 11.2 |
| Abatacept | 9/81 | 1.20 | 0.61 to 2.34 | 9/75 | 1.3 | 0.62 to 2.71 | 9/68 | 1.4 | 0.65 to 2.99 | | n.a. | |
| Rituximab | 42/192 | **4.04** | **2.32 to 7.03** | 22/90 | **5.42** | **2.77 to 10.61** | 21/86 | **4.99** | **2.43 to 10.26** | 22/104 | **3.72** | **1.21 to 11.48** |
| Belimumab | 1/27 | 0.71 | 0.19 to 2.68 | | n.a. | | | n.a. | | 1/27 | 1.07 | 0.21 to 5.37 |
| IL-6 inhibitors | 5/90 | 0.83 | 0.38 to 1.84 | 1/68 | 0.25 | 0.03 to 2.43 | 1/63 | 0.25 | 0.03 to 2.33 | 4/23 | 2.69 | 0.88 to 8.19 |
| IL-17/IL-23/IL-12+23 inhibitors | 1/115 | 0.25 | 0.03 to 2.04 | 1/112 | 0.26 | 0.03 to 2.06 | | n.a. | | | n.a. | |
| tsDMARDs | 15/145 | 1.60 | 0.91 to 2.8 | 15/142 | 1.75 | 0.99 to 3.12 | 13/118 | 1.57 | 0.75 to 3.27 | | n.a. | |
| Glucocorticoids (GCs) | | | | | | | | | | | | |
| No GCs | 165/2417 | 1 | Reference | 109/1721 | 1 | Reference | 78/863 | 1 | Reference | 38/551 | 1 | Reference |
| GCs 1–10 mg/day | 170/1062 | 1.43 | 0.98 to 2.09 | 89/567 | 1.36 | 0.76 to 2.45 | 78/464 | 1.34 | 0.66 to 2.74 | 75/469 | **1.69** | **1.11 to 2.57** |

Continued

**Table 2** Continued

| N deaths/patients (%) | All | | | Patients with inflammatory joint diseases (IJDs) | | | Only patients with rheumatoid arthritis | | | Patients with connective tissue diseases (CTDs) or vasculitis | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 384/3705 (10.4%) | | | 211/2348 (9.0%) | | | 166/1371 (12.1%) | | | 147/1157 (12.7%) | | |
| | N deaths/patients | OR | 95% CI | N deaths/patients | OR | 95% CI | N deaths/patients | OR | 95% CI | N deaths/patients | OR | 95% CI |
| GCs>10 mg/day | 49/226 | **1.69** | **1.18 to 2.41** | 12/60 | 1.55 | 0.67 to 3.57 | 10/44 | 1.59 | 0.6 to 4.18 | 34/137 | **1.93** | **1.11 to 3.36** |

Missing values were imputed via multiple imputation, patient numbers may thus be rounded. Effects significant at level α=0.05 are marked in bold. Patients were excluded from a particular analysis if the medication they received provided ≤20 patients for that analysis or if there were no deaths reported for that specific medication.
TNF; tumour necrosis factor; CTD, connective tissue diseases; CVD, cardiovascular disease; DMARD, disease-modifying antirheumatic drugs; GC, glucocorticoids; IL, interleukin; JIA, juvenile idiopathic arthritis; N, number; n.a., not applicable; tsDMARD, targeted synthetic disease-modifying antirheumatic drugs.

diseases and COVID-19. We found that moderate/high disease activity was significantly associated with COVID-19-related death, confirming recent recommendations regarding the importance of disease control in rheumatic diseases in the COVID-19 era.[1] Other factors associated with death were older age, male sex and the presence of comorbidities, which is consistent with reports from the general population.[8] Overall, compared with methotrexate monotherapy, most DMARDs were not associated with higher odds of death, although rituximab and sulfasalazine were notable exceptions. Prednisolone-equivalent dosages >10 mg/day and other immunosuppressive drugs (as opposed to immunomodulatory DMARDs) were also associated with COVID-19-related death.

In this cohort of patients with underlying rheumatic diseases, the COVID-19-related death rate was 10.5%, clearly higher than that reported in the general population in most countries. However, this study was not designed to calculate a precise point estimate for mortality. Reporting biases and population-related factors, including COVID-19 testing rates, could explain this figure and, importantly, it should not be taken as an estimate of the overall death rate among patients with rheumatic diseases and COVID-19.

The association of rituximab with poorer COVID-19-related outcomes is a previously unreported finding outside of case reports. Rituximab binds to CD20 on the surface of B-cells, effectively depleting this cell type, and interferes with antibody development. Therefore, B-cell depletion could potentially compromise antiviral immunity, including the development of SARS-CoV-2 antibodies.[19] With our data, it was not possible to determine the exact timing of infection following rituximab infusion, although all patients were clinically judged by their rheumatologist to have been exposed to the immunological effects of the drug at the time of COVID-19 diagnosis. The association between rituximab and COVID-19-related death could have also been influenced by the typical coadministration of methylprednisolone with rituximab.

A finding that merits further research is the higher odds of death found with sulfasalazine treatment. This association has also been reported in results from an international registry of patients with inflammatory bowel disease and COVID-19, where sulfasalazine or 5-aminosalicylate (5-ASA) use was associated with severe COVID-19 (adjusted OR of 3.1 (1.3 to 7.7)).[20] This finding is surprising as sulfasalazine is usually considered to have a low immunosuppressive effect. Prior research supports an immune regulatory effect driven by sulfasalazine or its metabolite 5-ASA against other RNA viruses.[21–24] However, causal interpretation of the association between sulfasalazine and COVID-19-related death should not be made. The perceived low immunosuppressive effect of sulfasalazine may have led rheumatologists to prescribe preferentially sulfasalazine over methotrexate in patients who were perceived to be at higher risk, for example, patients with pulmonary disease, smoking or recurrent chest infections. In an observational study like ours, this could lead to unmeasured confounding. A salient difference in sulfasalazine users in our study was a higher proportion of current or former smokers, compared with non-users. In the stratified analyses for chronic lung disease, the association between death and sulfasalazine was significant in both subgroups with and without chronic lung disease, while in the stratified analyses for smoking, the association between death and sulfasalazine was limited to ever smokers, so the factor 'smoking' could potentially be an effect modifier. Another potential explanation for this finding could be the

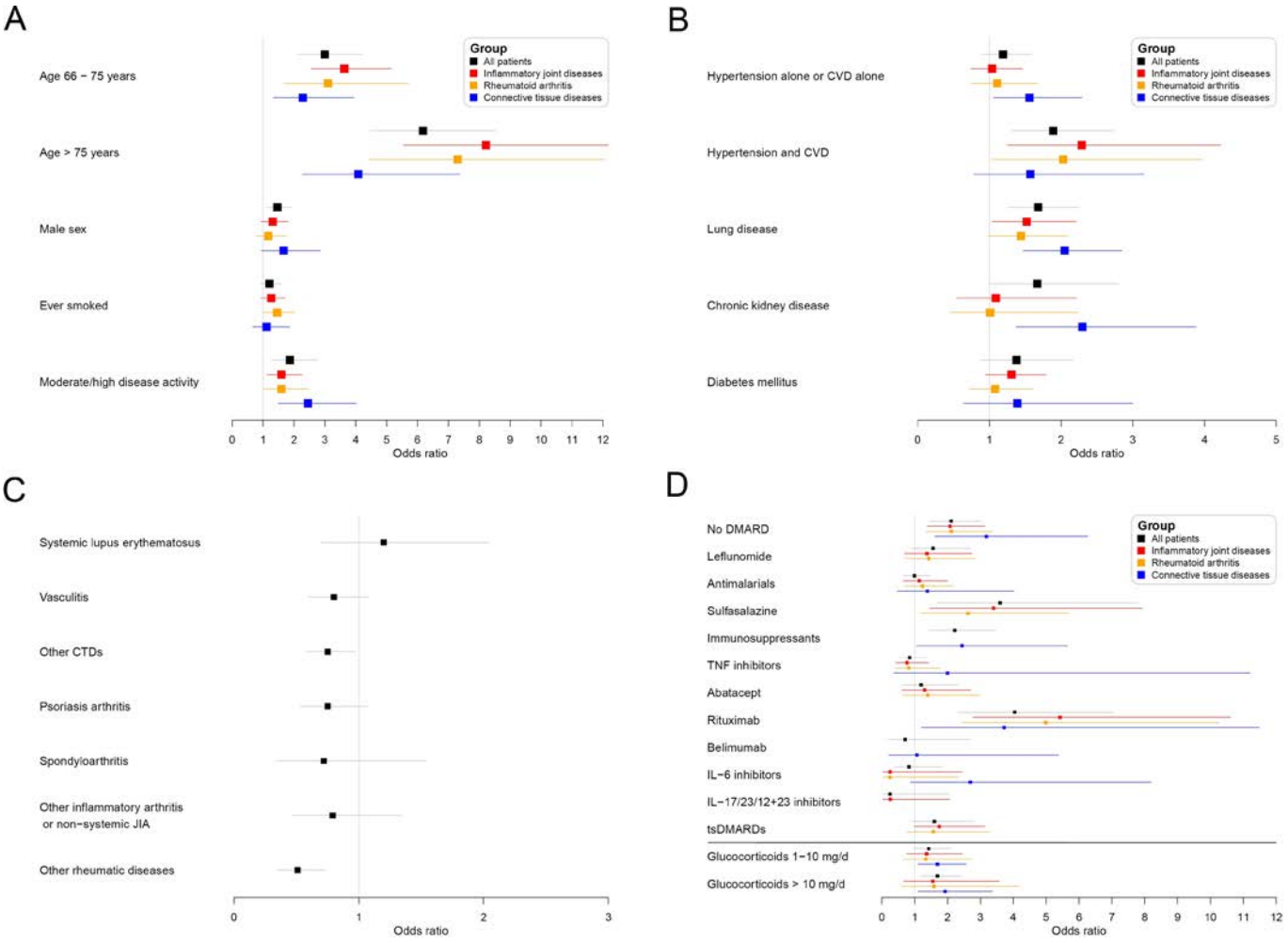


**Figure 3** Results of the main logistic regression analysis. Shown are multivariable-adjusted ORs for the outcome COVID-19-related death with 95% CIs, assessing the association with (A) general patient characteristics, (B) comorbidities, (C) rheumatic disease diagnoses (RMD) and (D) rheumatic disease medications. ORs are shown for four groups: all patients (black), patients with inflammatory joint disease (red), patients with rheumatoid arthritis (orange), and patients with a connective tissue disease or vasculitis (blue). For (C), only ORs for all patients are shown. The reference categories are as follows: (A) ≤65 years, females, never smoked, remission or low disease activity; (B) the non-presence of the specific comorbidities (for all effects); (C) rheumatoid arthritis (for all effects); (D) methotrexate monotherapy (for all effects except for glucocorticoids), no glucocorticoids (for glucocorticoid dosage groups). Patients receiving multiple csDMARDs or immunosuppressants (except glucocorticoids) were grouped according to the following hierarchy: immunosuppressants>sulfasalazine>antimalarials>leflunomide>methotrexate; patients receiving a b/tsDMARD were considered solely in the b/tsDMARD group; glucocorticoids were examined separately and categorised by prednisolone-equivalent dosage (1–10 mg/day and >10 mg/day). bDMARD, biological disease-modifying anti-rheumatic drug; csDMARD, conventional synthetic disease-modifying antirheumatic drug; CTD, connective tissue diseases; CVD, cardiovascular disease; JIA, juvenile idiopathic arthritis; tsDMARD, targeted synthetic disease-modifying anti-rheumatic drug.

merging of sulfasalazine combination therapy (with other csDMARDs) with sulfasalazine monotherapy; however, the increased odds for death persisted in the sulfasalazine monotherapy group and was not driven by the combination treatment (data not shown).

Despite the large overall sample size, for some therapies (eg, IL-6 and IL-17/IL-23/IL-12+23 inhibitors) the number of users was low and no firm conclusions could be made. IL-6 inhibitors have been used to counteract the hyperinflammatory state produced by COVID-19, with mostly disappointing randomised trial results.[25 26] Their efficacy is still being investigated in ongoing trials, but it is reassuring that they were not associated with COVID-19-related death in our analyses. Previous studies had shown an association between TNF inhibitors and a decreased risk of sepsis and mortality in patients with RA after serious infection compared with csDMARDs.[27 28] We could not confirm such an association after stratification by disease and adjustment for disease activity. However, the data indicate that some associations may exist among patients diagnosed with IJD other than RA (a subgroup comprising predominantly patients with axial SpA and PsA), in whom male sex and diabetes mellitus were associated with a higher odds of death, and TNF inhibitor use was associated with a lower odds of death (univariable analysis, data not shown). Due to a small number of deceased patients in this subgroup with non-RA subtypes of IJD (n=37 deaths), these effects could not be assessed in a multivariable model and this should be investigated in the future when higher case numbers allow a more stable assessment.

This study has limitations. As a cross-sectional, case-reporting registry, it may be subject to selection bias if more severe cases

are more likely to come to the rheumatologists' attention and therefore to be reported. There is an absence of a population-based comparator, and we are unable to make comparisons between those with and without COVID-19. Moreover, we caution against interpreting our estimates causally. There is likely unmeasured confounding dependent on the particularities of health systems and case reporting differences. We tried to address this by limiting the research questions to those that could be answered with this dataset and by accounting for potential confounders in our analyses. The high number of variables compared with outcome events in the subgroup models may result in biased estimates.[29 30] However, the consistency between the main model and the sensitivity analyses (including using a lower number of variables) do not indicate an issue with overfitting.

In conclusion, people with rheumatic diseases with higher disease activity have higher odds of COVID-19-related death, highlighting the importance of disease control, preferably by managing DMARDs effectively without increasing glucocorticoids. Future studies should address the observed association of rituximab and sulfasalazine with poor outcomes. Finally, as in the general population, older age, male sex and/or the presence of comorbidities increase the odds of COVID-19-related death.

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**Correction notice** This article has been corrected since it published Online First. The collaborator names have been updated.

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CLINICAL SCIENCE

# Use of non-steroidal anti-inflammatory drugs and risk of death from COVID-19: an OpenSAFELY cohort analysis based on two cohorts

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## ABSTRACT

**Objectives** To assess the association between routinely prescribed non-steroidal anti-inflammatory drugs (NSAIDs) and deaths from COVID-19 using OpenSAFELY, a secure analytical platform.

**Methods** We conducted two cohort studies from 1 March to 14 June 2020. Working on behalf of National Health Service England, we used routine clinical data in England linked to death data. In study 1, we identified people with an NSAID prescription in the last 3 years from the general population. In study 2, we identified people with rheumatoid arthritis/osteoarthritis. We defined exposure as current NSAID prescription within the 4 months before 1 March 2020. We used Cox regression to estimate HRs for COVID-19 related death in people currently prescribed NSAIDs, compared with those not currently prescribed NSAIDs, accounting for age, sex, comorbidities, other medications and geographical region.

**Results** In study 1, we included 536 423 current NSAID users and 1 927 284 non-users in the general population. We observed no evidence of difference in risk of COVID-19 related death associated with current use (HR 0.96, 95% CI 0.80 to 1.14) in the multivariable-adjusted model. In study 2, we included 1 708 781 people with rheumatoid arthritis/osteoarthritis, of whom 175 495 (10%) were current NSAID users. In the multivariable-adjusted model, we observed a lower risk of COVID-19 related death (HR 0.78, 95% CI 0.64 to 0.94) associated with current use of NSAID versus non-use.

**Conclusions** We found no evidence of a harmful effect of routinely prescribed NSAIDs on COVID-19 related deaths. Risks of COVID-19 do not need to influence decisions about the routine therapeutic use of NSAIDs.

## Key messages

**What is already known about this subject?**

- There have been concerns that non-steroidal anti-inflammatory drugs (NSAIDs) may increase the risk of COVID-19 disease. Recent observational studies reported no evidence of a harmful effect of NSAID use on COVID-19 severity among patients with COVID-19.
- However, most studies were of much smaller sample size, not general population based or did not specifically investigate individual NSAIDs (eg, naproxen and ibuprofen).
- In addition, limited clinical data are available to advise patients using long-term NSAID treatment (including people with rheumatoid arthritis and osteoarthritis) whether the treatment should be continued or stopped in the context of COVID-19 pandemic.

**What does this study add?**

- We identified two study populations (2 463 707 people who ever used NSAIDs in the past 3 years from the general population and 1 708 781 people with rheumatoid arthritis/osteoarthritis) in England using OpenSAFELY platform. We then grouped them into current users and non-users, respectively, in each study population.
- In both populations, no association between NSAIDs and COVID-19 related death was found.

**How might this impact on clinical practice or future developments?**

- This study does not support the hypothesis of any harmful effect of NSAIDs on COVID-19 related deaths among regular NSAID users.
- Treatment decisions about the routine use of NSAIDs do not need to be influenced by fears of an effect on COVID-19 outcomes.

## INTRODUCTION

COVID-19, caused by the SARS-CoV-2, has been diagnosed in approximately 18 million patients with >690 000 deaths in >200 countries as of 5 August 2020.[1]

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed for relief of pain and inflammation with nearly 11 million NSAID prescriptions dispensed in primary care in England in the last 12 months.[2] Additionally, some NSAIDs (eg, ibuprofen and aspirin) are available for sale without a prescription with a single brand of ibuprofen alone having sales of approximately £100 million per annum.[3] Nine non-interventional studies have suggested that NSAIDs may be associated with increased risk of complications of lower respiratory tract infections[4–12]; though there is evidence that indometacin may have protective antiviral effects reported from a single animal study.[13]

There is now a debate over whether NSAIDs may worsen the prognosis of COVID-19. On 14 March, it was recommended in France that patients should avoid NSAID use due to an apparent worsening of COVID-19 in those taking ibuprofen, based on unpublished reports.[14] This gained worldwide attention and resulted in the National Health Service (NHS) England medical director issuing a directive that paracetamol should be used in preference to NSAIDs[14] for symptoms of COVID-19. Subsequent reviews by USA, UK and EU drug regulators[15–17] recommended that individuals currently using NSAIDs for the management of chronic diseases should continue the treatment while calling for more evidence of the impact of NSAIDs in patients with COVID-19. Two systematic reviews highlighted a lack of studies investigating the effect of NSAIDs on COVID-19, demonstrating the urgent need of new studies.[18 19] One cohort study was recently conducted to investigate such association, but individual NSAIDs were not specifically investigated.[20]

We therefore investigated the association between NSAID use and deaths from COVID-19 using linked data from >17 million patients in England. We further examined whether the association varied by types of NSAID.

## METHODS

### Study design

We conducted two cohort studies using primary care electronic health record data linked to death data from the Office for National Statistics between 1 March 2020 and 14 June 2020.

### Data source

Primary care records managed by the software provider The Phoenix Partnership (TPP) were linked to Office for National Statistics death data through OpenSAFELY, a data analytics platform created by our team on behalf of NHS England.[21] The dataset analysed within OpenSAFELY is based on 24 million people currently registered with primary care practices using The Phoenix Partnership SystmOne software, representing 40% of the English population. It includes pseudonymised data such as coded diagnoses, prescribed medications and physiological parameters.

### Study populations

We identified two cohorts, anticipating that underlying factors influencing NSAID use and therefore potential biases would differ between them. The first cohort was all people with ≥1 oral NSAID prescription within the 3 years before study start (1 March 2020), identified from the general population. It was chosen to minimise confounding by restricting to people who were currently prescribed NSAIDs and those who recently stopped NSAIDs as their characteristics were likely to be more comparable than never-users. The second cohort was all people with a diagnosis of rheumatoid arthritis (RA)/osteoarthritis (OA) before study start. It was chosen because they were potential NSAID users with similar underlying diseases to reduce confounding by indication. A patient could be included in both cohorts.

In both cohorts, people with missing data for gender, index of multiple deprivation, <1 year of primary care records or aged <18 or >110 years were excluded. Aspirin is used at lower doses as an antiplatelet to prevent cardiovascular disease,[22] indicating aspirin users constitute a different population from other NSAID users. We therefore excluded people ever prescribed aspirin in the 10 years before study start or a record of either stroke or myocardial infarction before study start. We excluded people with a record of gastrointestinal bleeding or current asthma before the study start, as they are contraindications to NSAIDs.[22]

### Exposures

In the main analysis, we defined current NSAID users as those ever prescribed NSAID in the 4 months prior to study start, and non-users are those with no record of NSAID prescription in the same time period.

We examined whether the association varied by types of NSAID, specifically: (1) naproxen dose (categorised as non-use, high-dose naproxen (500 mg), low-dose naproxen (250 mg) and other NSAIDs based on the strength of the formulation), (2) COX-2 specific NSAIDs (categorised as non-use, COX-2 specific (celecoxib/etoricoxib) and non-specific NSAIDs) and (3) ibuprofen (categorised as non-use, ibuprofen and other NSAIDs).

### Outcomes

Follow-up for each cohort began on the 1 March 2020 and ended either on date of death or study end date (14 June 2020). If people in the non-user group received a NSAID prescription after 1 March 2020, they were censored at the date of this prescription (online supplemental figure S1).

The outcome was COVID-19 related death as registered in Office for National Statistics data using International Classification of Diseases (ICD)-10 codes U07.1 ('COVID-19, virus identified') and U07.2 ('COVID-19, virus not identified') listed either as the underlying or any contributing cause of death. The latter ICD-10 code is used when laboratory testing is inconclusive or unavailable.[23]

### Covariates

Figure 1 presents the final list of potential confounders. Our methodology for creating codelists for variables has been previously described.[21] All codelists for identifying exposures, covariates and outcomes are openly shared at https://codelists.opensafely.org/.

### Statistical methods

Baseline characteristics in each cohort were summarised using descriptive statistics, stratified by exposure status. Time to COVID-19 related death was displayed in Kaplan-Meier plots. We present adjusted cumulative mortality curves and the difference between curves using the Royston-Parmar model. We estimated HRs with 95% CIs for the association between current NSAID use and COVID-19 related death using Cox regression with time since cohort entry as the underlying timescale. We accounted for competing risk by modelling the cause-specific hazard (ie, censoring non-COVID-19 deaths). We used graphical methods and tests based on Schoenfeld residuals to explore violations of the proportional hazards assumption.

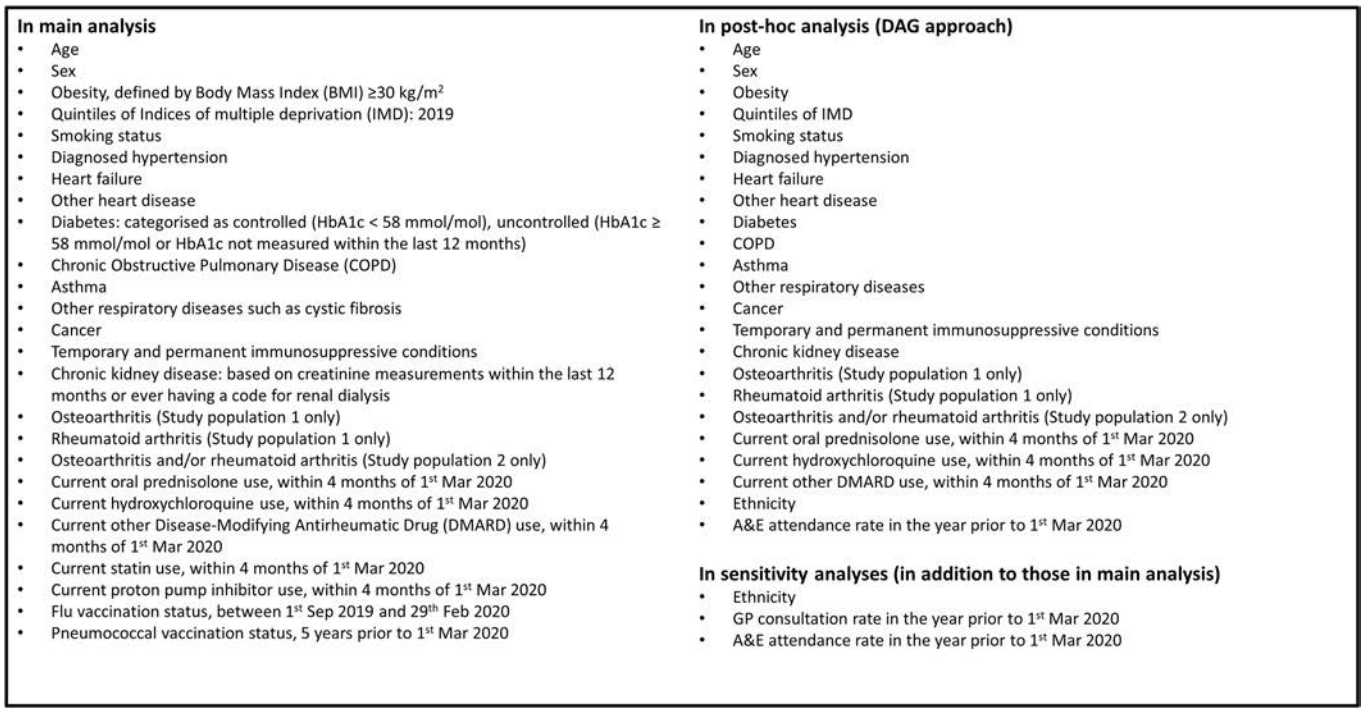


**Figure 1** Prespecified hypothetical confounders. A&E, accident & emergency; DMARD, disease-modifying antirheumatic drugs; HbA1c, hemoglobin A1c; GP, general practice.

Unadjusted models, models adjusted for age (using restricted cubic splines) and sex and multivariable-adjusted models including covariates listed in figure 1 were fitted. We stratified the multivariable-adjusted models by geographical regions, defined by Sustainability and Transformation Partnerships,[24] to account for between-region variations. We evaluated the variation by age (under and 70+ years old) and performed likelihood ratio tests to analyse effect modification.

### Quantitative bias analysis

We used e-value formulae to calculate the minimum necessary strengths of association between an unmeasured confounder and exposure or outcome, conditional on measured covariates, to fully explain observed non-null adjusted associations (ie, to move the observed non-null association to the null).[25]

### Sensitivity analyses

Table 1 shows the list of sensitivity analyses.

### Software and reproducibility

Data management was performed using Python V.3.8 and SQL, with analysis carried out using Stata V.16.1. All study analyses were preplanned unless otherwise stated. All code for data management and analyses in addition to the

**Table 1** List of sensitivity analyses

| Sensitivity analysis | Justification |
|---|---|
| 1. Additionally adjusted for ethnicity in multivariable-adjusted models. | In the main analysis, we did not adjust for ethnicity as it was not anticipated to be a strong confounder and due to a sizeable proportion of individuals with missing ethnicity (~23%). We undertook complete case analysis to address missing data. |
| 2. Additionally adjusted for the number of primary care consultations and A&E attendance in the past year in multivariable-adjusted models. | To explore the impact of healthcare-seeking behaviours. |
| 3. For covariate of diabetes severity, we separated people with diabetes diagnosis and HbA1c measures ≥58 mmol/mol and those with diabetes diagnosis but without HbA1c measures in the past year into two different categories. | People with a diabetes diagnosis but not having HbA1c measures in the past year are likely to have uncontrolled diabetes due to their potential lack of monitoring and management of diabetes. Therefore, we classified these people as uncontrolled diabetes in the main analysis. This is an analysis to test the sensitivity of the results. |
| 4. Repeated main analysis with a choice of covariates selected by a DAG approach (post hoc analysis). | To test the robustness of the results by choosing a set of covariates that are confounders with the use of a structured visual presentation (online supplemental figure S2). |
| 5. Repeated main analysis varying the definition of currently prescribed an NSAID to within 2 months of 1 March 2020. | To assess the sensitivity of exposure definition. |
| 6. Repeated main analysis excluding indometacin from all NSAIDs as the exposure of interest. | Indometacin was the only NSAID that was suggested to have antiviral activity against SARS virus.[13] |
| 7. Repeated main analysis without censoring people who were prescribed NSAIDs after study start date in the non-use group. | To examine data as an intention-to-treat analysis, in order to limit potential bias due to informative censoring. |
| 8. Repeated main analysis excluding people ever prescribed aspirin before study start date. | To assess the sensitivity of exclusion criteria. |

DAG, directed acyclic graph; NSAID, non-steroidal anti-inflammatory drug.

prespecified protocol are archived at: https://github.com/opensafely/nsaids-covid-research.

### Patient and public involvement

Patients were not formally involved in developing this specific study design that was developed rapidly in the context of a global health emergency. We have developed a publicly available website https://opensafely.org/ through which we invite any patient or member of the public to contact us regarding this study.

## RESULTS

Online supplemental figure S3 shows the flow chart of inclusion of participants. A total of 561 027 (13%) individuals were included in both study populations. Of them, 175 495 (25%) were current NSAID users and 385 532 (11%) were non-users.

## MAIN ANALYSIS

### Study population 1: general population

#### Patient characteristics

We included 536 423 current NSAID users and 1 927 284 non-users (table 2). Median age was 53 years (IQR 42–64) among current users and 49 years (IQR 36–60) among non-users. More women were current users (59.2%) than non-users (56.7%).

Current users were more likely to be obese, former smokers and have a medical history of hypertension, diabetes, other respiratory diseases, cancer, chronic kidney disease, OA and RA than non-users. Current users were also more likely to have a prescription for statins, proton pump inhibitors and disease-modifying antirheumatic drugs and to have had more primary care consultations and vaccinations than non-users.

#### Unadjusted and multivariable results

Online supplemental figures S4 and S5 present time to COVID-19 related death in Kaplan-Meier plots and adjusted cumulative mortality plots. We identified 832 COVID-19 related deaths in the general population (online supplemental table S1). The unadjusted HR for current NSAID use was 1.26 (95% CI 1.08 to 1.47), compared with non-use in the unadjusted model (figure 2). In the multivariable-adjusted model, we observed no evidence of difference in risk (HR 0.96, 95% CI 0.80 to 1.14). There was no evidence suggesting that the HR differed by age in all adjusted models (online supplemental table S2). We did not detect deviations from the proportional hazards assumption (online supplemental table S3 and figure S6).

### Study population 2: RA/OA population

#### Patient characteristics

We included 175 495 current NSAID users and 1 533 286 non-users (table 2). A higher proportion of people aged 70+ years were included in this population than the general population. Median age was 63 years (IQR 55–71) among current users and 68 years (IQR 58–76) among non-users. Relative to current users, non-users were older at study start date. Approximately 60% of individuals were women in both groups.

Current users were more likely to be obese, more deprived, former/current smokers and to have had more primary care consultations and a prescription for proton-pump inhibitors and disease-modifying antirheumatic drugs than non-users. However, non-users were more likely to have comorbidities than current users.

### Unadjusted and multivariable results

Online supplemental figures S7 and S8 present time to COVID-19 related death in Kaplan-Meier plots and adjusted cumulative mortality curves, respectively. We identified 2573 COVID-19 related deaths in the RA/OA population (online supplemental table S1). The unadjusted HR for current use was 0.43 (95% CI 0.36 to 0.52), compared with non-use (figure 3). In the multivariable model, we observed a lower risk of COVID-19 related death associated with current use (HR 0.78, 95% CI 0.64 to 0.94). Post hoc analyses, after adjustment for age and sex, showed most variables had minimal impact, though adjustment for PPI moved the estimate away from the null (online supplemental table S4). There was no evidence suggesting that HR differed by age in all adjusted models. We did not detect deviations from the proportional hazards assumption (online supplemental table S3 and figure S9).

## ANALYSES INVESTIGATING DIFFERENT TYPES OF NSAIDS

Online supplemental tables S5–S10 present the baseline characteristics, stratified by different types of NSAIDs. Online supplemental figures S10 and S11 present time to COVID-19 related deaths by types of NSAIDs in Kaplan-Meier plots. There was no evidence that the association with COVID-19 death varied by: (1) naproxen dose, (2) COX-specific status and (3) ibuprofen versus other NSAIDs in either study population (figures 2 and 3 and online supplemental tables S11–S13).

## SENSITIVITY ANALYSES

After we excluded people who were ever prescribed aspirin, we observed no difference in risk of COVID-19 related death associated with current use compared with non-use (HR 0.84, 95% CI 0.69 to 1.02) in RA/OA population (online supplemental table S14). In the post hoc analysis when we used a directed acyclic graph (DAG) approach to select covariates, we observed a marginal decreased risk of COVID-19 in the complete case analysis, additionally adjusted for ethnicity (HR 0.79, 95% CI 0.64 to 0.99) (online supplemental table S15). The results of all other sensitivity analyses were broadly similar to those of the main analyses (online supplemental tables S16–S21).

## QUANTITATIVE BIAS ANALYSIS

To fully explain the multivariable-adjusted HR (0.78) or the upper bound of the 95% CI (0.94) in the RA/OA population, an unmeasured confounder would need to be associated (conditional on measured covariates) with either non-use, relative to current use or COVID-19 mortality by at least risk ratio (RR) of 1.88 (effect estimate) or 1.29 (upper bound) and with both non-use and COVID-19 mortality by at least RR of 1.28 (effect estimate) or 1.06 (upper bound) (online supplemental figure S12).

## DISCUSSION

### Summary

Based on routinely collected data, our study showed no overall increased risk of COVID-19 related death associated with current NSAID use in adults, compared with non-use. This was consistently seen across all analyses.

In this study, we used two different populations to explore the potential impact of confounding. Current users were generally older and had more comorbidities than non-users in the general population cohort. As expected, this was associated with an increased risk of COVID-19 related death in current users compared with non-users in the unadjusted model. In contrast, current NSAID users were younger and had more comorbidities than non-users in the RA/OA population, associated with a decreased risk of COVID-19

**Table 2** Demographic and clinical characteristics

| | Study population 1: general population (people prescribed NSAIDs in the past 3 years) | | Study population 2: patients with rheumatoid arthritis or osteoarthritis | |
|---|---|---|---|---|
| | Non-use of NSAIDs | Current use of NSAIDS | Non-use of NSAIDs | Current use of NSAIDS |
| Total | **1 927 284** | **536 423** | **1 533 286** | **175 495** |
| Age as of 1 March 2020 | | | | |
| 18–<40 | 598 513 (31.1) | 115 858 (21.6) | 32 958 (2.1) | 4433 (2.5) |
| 40–<50 | 397 201 (20.6) | 103 076 (19.2) | 97 870 (6.4) | 15 813 (9.0) |
| 50–<60 | 423 937 (22.0) | 133 066 (24.8) | 292 186 (19.1) | 45 397 (25.9) |
| 60–<70 | 283 639 (14.7) | 106 205 (19.8) | 416 489 (27.2) | 56 947 (32.4) |
| 70–<80 | 169 281 (8.8) | 62 221 (11.6) | 436 477 (28.5) | 41 350 (23.6) |
| 80+ | 54 713 (2.8) | 15 997 (3.0) | 257 306 (16.8) | 11 555 (6.6) |
| Median, IQR | 49 (36–60) | 53 (42–64) | 68 (58–76) | 63 (55–71) |
| Sex | | | | |
| Female | 1 093 581 (56.7) | 317 341 (59.2) | 951 417 (62.1) | 110 526 (63.0) |
| Body mass index | | | | |
| <18.5 | 26 435 (1.4) | 6041 (1.1) | 19 616 (1.3) | 1260 (0.7) |
| 18.5–24.9 | 484 862 (25.2) | 114 657 (21.4) | 379 233 (24.7) | 31 531 (18.0) |
| 25–29.9 | 577 087 (29.9) | 159 573 (29.7) | 518 602 (33.8) | 55 387 (31.6) |
| 30–34.9 | 333 254 (17.3) | 106 314 (19.8) | 298 505 (19.5) | 40 513 (23.1) |
| 35–39.9 | 138 059 (7.2) | 50 406 (9.4) | 119 286 (7.8) | 20 062 (11.4) |
| 40+ | 71 503 (3.7) | 30 438 (5.7) | 58 801 (3.8) | 12 396 (7.1) |
| Missing | 296 084 (15.4) | 68 994 (12.9) | 139 243 (9.1) | 14 346 (8.2) |
| Ethnicity | | | | |
| White | 1 236 854 (64.2) | 357 651 (66.7) | 1 095 982 (71.5) | 125 073 (71.3) |
| Mixed | 20 556 (1.1) | 4696 (0.9) | 6563 (0.4) | 830 (0.5) |
| Asian/Asian British | 151 533 (7.9) | 33 010 (6.2) | 51 587 (3.4) | 6969 (4.0) |
| Black | 49 618 (2.6) | 10 527 (2.0) | 17 645 (1.2) | 2106 (1.2) |
| Other | 30 214 (1.6) | 6925 (1.3) | 10 916 (0.7) | 1241 (0.7) |
| Missing | 438 509 (22.8) | 123 614 (23.0) | 350 593 (22.9) | 39 276 (22.4) |
| Index of multiple deprivation | | | | |
| 1 (least deprived) | 388 369 (20.2) | 107 541 (20.0) | 313 701 (20.5) | 30 797 (17.5) |
| 2 | 387 428 (20.1) | 108 997 (20.3) | 309 372 (20.2) | 32 946 (18.8) |
| 3 | 382 357 (19.8) | 107 626 (20.1) | 307 669 (20.1) | 34 597 (19.7) |
| 4 | 384 598 (20.0) | 106 598 (19.9) | 303 859 (19.8) | 36 682 (20.9) |
| 5 (most deprived) | 384 532 (20.0) | 105 661 (19.7) | 298 685 (19.5) | 40 473 (23.1) |
| Smoking status | | | | |
| Never | 841 256 (43.6) | 220 293 (41.1) | 672 833 (43.9) | 70 283 (40.0) |
| Former | 665 068 (34.5) | 207 354 (38.7) | 692 164 (45.1) | 80 983 (46.1) |
| Current | 389 340 (20.2) | 103 258 (19.2) | 164 464 (10.7) | 23 913 (13.6) |
| Missing | 31 620 (1.6) | 5518 (1.0) | 3825 (0.2) | 316 (0.2) |
| Comorbidities | | | | |
| Hypertension | 353 803 (18.4) | 128 078 (23.9) | 625 247 (40.8) | 66 098 (37.7) |
| Heart failure | 9512 (0.5) | 2433 (0.5) | 36 888 (2.4) | 1413 (0.8) |
| Other heart disease | 27 881 (1.4) | 8726 (1.6) | 57 976 (3.8) | 4202 (2.4) |
| Diabetes | | | | |
| Controlled (HbA1c <58 mmol/mol) | 122 653 (6.4) | 42 132 (7.9) | 177 397 (11.6) | 19 535 (11.1) |
| Uncontrolled (HbA1c ≥58 mmol/mol) | 50 268 (2.6) | 16 504 (3.1) | 58 452 (3.8) | 6286 (3.6) |
| HbA1c not measured | 4536 (0.2) | 1303 (0.2) | 3695 (0.2) | 419 (0.2) |
| COPD | 42 636 (2.2) | 15 435 (2.9) | 85 858 (5.6) | 8373 (4.8) |
| Other respiratory diseases | 17 270 (0.9) | 6194 (1.2) | 38 248 (2.5) | 3435 (2.0) |
| Cancer | 95 315 (4.9) | 32 128 (6.0) | 174 647 (11.4) | 15 940 (9.1) |
| Immunosuppression | 9285 (0.5) | 2918 (0.5) | 8498 (0.6) | 1009 (0.6) |
| Chronic kidney disease | 51 642 (2.7) | 17 570 (3.3) | 164 985 (10.8) | 11 148 (6.4) |
| Osteoarthritis | 367 954 (19.1) | 162 676 (30.3) | 1 473 833 (96.1) | 162 676 (92.7) |
| Rheumatoid arthritis | 28 690 (1.5) | 21 526 (4.0) | 95 056 (6.2) | 21 526 (12.3) |
| Primary care consultations | | | | |
| Median, IQR | 5 (2–10) | 7 (4–13) | 6 (3–11) | 8 (5–14) |
| Min, Max | 0, 626 | 0, 576 | 0, 468 | 0, 360 |

Continued

**Table 2** Continued

| | Study population 1: general population (people prescribed NSAIDs in the past 3 years) | | Study population 2: patients with rheumatoid arthritis or osteoarthritis | |
|---|---|---|---|---|
| | Non-use of NSAIDs | Current use of NSAIDS | Non-use of NSAIDs | Current use of NSAIDS |
| A&E attendance | | | | |
| Median, IQR | 0 (0–0) | 0 (0–0) | 0 (0–0) | 0 (0–0) |
| Min, Max | 0, 118 | 0, 152 | 0, 71 | 0, 63 |
| Vaccination | | | | |
| Influenza | 435 383 (22.6) | 162 082 (30.2) | 806 064 (52.6) | 86 515 (49.3) |
| Pneumococcal | 116 464 (6.0) | 44 902 (8.4) | 193 145 (12.6) | 24 744 (14.1) |
| Medications | | | | |
| Statin | 223 221 (11.6) | 87 169 (16.3) | 415 120 (27.1) | 47 020 (26.8) |
| Proton-pump inhibitors | 268 934 (14.0) | 342 266 (63.8) | 371 464 (24.2) | 137 180 (78.2) |
| Oral prednisolone | 39 081 (2.0) | 16 084 (3.0) | 61 256 (4.0) | 8265 (4.7) |
| Hydroxychloroquine | 8074 (0.4) | 6680 (1.2) | 16 783 (1.1) | 5104 (2.9) |
| Other DMARDs | 20 770 (1.1) | 16 857 (3.1) | 48 819 (3.2) | 12 753 (7.3) |

A&E, accident & emergency; COPD, chronic obstructive pulmonary disease; DMARDs, disease-modifying antirheumatic drugs; HbA1c, hemoglobin A1c; NSAIDs, non-steroidal anti-inflammatory drugs.

related death in the unadjusted model. Notably, both associations were largely removed on adjustment for age. We observed a small decreased risk of COVID-19 related death among current users in the RA/OA population but not in the general population in the multivariable-adjusted models. In a post hoc analysis informed by a DAG that captures the complexity of relationships between variables, this protective effect was somewhat attenuated, suggesting it is not a robust finding and is subject to model variable selection. Moreover, our main analysis in the RA/OA population might also be subject to residual confounding. As demonstrated in quantitative bias analysis, an unmeasured confounder of only moderate strength could potentially fully explain this observed association. As we consistently found no evidence of harmful effect of NSAIDs on COVID-19 related death, using two populations provides a useful context for result interpretation.

## Findings in context

It was postulated that NSAIDs might delay diagnosis and thus clinical care by masking the symptoms of a worsening infection.[4 8–10 26] In vivo and in vitro cellular studies show that NSAIDs weaken the immune response to pathogens by limiting the local recruitment of innate immune cells and reducing antibody synthesis, but the immunomodulatory effects of NSAIDs are not fully understood.[27 28] Notably, these proposed mechanisms are not specific to COVID-19. Recently, it has been suggested that ibuprofen upregulates ACE 2,[29] which has a role in binding SARS-CoV-2 to target cells and could increase the risk of developing severe COVID-19 disease through this route.[30] Some animal studies reported that administration of soluble recombinant ACE 2 might alleviate lung injury in people with respiratory infection.[31 32] It remains unknown whether the findings can be generalised to humans.

In line with our results, five observational studies reported no evidence of a harmful effect of NSAID use on COVID-19

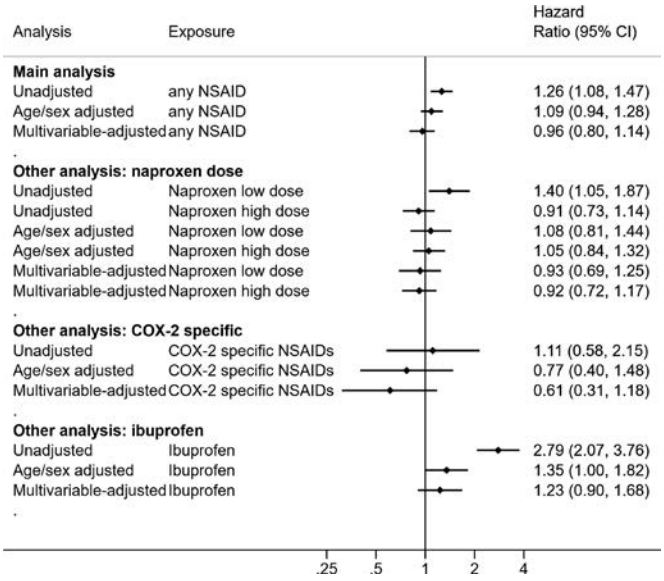


**Figure 2** HRs of the association between current use of NSAIDs and COVID-19 related deaths in the general population. NSAIDs, non-steroidal anti-inflammatory drugs.

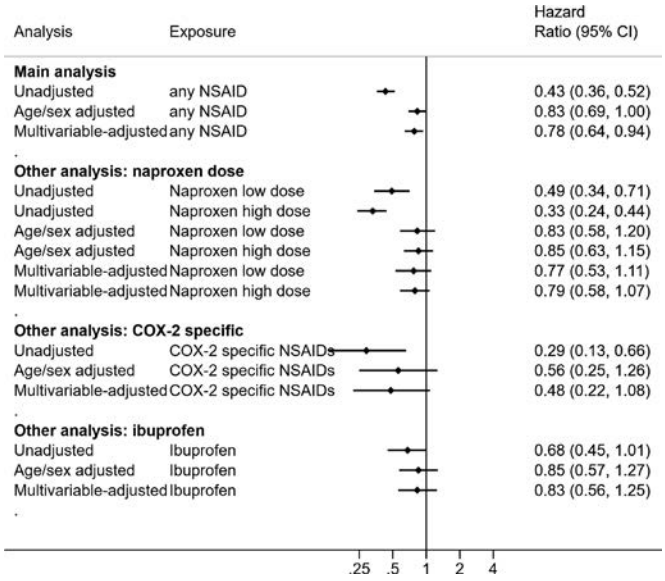


**Figure 3** HRs of the association between current use of NSAIDs and COVID-19 related deaths in the rheumatoid arthritis or osteoarthritis population. NSAIDs, non-steroidal anti-inflammatory drugs.

severity among patients with COVID-19[33–36] but most were of much smaller sample size and not all were general population based, limiting generalisability.[34] A case–control study that investigated the association between renin–angiotensin–aldosterone system blockers and COVID-19 diagnosis found no association between NSAIDs and COVID-19 diagnosis.[37] In contrast, a US cohort study reported a lower odds of mortality associated with NSAID use prior to hospitalisation among patients with COVID-19 (adjusted OR 0.56, 95% CI 0.40 to 0.82).[38] However, patient characteristics, stratified by NSAID exposure and the covariates adjusted for, were not clear. A recent cohort study demonstrated that NSAIDs were not associated with 30-day mortality or other severe COVID-19 outcomes in Danish people who tested positive for SARS-CoV-2.[20] This study was well conducted with robust methodology and of large sample size but it might still be subject to potential issues around selective testing for COVID-19. Furthermore, specific types of NSAIDs were not explored in the analyses, limiting the interpretation of the results.

Notably, we assessed exposure as NSAID use prior to the outbreak in England to establish who were current users, but we did not evaluate any potential therapeutic role of NSAIDs to treat patients with COVID-19. While our study mainly focused on current NSAID use for routine clinical care, there are some ongoing clinical trials investigating the role of NSAIDs in management of COVID-19. They are due to complete later this year or next year (NCT04325633[39]; NCT04382768[40]; NCT04334629[41]; and NCT04344457).[42]

### Strengths and limitations

The greatest strength of this study was the power we had to examine the association between NSAIDs and COVID-19 death, particularly on types of NSAID as our dataset included medical records from 24 million individuals. We also used two different study populations for comparisons to understand the impact of confounding by indication. The breadth of data available in primary care allows us to account for a wide range of potential confounders. We prespecified our analysis plan and have openly shared all analytical code.

We recognise possible limitations. First, we do not know whether patients truly took the medications as prescribed. Second, the supply of NSAIDs 'over the counter' is not captured . However, 'over the counter' purchases are likely to be for ibuprofen, used for acute, irregular conditions and may mean some non-users were in fact taking ibuprofen. This would tend to bias results towards the null. However, this is unlikely to impact the result in the RA/OA population as GPs in England prescribe NSAIDs for long-term conditions such as RA/OA.[43] In our study, information on indications is not readily available; therefore, we cannot distinguish whether the NSAID use was for long-term or short-term conditions for further investigation. Notably, our results from the RA/OA population can generalise the findings to long-term NSAID users as these people receive prescriptions regularly to manage their medical condition. Additionally, we do not capture all additional medicines commonly used in the treatment of RA. In England, a small number of medicines for long-term conditions are supplied routinely by hospitals directly to patients.[44] This includes biological treatments such as adalimumab and infliximab, and we have advocated for the release of these data but access remains restricted.[45 46] Access to these data is important, as biological treatments might be preferentially prescribed in patients with more comorbidities, resulting in unmeasured confounding in our RA/OA population. Notably, our outcome reflected the probability of both COVID-19 infection and, once infected, COVID-19 mortality. If there was a strong harmful effect of NSAIDs on either of these endpoints, we would have observed a higher hazard of COVID-19 mortality among current users compared with non-users. However, we acknowledge that behavioural differences between our comparison groups may have led to a difference in the risk of infection, for example, if the NSAID exposed group were more risk avoidant. This could have attenuated any increased risk of harmful outcomes if differences in risk behaviour were substantial.

## CONCLUSIONS

We found no evidence of a harmful effect of routinely prescribed NSAIDs on COVID-19 related death. People currently prescribed NSAIDs for their long-term conditions should continue their treatment as part of their routine care.

### Information governance

NHS England is the data controller; TPP is the data processor; and the key researchers on OpenSAFELY are acting on behalf of NHS England. This implementation of OpenSAFELY is hosted within the TPP environment, which is accredited to the ISO 27001 information security standard and is NHS IG Toolkit compliant[47 48]; patient data have been pseudonymised for analysis and linkage using industry standard cryptographic hashing techniques; all pseudonymised datasets transmitted for linkage onto OpenSAFELY are encrypted; access to the platform is via a virtual private network connection, restricted to a small group of researchers, their specific machine and IP address; the researchers hold contracts with NHS England and only access the platform to initiate database queries and statistical models; all database activity is logged; only aggregate statistical outputs leave the platform environment following best practice for anonymisation of results such as statistical disclosure control for low cell counts.[49] The OpenSAFELY research platform adheres to the data protection principles of the UK Data Protection Act 2018 and the EU General Data Protection Regulation 2016. In March 2020, the Secretary of State for Health and Social Care used powers under the UK Health Service (Control of Patient Information) Regulations 2002 to require organisations to process confidential patient information for the purposes of protecting public health, providing healthcare services to the public and monitoring and managing the COVID-19 outbreak and incidents of exposure.[50] Taken together, these provide the legal bases to link patient datasets on the OpenSAFELY platform. General practices (GP), from which the primary care data are obtained, are required to share relevant health information to support the public health response to the pandemic and have been informed of the OpenSAFELY analytics platform.

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**Data availability statement** All data relevant to the study are included in the article or uploaded as supplementary information. All code for data management and analyses in addition to the prespecified protocol are archived at: https://github.com/opensafely/nsaids-covid-research. All codelists for identifying exposures, covariates and outcomes are openly shared at https://codelists.opensafely.org/. Access to the platform is via a virtual private network connection, restricted to a small group of researchers. All data relevant to the study are included in the article or uploaded as supplementary information



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# SARS-CoV-2 vaccine hesitancy among patients with rheumatic and musculoskeletal diseases: a message for rheumatologists

SARS-CoV-2 vaccines appear to be the most promising strategy for fighting the virus and protecting also those who might be at higher risk of severe COVID-19, such as patients with rheumatic and musculoskeletal diseases (RMDs). However, vaccine hesitancy might greatly impair the possibility to reach herd immunity and curtail the virus.[1 2] As underlined by some studies performed before vaccine availability, a non-negligible proportion of subjects among the general population would have refused vaccination against COVID-19.[3 4]

During the first weeks of the ongoing vaccination campaign, we proposed an online survey to adult patients with RMDs residing in the Lazio region followed up at our tertiary referral centre in Rome, Italy. Healthy controls (HCs) were recruited using the 'best friend' system. Participants had to report on eight different domains with two possible answers: 'yes' or 'no' (table 1).

Only for the item 'Willingness to receive COVID-19 vaccination', answers were 'yes' or 'no/don't know', with the possibility to give an explanation in case of a negative answer.

For statistical analyses, Mann-Whitney test, $\chi^2$ test and multivariable logistic regression models, also with interaction terms, were used (two-sided, significance level <0.05). Covariates were selected according to a clinical criterion. The analysis was performed by RStudio software.

In all, 626 (75%) of 830 patients with RMDs and 345 (93%) of 370 HCs completed the survey. RMDs included rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, ankylosing spondylitis, systemic sclerosis, Sjögren's syndrome, fibromyalgia, undifferentiated connective tissue diseases, vasculitis, myositis, antiphospholipid syndrome, and miscellaneous rare diseases including mixed connective tissue disease, Behçet's disease, adult-onset Still's disease and IgG4-related disease (online supplemental figure 1). Clinical and demographic features as well as willingness to receive COVID-19 vaccine according to specific RMDs are shown in online supplemental table 1. Acceptance to receive COVID-19 vaccine was reported by 284 (82.3%) of 345 HCs and 344 (54.9%) of 626 patients with RMDs, which is a lower proportion in comparison with a smaller RMD cohort from Lombardy, a region more severely affected by SARS-CoV-2 pandemic compared with Lazio.[5] Multivariable analysis confirmed that patients with RMDs were less willing to receive COVID-19 vaccine compared with HCs (table 1), although they were more likely to perceive themselves at risk of becoming infected with SARS-CoV-2 and developing a severe COVID-19. No differences emerged concerning fear of adverse events (AEs) or distrust for vaccines. Patients with RMDs did not show a generalised hostility to immunisation practices as they had mostly received vaccines against pneumococcus and influenza in the past. Of note, patients with RMDs refusing vaccination would be significantly more willing than HCs to reconsider their decision if more medical education was provided (table 1).

Among patients with RMDs, individual variables significantly associated with willingness to receive the COVID-19 vaccine included older age (p=0.0009) and male sex (p=0.0009), a finding not unexpected because the risk of developing severe COVID-19 is higher for elderly men who might consequently be more inclined to vaccinate.[6] As previously reported,[5] acceptance of vaccination was associated with higher levels of education, whereas neither comorbidities nor ongoing immune suppressive therapy had any effect (online supplemental table 1). Interaction tests did not reveal a more pronounced willingness in specific subgroups of patients with

**Table 1** Multivariable models predicting willingness to receive COVID-19 vaccination and other SARS-CoV-2 and/or vaccine-related outcomes in patients with RMDs and healthy controls

| | Domain 1 Willingness to receive COVID-19 vaccination | | Domain 2 Perceive oneself at risk of becoming infected with SARS-CoV-2 | | Domain 3 Perceive oneself at risk of developing severe COVID-19 | | Domain 4 Fear of COVID-19 vaccine-related AEs | |
|---|---|---|---|---|---|---|---|---|
| | OR (95% CI) | P value | OR (95% CI) | P value | OR (95% CI) | P value | OR (95% CI) | P value |
| Patients | | | | | | | | |
| Rheumatologic Patients | **0.24** (0.17 to 0.34) | <0.0001 | **11.3** (8.0 to 15.9) | <0.0001 | **11.06** (7.8 to 15.6) | <0.0001 | 0.95 (0.52 to 1.7) | 0.865 |
| Healthy controls | Ref. | – | Ref. | – | Ref. | – | Ref. | – |
| Age | 1.01 (1.00 to 1.02) | <0.001 | 1.04 (1.03 to 1.05) | <0.0001 | 1.04 (1.03 to 1.06) | <0.0001 | 1.00 (0.98 to 1.02) | 0.880 |
| Sex | | | | | | | | |
| Female | 0.67 (0.47 to 0.95) | <0.01 | 1.74 (1.18 to 2.56) | <0.001 | 1.61 (1.1 to 2.36) | <0.1 | 2.25 (1.04 to 4.86) | 0.039 |
| Male | Ref. | – | Ref. | – | Ref. | – | Ref. | – |
| | **Domain 5 Distrust of COVID-19 vaccine** | | **Domain 6 Willingness to reconsider decision pending more information** | | **Domain 7 Influenza vaccination received in 2020** | | **Domain 8 Pneumococcal vaccination received in 2020** | |
| | OR (95% CI) | P value | OR (95% CI) | P value | OR (95% CI) | P value | OR (95% CI) | P value |
| Patients | | | | | | | | |
| Rheumatologic Patients | 1.29 (0.20 to 8.15) | 0.781 | **3.08** (2.19 to 4.34) | <0.0001 | **1.60** (1.18 to 2.16) | 0.002 | **2.23** (1.34 to 3.73) | 0.002 |
| Healthy controls | Ref. | – | Ref. | – | Ref. | – | Ref. | – |
| Age | 0.99 (0.94 to 1.05) | 0.930 | 0.99 (0.98 to 1.00) | 0.250 | 1.05 (1.04 to 1.06) | <0.0001 | 1.06 (1.04 to 1.08) | <0.0001 |
| Sex | | | | | | | | |
| Female | 0.30 (0.05 to 1.57) | 0.155 | 1.37 (0.96 to 1.95) | 0.078 | 1.04 (0.75 to 1.45) | 0.778 | 0.89 (0.56 to 1.41) | 0.630 |
| Male | Ref. | – | Ref. | – | Ref. | – | Ref. | – |

AEs, adverse events; RMD, rheumatic and musculoskeletal disease.

RMDs (online supplemental table 2). In more than half of the cases, the reason for refusal was disease-linked (28.4%, fear of AEs related to disease; 25.6%, fear of disease worsening; 43.5%, fear of AEs regardless of the disease; 2.7%, distrust in COVID-19 vaccine).

In conclusion, patients with RMDs may change their attitude to COVID-19 vaccination if properly informed about risks and benefits by their trusted specialist. Hence, rheumatologists should inform their patients transparently considering their doubts and concerns during follow-up visits or organise dedicated online patient meetings to influence the patient's health-related choices. Patient associations might be involved to give evidence-based advice to patients with RMDs.

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## Experience with milatuzumab, an anti-CD74 antibody against immunomodulatory macrophage migration inhibitory factor (MIF) receptor, for systemic lupus erythematosus (SLE)

Milatuzumab (hLL1) is a humanised IgG1κ antibody that reacts with a cell surface epitope of human CD74, the human leukocyte antigen (HLA) class-II associated invariant chain present on antigen-presenting cells (APCs), including B cells and dendritic cells.[1] Although initially studied for oncologic malignancies,[2–5] dysregulation of APCs may also occur in non-malignant disorders, and several preclinical studies showed that milatuzumab modestly inhibited B cell proliferation, enhanced spontaneous migration, alterations of adhesion molecule expression and chemotaxis important for lymphocyte recruitment,[6] and also reduced production of interferon-α in stimulated peripheral blood mononuclear cells isolated from healthy donors and patients with systemic lupus erythematosus (SLE) (unpublished results). Migration inhibitory factor (MIF) is a cytokine that activates a multicomponent receptor comprising the CD74 ligand-binding protein. Preliminary work has suggested that MIF plays a role in the inflammatory process of rheumatoid arthritis.[7] Anticipating milatuzumab could potentially help control underlying immune responses responsible for autoimmunity, a Phase Ib study supported in part by the U.S. Department of Defense (Grant W81XWH-13-1-0392) was undertaken in SLE (Clinicaltrials.gov identifier: NCT01845740). All patients had positive antinuclear antibody (ANA) (titer ≥1:80) and moderately active SLE in at least two organ domains (BILAG B's), or with more severe activity restricted to one organ domain (BILAG A), despite maintenance corticosteroids (at least 5 mg/day prednisone, or equivalent) and any other standard SLE medications, including immunosuppressives and antimalarial drugs. Patients were to receive milatuzumab administered by subcutaneous injection once weekly for four consecutive weeks. Disease activity was assessed every 4 weeks by British Isles Lupus Assessment Group (BILAG2004) and Systemic Lupus Erythematosus

**Table 1** Patient cases demonstrating evidence of efficacy with lasting changes

| Patient | Outcome |
|---|---|
| 253-001 | Active lupus despite prednisone and rituximab. SLEDAI decreased from 10 to 2 with disappearance of rash and arthritis for 1 year. Subsequent severe relapse with arthritis, serositis, anti-ds DNA (ELISA) over 1000 and hospitalisation. |
| 253-004 | Active lupus despite prednisone and quinacrine. SLEDAI decreased from 10 to 2 at week 48. Mucocutaneous and musculoskeletal BILAGs went from B to C and B to D, respectively. There have been no relapses over the last 5 years and patient feels well. |
| 253-008 | Active lupus despite prednisone and hydroxychloroquine. SLEDAI improved from 6 to 0 at week 24 with disappearance of arthritis and rash. Mucocutaneous and musculoskeletal BILAGs both went from B to D. Patient passed away 1 year later from a myocardial infarction with no disease activity. |
| 253-017 | Active lupus despite methotrexate, prednisone and hydroxcyloroquine. SLEDAI decreased from 15 to 8 at week 48. Musculoskeletal BILAG improved from B to C while mucocutaneous and cardiorespiratory scores improved from B to D. Had one course of retreatment. Five years later, she has only mild synovitis. |
| 253-018 | Active lupus despite prednisone and hydroxychloroquine. SLEDAI decreased from 15 to 8 with one course of retreatment, with constitutional, mucocutaneous and cardiorespiratory BILAGs decreased from B to D. Five years later, she has no evidence of disease activity. |
| 253-020 | Active lupus despite abatacept, prednisone and mycophenolate mofetil. SLEDAI decreased from 10 to 2 with disappearance of rash, arthritis and normalisation of anti-ds DNA antibodies and complement. After recurrence a year later, patient was started on belimumab. |
| 253-021 | Active lupus despite prednisone and rituximab. SLEDAI went from 6 to 0 after one course of retreatment, with mucocutaneous and musculoskeletal BILAGs scores decreased from B to D. After 4 years, she has rare rashes with arthralgias. |

Agents given in year prior to entry listed.
All seven responded within 30 days of study entry.
BILAG, British Isles Lupus Assessment Group; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

Disease Activity Index (SELENA-SLEDAI) until at least 12 weeks, then continuing in patients with an improved response or on-going stable disease until relapse or up to 48 weeks. Milatuzumab appeared safe with manageable toxicity limited to mild–moderate injection site or constitutional reactions in the first cohort of 10 patients treated with 250 mg doses, with suppression of moderately active disease activity extending 24 weeks in most patients.[8 9] Since there was evidence of treatment efficacy already at this first planned dose level, a double-blind, placebo-controlled, expansion phase was initiated to confirm the activity. A total of 30 patients were planned to be randomised 1:1:1 to receive milatuzumab at doses of 250 mg, 150 mg or placebo, but the results were statistically inconclusive since the study was terminated early due to poor enrollment after only 12 additional patients had been entered.

All except one patient in the study were treated at Cedars-Sinai Medical Center. While we found that the antibody appeared to have at least modest activity in some patients, most improvements were transient and had reverted to baseline within 12 weeks. However, we noted clearer evidence of efficacy with lasting changes in seven of our patients who received milatuzumab within 30 days and had not responded to standard of care therapies at screening (table 1) and concluded that anti-CD74 approaches in SLE or other autoimmune conditions may be promising, requiring further investigation of milatuzumab.

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**Contributors** All authors were involved with the design, writing, analysis and collection of data.

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# Validation of the 2019 ACR/EULAR criteria for IgG4-related disease in a Japanese kidney disease cohort: a multicentre retrospective study by the IgG4-related kidney disease working group of the Japanese Society of Nephrology

IgG4-related disease (IgG4-RD) is a fibroinflammatory condition that can affect various organs. The kidney is one of the organs most frequently affected and IgG4-related tubulointerstitial nephritis (TIN) is the most dominant feature.[1] However, several radiologically characteristic lesions within the kidney have also been shown to be diagnostic for IgG4-RD affecting the kidney, in the setting of definitively diagnosed IgG4-related lesions in extrarenal organs.[2] Therefore the term 'IgG4-related kidney disease (IgG4-RKD)' has been proposed as a comprehensive term for the renal lesions associated with IgG4-RD.[2 3]

In 2011, the IgG4-RKD working group of the Japanese Society of Nephrology proposed diagnostic criteria for IgG4-RKD.[4] Recently, we validated those criteria in a Japanese kidney cohort and developed a revised version.[5] On the other hand, the 2019 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for IgG4-RD (the ACR/EULAR criteria) were proposed in 2019.[6] According to the latter criteria, exclusion criteria should be applied first to any potential IgG4-RD case. Then, inclusion criteria consisting of eight weighted domains are applied to any case that does not satisfy any of the exclusion criteria, and if the total inclusion points score is ≥20, the case can be classified as 'IgG4-RD'. We validated the ACR/EULAR criteria in the Japanese kidney cohort used in our validation study for IgG4-RKD 2011.[5] Briefly, the cohort comprised Japanese patients diagnosed as having renal injury on the basis of urinalysis, radiographic findings and/or function tests between April 2012 and May 2019, in whom serum IgG4 values and/or data for immunohistological staining of IgG4 in renal biopsy samples were known and for whom sufficient clinical information was available. These patients were classified as IgG4-RD or mimickers based on the ACR/EULAR criteria, and the results were evaluated by expert opinion.

Among the 105 patients included, the expert panel diagnosed 55 as true IgG4-RKD and 50 as mimickers. One patient in each group was used for validation of the ACR/EULAR criteria. The clinical and renal pathological features of each group are shown in table 1. In the IgG4-RKD group, renal biopsy was performed in 51 patients and IgG4-TIN was evident in 48 of them (tissue samples being inadequate in 3). Of the 48 patients with biopsy-proven IgG4-RKD, 34 had extrarenal lesions. Among 14 patients who had only renal lesions, 13 had at least one of the following items: storiform fibrosis demonstrated by renal biopsy, hypocomplementaemia or bilateral renal cortex low-density areas demonstrated by radiology. In seven patients for whom renal histology confirmation was not possible (unavailable in four and inadequate in three), diagnosis of IgG4-RKD was based on radiologically evident bilateral renal cortex low-density areas, in the setting of biopsy-proven IgG4-related extrarenal lesions (n=6) or a definite diagnosis of autoimmune pancreatitis (n=1).

Four of the 55 IgG4-RKD patients and 24 of the 50 mimickers had exclusion criteria. Of the remaining cases, 50 of 51 IgG4-RKD patients and 1 of 26 mimickers had an inclusion criterion score of ≥20 points (figure 1 and online supplemental table 1). One IgG4-RKD patient, whose autoimmune pancreatitis was the focal swelling type, was misclassified as non-IgG4-RKD. As a result, 50 of the 55 IgG4-RKD patients were classified as IgG4-RKD and 49 of the 50 mimickers were classified as non-IgG4-RKD (sensitivity 90.9%, specificity 98.0%, positive predictive value 98.0% and negative predictive value 90.7%).

Many IgG4-RKD patients had extrarenal lesions and IgG4-positive cell-rich TIN associated with other diseases was effectively excluded on the basis of exclusion criteria. In conclusion, the ACR/EULAR criteria showed an excellent test performance for IgG4-RKD in Japanese patients, although further validation studies of other racial groups will be necessary.

**Table 1** Data are available on reasonable request

| | IgG4-RKD (n=55) | Mimicker (n=50) | P value |
|---|---|---|---|
| Age at diagnosis of the kidney disease, mean±SD (years) | 69.9±9.4 | 56.7±17.4 | <0.001 |
| Male (%) | 76.4 | 44 | 0.001 |
| Allergy (%) | 27.5 | 36.7 | 0.393 |
| Serum IgG4 (mg/dL), mean±SD | 1028±796 | 226±261 | <0.001 |
| Elevated serum IgG4 (≥135 mg/dL), n/total (%) | 54/55 (98.2) | 18/50 (36.0) | <0.001 |
| Hypocomplementaemia, n/total (%) | 39/55 (70.1%) | 7/42 (16.7%) | <0.001 |
| Renal pelvis thickening/soft tissue, n/total (%) | 5/55 (9%) | 1/50 (2%) | 0.20 |
| Bilateral renal cortex low-density areas, n/total (%) | 29/55 (52.7%) | 7/50 (14.0%) | <0.001 |
| Extrarenal organ(s) involvement, n/total (%) | 41/55 (74.5) | 20/50 (40.0) | <0.001 |
| Renal biopsy, performed, n/total (%) | 51/55 (92.7) | 50/50 (100) | 0.120 |
| Dense IgG4+PC, n/total (%) | 48/51 (94.1) | 13/40 (32.5) (not evaluated in 10) | <0.001 |
| Storiform fibrosis in the renal pathology, n/total (%) | 28/51 (54.9) | 3/50 (6) | <0.001 |
| Renal pathological diagnosis (n) | | | |
| | IgG4-TIN (48)<br>with MN (4)<br>with FSGS (1)<br>with mesPGN (2)<br>Inadequate tissue (3) | AAV (8)<br>MPA (5), EGPA (3)<br>Idiopathic TIN (5)<br>Drug-induced TIN (5)<br>Nephrosclerosis (4)<br>Sjögren's syndrome (4)<br>Sarcoidosis (3)<br>MCD (3)<br>Necrotising GN without ANCA (3)<br>MN (3)<br>Others* (12) | |

Dense IgG4+PC: dense lymphoplasmacytic infiltration with infiltrating IgG4-positive plasma cells >10/high power field and/or ratio of IgG4-positive plasma cells >40% in the renal pathology.
Others*: TIN and uveitis syndrome (n=2), TIN associated with inflammatory bowel disease (n=2), TIN with IgM-positive plasma cells (n=2), TIN associated with infection (n=2), IgA nephropathy (n=1), diabetic nephropathy (n=1), malignant lymphoma (n=1) and antibody-mediated rejection after renal transplantation (n=1).
AAV, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis; EPGA, eosinophilic granulomatosis with polyangiitis; FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; IgG4-TIN, IgG4-related tubulointerstitial nephritis; MCD, multicentric Castleman's disease; mesPGN, mesangial proliferative glomerulonephritis; MN, membranous nephropathy; MPA, microscopic polyangiitis; TIN, tubulointerstitial nephritis.

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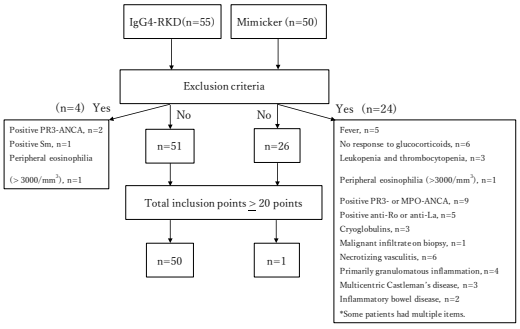


**Figure 1** Performance of the American College of Rheumatology/European League Against Rheumatism classification criteria for IgG4-related disease in a Japanese kidney cohort. ANCA, antineutrophil cytoplasmic antibody; IgG4-RKD, IgG4-related kidney disease; MPO, myeloperoxidase; PR-3, proteinase 3.

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## Bone loss in patients with SAPHO syndrome: a preliminary study

Synovitis, acne, palmoplantar pustulosis, hyperostosis and osteitis (SAPHO) syndrome is a rare entity that involves the skin, bones and joints. The estimated prevalence of SAPHO syndrome is lower than 1/10 000.[1] The real prevalence may be underestimated because of lack of typical symptoms.[2] Bone is one of the critical affected organs for SAPHO. The most common site is the anterior chest wall (65%–90%), followed by the thoracic spine.[1] However, the effects of SAPHO syndrome on bone loss or osteoporosis have not been clarified. We performed a case–control study to show bone loss in patients with SAPHO syndrome in a Chinese population.

From June 2014 to August 2019, a total of 27 new-onset SAPHO patients were included in the study after excluding patients who had been diagnosed for more than 6 months and who were younger than 25 years old. The diagnosis of SAPHO syndrome was based on clinical symptoms and radiological examinations.[2] The diagnosis is based on the presence of at least one of four features: (1) osteoarticular manifestations with severe acne; (2) osteoarticular manifestations with palmoplantar pustulosis; (3) hyperostosis with or without skin lesions; (4) recurrent multifocal chronic osteomyelitis involving the axial or peripheral skeleton, with or without skin lesions. Two or three age-matched and gender-matched control subjects who underwent chest CT scan for physical examinations were matched for each patient.

**Table 1** Prevalence of bone loss

| | Total population | | | Women | | | Men | | |
|---|---|---|---|---|---|---|---|---|---|
| | SAPHO (n=27) | Control (n=70) | P value | SAPHO (n=15) | Control (n=42) | P value | SAPHO (n=12) | Control (n=28) | P value |
| Age | 44.6±10.4 | 46.4±9.8 | >0.05 | 46.3±10.6 | 47.9±9.3 | >0.05 | 42.3±10.1 | 44.1±10.1 | >0.05 |
| BMI | 22.6±3.9 | 23.7±3.4 | >0.05 | 21.4±3.5 | 22.4±4.3 | >0.05 | 22.5±4.1 | 23.2±3.6 | >0.05 |
| Disease duration, months | 5.4 (2.0–12.0) | – | | 5.8 (1.0–12.0) | – | | 5.2 (2.0–9.0) | – | |
| Skin manifestations (n) | 19 (70.4%) | – | | 10 (66.7%) | – | | 9 (75.0%) | – | |
| Osteoarticular manifestations (n) | 21 (77.8%) | – | | 12 (80.0%) | – | | 9 (75.0%) | – | |
| Anterior chest wall | 17 (63.0%) | – | | 9 (60.0%) | – | | 8 (66.7%) | – | |
| Peripheral joints | 3 | – | | 1 | – | | 2 | – | |
| Spine | 10(37.0%) | – | | 6 (40.0%) | – | | 4 (33.3%) | – | |
| Steroids using (n) | 3 (11.1%) | – | | 2 (10.3%) | – | | 1 (8.3%) | – | |
| CRP elevated (n) | 12 (44.4%) | – | | 7 (46.7%) | – | | 5 (41.7%) | – | |
| ESR elevated (n) | 10 (37.0%) | | | 6 (40.0%) | | | 4 (33.3%) | | |
| Prevalence of osteoporosis (n) | | | <0.001 | | | <0.001 | | | >0.05 |
| Normal BMD | 12 (44.4%) | 60 (85.7%) | | 6 (40.0%) | 38 (90.5%) | | 6 (50.0%) | 22 (78.6%) | |
| Osteopenia | 4 (14.8%) | 4 (5.7%) | | 3 (20.0%) | 3 (7.1%) | | 1 (8.3%) | 1 (3.6%) | |
| Osteoporosis | 11 (40.7%) | 6 (8.6%) | | 6 (40.0%) | 1 (2.4%) | | 5 (41.7%) | 5 (17.6%) | |
| Prevalence of bone loss (n) | | | <0.001 | | | <0.001 | | | 0.07 |
| Normal BMD | 12 (44.4%) | 60 (85.7%) | | 6 (40.0%) | 38 (90.5%) | | 6 (50.0%) | 22 (78.6%) | |
| Bone loss | 15 (55.6%) | 10 (14.3%) | | 9 (60.0%) | 4 (9.5%) | | 6 (50.0%) | 6 (21.4%) | |
| Diabetes (n) | 1 | 2 | >0.05 | 0 | 1 | | 1 | 1 | >0.05 |

The osteoporosis or bone loss was evaluated based on the CT attenuation (Hounsfield units, HU) of thoracic spine 10–12 (T10–T12) and lumbar spine (L1). Normal bone mineral density (BMD) was considered when HU was more than 160; osteopenia and osteoporosis were defined when HU was ranged from 135 to 160 and less than 135. Bone loss was defined as osteoporosis or osteopenia. Steroids using means receiving long term (>2 weeks) systematic therapy. Elevated C reactive protein (CRP) was defined when CRP was larger than 8.0 mg/L. Elevated erythrocyte sedimentation rate (ESR) was defined when ESR was >20 mm/hour. The continuous data were shown as mean±SD or range, and was analysed by independent-sample t-test. Qualitative data were shown as number (percentage), and was compared by $\chi^2$ test or Fisher's exact test.
BMI, body index mass; SAPHO, synovitis, acne, palmoplantar pustulosis, hyperostosis and osteitis.

The demographic data, medical history, clinical symptoms, erythrocyte sedimentation rate and C reactive protein were collected.

All CT scan was obtained from multi-detector CT system (GE Healthcare, Tokyo, Japan). The images were reconstructed in the work station using a 0.625 mm section thickness and 0.5 mm increments. CT attenuation (Hounsfield units, HU) of thoracic spine 10–12 (T10–T12) and lumbar spine (L1) was measured on the region of interests (ROIs) avoiding erosion and sclerosis. Five ROIs were measured for each vertebral body (central section and extended two upper and lower sections) and the average of CT attenuation was obtained. Bone loss and osteoporosis were defined based on CT attenuation.[3] Briefly, normal bone mineral density was considered when HU was larger than 160; osteopenia and osteoporosis were defined when HU was ranged from 135 to 160 and less than 135.

70.4% and 77.8% patients had skin lesions and osteoarticular manifestations, respectively (table 1). The CT values of vertebral bodies in SAPHO patients were all significantly lower than those in control ($p<0.01$ or $p<0.05$) (figure 1A,B). Similar results were observed when considering the effect of gender and age (figure 1C,D). The prevalence of bone loss was 55.6% and osteoporosis was 40.7% in total SAPHO population. The prevalence of osteoporosis or bone loss in SAPHO group was significantly higher than those in control for total population and women (table 1, all $p<0.001$). Univariate and multivariate logistic regression analysis both showed that the risk of bone loss and osteoporosis in SAPHO patients (online supplemental table 1) were both higher than the control (OR=11.52, 95% CI 3.10 to 42.81; OR=20.59, 95% CI 4.18 to 101.17). Subgroup analysis showed similar trends in men and women (online supplemental table 2).

The bone loss in SAPHO patients may be caused by the overexpressed proinflammatory cytokines.[4] In addition, glucocorticoid using which may induce bone loss are also widely used in SAPHO patients. Our patients were new-diagnosed SAPHO. Only three patients received long term (>2 weeks) systematic therapy of steroids. The influence of pharmacological treatment on bone may be little in our study. SAPHO syndrome may directly induce bone loss. The prevalence of osteoporosis in SAPHO patients is higher than a recent national quantitative CT (qCT) survey in China.[5] The bone loss in SAPHO patients needs to be paid attention to because SAPHO usually occurs in young adult (mean age 37–48).[1] Bisphosphonates that have been widely used to treat osteoporosis may be potential drugs for SAPHO.[6] The sample size is small because the rarity of the SAPHO syndrome. Our study is just a preliminary exploration. Further studies are needed.

In conclusion, our study reports that CT attenuations of vertebral bodies in SAPHO patients are lower than those in control. The prevalence of bone loss or osteoporosis in SAPHO patients is higher than that in general population. Osteoporosis should be paid attention to in the management of SAPHO syndrome.

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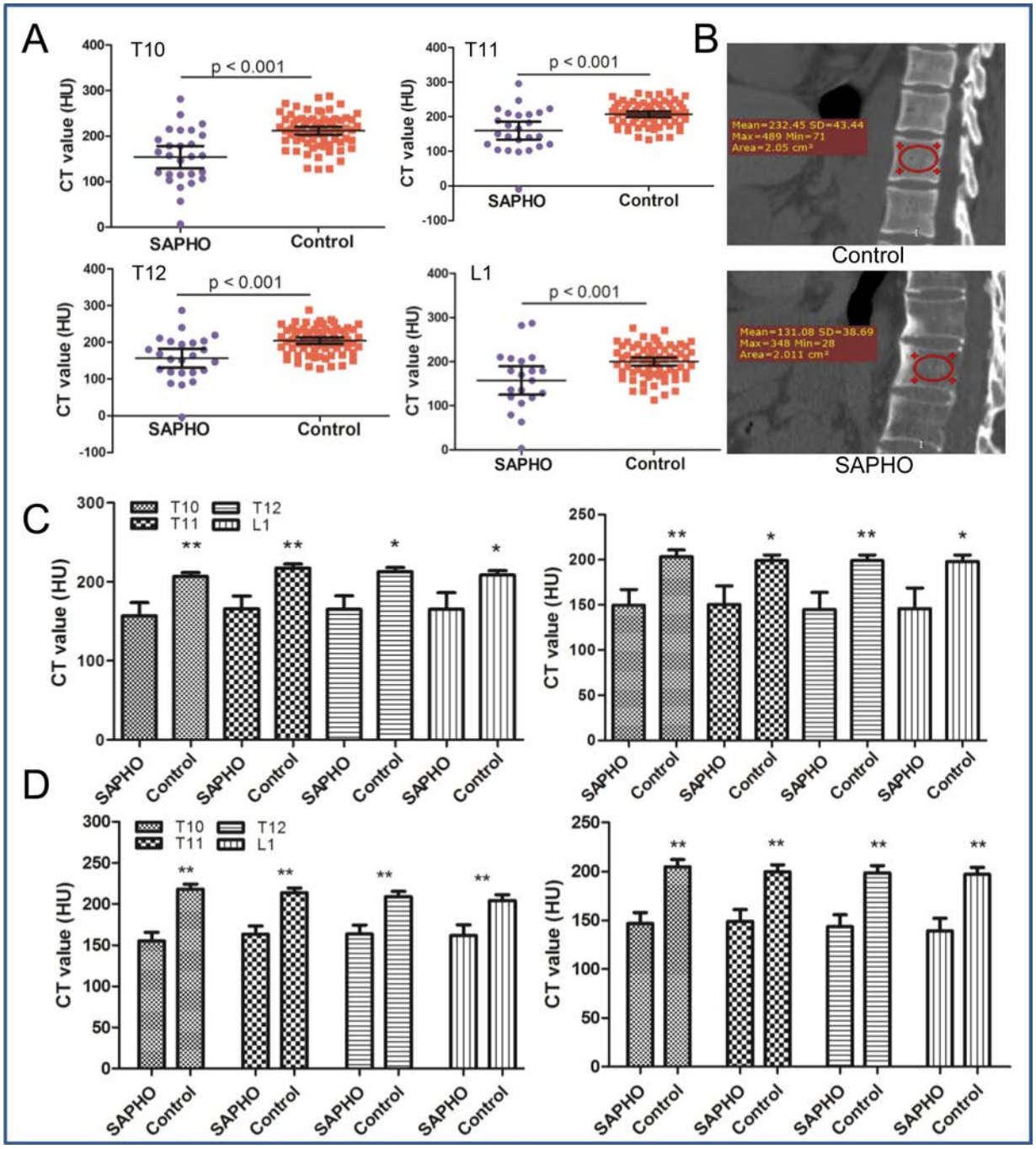


**Figure 1** CT attenuation (Hounsfield units, HU) in thoracic spine 10–12 (T10–T12) and lumbar spine (L1) (A), and sagittal CT images of T12 in patients with synovitis, acne, palmoplantar pustulosis, hyperostosis and osteitis (SAPHO) syndrome and age/gender-matched control (two 64 years old men) (B). Cortical sclerosis and osteoporosis both occurred in T12 in a SAPHO patient. CT values of T10–T12 and L1 in women (left) and men (right) (C). Age-adjusted CT values of T10–T12 and L1 in women (left) and men (right) (D). SAPHO versus control: **p<0.01; *p<0.05. Data are shown as mean with 95% CI (A) and mean with SE (C, D). The data were analysed using Mann-Whitney U test (A, C).

**Correction notice** This article has been corrected since it published Online First. The co-corresponding author has been added.

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# Non-causal association of gut microbiome on the risk of rheumatoid arthritis: a Mendelian randomisation study

I read with great interest the article by Alpizar-Rodriguez *et al* regarding the risk of intestinal dysbiosis, particularly *Prevotella* spp enrichment, in preclinical rheumatoid arthritis (RA).[1] Immune response in gut is assumed to be one of the triggers of development of RA.[2] However, it is hard to assess causal association by case–control study due to limitations such as latent confounding factors; *dysbiosis first or RA first*. Therefore, to investigate causal effect of gut microbiome on the development of RA, I conducted Mendelian randomisation (MR) analysis.[3] MR is useful to investigate causal association among phenotypes and/or biomarkers because it is based on genetic variation to mimic the design of randomised controlled trials. In MR, single nucleotide polymorphisms (SNP) are expected to be random and causally upstream of the exposure; thus, SNP are used as instrumental variables (IVs) in MR.

I used the publicly available two data sets of genome-wide association studies (GWASs) for gut microbiome (totally 3326 individuals) of European ancestry as the exposure[4 5] and one data set of GWAS for RA (19 234 cases and 61 565 controls) of European and Asian ancestries as the outcome,[6] respectively. To improve inference, selection of genetic variants associated with gut microbiome as IVs was based on linkage disequilibrium $R^2$ of 0.001, clumping distance of 10 000 kb and p value threshold of 5.00E−08 (genome-wide significance). Then, I examined the association between single SNP and risk of RA. Finally, by combining them using MR analysis, I estimated the causal association between gut microbiome and risk of RA. The effect size was shown by beta coefficient or OR. I assessed heterogeneity across SNPs by Cochran's Q statistics. To explore whether single SNPs drives causal association, I performed a leave-one-out analysis. All MR analyses were performed in MR Base platform (http://www.mrbase.org/; App version: 1.2.2 3a435d) and R V.3.6.1.

I obtained 26 SNPs as IVs from gut microbiome GWASs (online supplementary table 1). Among them, rs1230666 (*MAGI3*) was also strongly associated with the risk of RA (figure 1A, online supplementary table 1), implying this single IV might bias the result of MR. Correspondingly, although the inverse variance weighted (IVW) and MR Egger methods showed decrease in bacterial taxa in gut microbiome reduced the risk of RA, this result might be biased by single rs1230666 according to heterogeneity p value of both IVW and MR Egger methods (<0.05, table 1) and scatter plots of genetic associations with gut microbiome against the genetic associations with RA (figure 1B). Indeed, leave-one-out sensitivity analysis demonstrated IVW method without rs1230666 lost significance (figure 1C).

Therefore, I conducted sensitivity analysis without rs1230666. As a result, association p value derived from IVW, MR Egger and weighted median methods were not significant (p = 0.286, p = 0.057, p = 0.166, respectively, table 1) with no evidence of heterogeneity (heterogeneity p value>0.05, table 1), implying gut microbiome might not have causal effect for risk of RA. According to other sensitivity analysis to assess violations of assumptions, test for directional horizontal pleiotropy by the MR-Egger regression showed that directional pleiotropy was unlikely to bias the results of both the former and later analysis using 26 and 25 IVs, respectively (intercept=0.009, p=0.614; intercept=−0.003, p=0.548; respectively), indicating no evidence of pleiotropy.

The current study suggested that dysbiosis might be secondary phenomenon rather than triggers in the pathogenesis of RA. Even after taking into consideration of limitation of MR analysis that power of the test could be insufficient when SNPs have weak association with exposure, the impact of gut microbiome as triggers of the development in RA might be small.

**Jun Inamo**

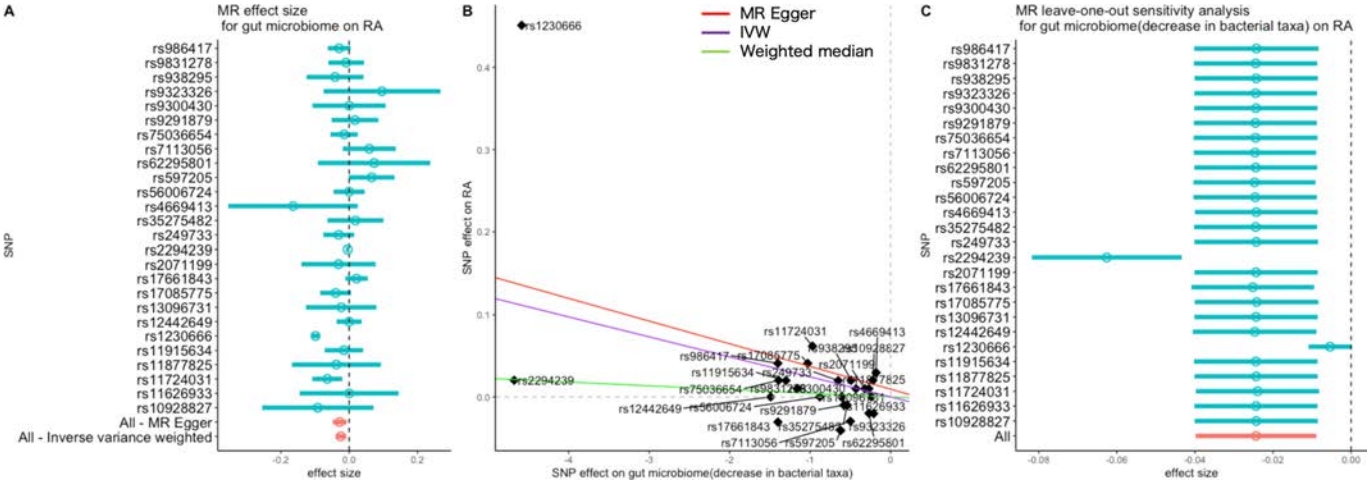


**Figure 1** MR of the causal effect of gut microbiome and risk of RA. (A) Forest plot of the causal effects of gut microbiome (decrease in bacterial taxa) SNPs on RA. The causal effect of gut microbiome on RA is estimated using each SNP singly using the Wald ratio, and represented in a forest plot. The MR estimate using all SNPs using the MR Egger and IVW methods are also shown. Each point represents effect estimates and bar represents 95% CI. (B) Scatter plots of genetic associations with gut microbiome against the genetic associations with RA. SNP effects on the RA are plotted against SNP effects on the gut microbiome. The slope of the line represents the causal association, and each method has a different line. (C) Leave-one-out sensitivity analysis is performed to ascertain if an association is being disproportionately influenced by a single SNP. Each turquoise point in the forest plot represents the MR analysis (using IVW) excluding that particular SNP. The overall analysis including all SNPs is also shown for comparison. IVW, inverse variance weighted; MR, Mendelian randomisation; RA, rheumatoid arthritis; SNP, single nucleotide polymorphism.

**Table 1** The MR estimates from each method of the causal effect of gut microbiome on RA risk

| Method | Number of SNPs | OR (95% CI) | Association p value | Cochrane Q statistic | Heterogeneity p value | *Number of SNPs | *OR (95% CI) | *Association p value | *Cochrane Q statistic | *Heterogeneity p value |
|---|---|---|---|---|---|---|---|---|---|---|
| MR Egger | 26 | 0.97 (0.95 to 0.99) | 0.013 | 306.4 | 8.78E–51 | 25 | 1.00 (0.99 to 1.00) | 0.286 | 29.8 | 1.54E–01 |
| IVW | 26 | 0.98 (0.96 to 0.99) | 0.002 | 309.7 | 6.79E–51 | 25 | 0.99 (0.99 to 1.00) | 0.057 | 30.3 | 1.75E–01 |
| Weighted median | 26 | 1.00 (0.98 to 1.00) | 0.143 | N/A | N/A | 25 | 1.00 (0.99 to 1.00) | 0.166 | N/A | N/A |

*Sensitivity analysis without rs1230666.
IVW, inverse variance weighted; MR, Mendelian randomisation; N/A, not applicable; RA, rheumatoid arthritis; SNP, single nucleotide polymorphism.

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**Contributors** All of conceptualisation, formal analysis and writing were conducted by JI.

**Competing interests** None declared.

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# Response to: 'Non-causal association of gut microbiome on the risk of rheumatoid arthritis: a Mendelian randomisation study' by Inamo

We are grateful to Dr Inamo[1] for his interest in our article. We agree and acknowledged in our manuscript that our study design did not allow us to assess a causal association. Our study described an increased relative abundance in *Prevotella* spp. in individuals in 'preclinical rheumatoid arthritis (RA) stages' using participants enrolled in a first-degree relatives of patients with RA (FDR-RA) cohort.[2] The microbiota of individuals in preclinical RA stages were significantly altered compared with FDR-RA controls. In particular, bacteria of the Prevotellaceae family and associated taxa were enriched among individuals in preclinical stages of RA.

Dr Inamo concludes from his analysis that intestinal dysbiosis is probably only secondary phenomenon and unlikely to trigger the pathogenesis of RA. We respectfully disagree with this conclusion for several reasons:

- As Inamo points out, it is indeed impossible to make causal inferences from cross-sectional studies. Furthermore, the author rightfully notices that the scientific community still does not know whether 'dysbiosis comes first or RA comes first', which is precisely the reason we focused our analysis not on patients with RA but on individuals in different preclinical stages of the disease. While our findings are certainly not yet a proof for a causal role of intestinal dysbiosis in RA development, the demonstration of a large proportion of individuals in preclinical stages of RA with a significant dysbiosis is certainly consistent with the mucosal origins hypothesis of RA development.[3]
- Inamo used Mendelian randomisation analyses to assess causal association of dysbiosis with RA, taking advantage of two large datasets of genome-wide association studies (GWASs).[4 5] Inamo obtained 26 SNPs from gut microbiome GWASs associated with reduced bacterial taxa (see online supplementary table 1, Inamo's correspondence letter) . However, recent data strongly suggest only a minor influence of genetics on microbiota composition.[6] Furthermore, no studies have ever found significant differences in alpha or beta diversities between RA cases and controls, despite significant differences in specific bacterial taxa (ie, *Prevotella copri*).[2 7] Thus, the exposure analysed by the author does not represent a relevant measure of dysbiosis in RA. It would have been more appropriate for Inamo to analyse, for instance, *Prevotella* spp. abundance instead of reduced bacterial taxa.

To formally establish a causal role of intestinal dysbiosis in RA development, longitudinal studies prior to the onset of RA are required to demonstrate that specific dysbiosis precedes the development of RA, which then would have to be further validated by relevant *in vivo* studies and microbiome-centred intervention trials.

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# Chronic hydroxychloroquine exposure and the risk of Alzheimer's disease

Hydroxychloroquine is an immunomodulatory agent which is commonly used to treat malaria and autoimmune diseases.[1] The association between hydroxychloroquine therapy and subsequent comorbidities has been extensively addressed.[1 2] Recently, a cohort study conducted in the UK by Fardet *et al* published in Annals of the Rheumatic Diseases reported that long-term use of hydroxychloroquine was not associated with the risk of Alzheimer's disease when compared with non-use of hydroxychloroquine (adjusted HR=0.81, 95% CI=0.58 to 1.12, p=0.20).[3] One comment published in Annals of the Rheumatic Diseases reported that due to conflicting results between observational studies regarding the impact of hydroxychloroquine on Alzheimer's disease, the relationship cannot be determined currently.[4]

In order to test such an association in a different population, a preliminary case-control study was conducted using the 2005–2012 database of the Taiwan National Health Insurance Program with 23 million people living in Taiwan.[5] People aged≥65 years with newly diagnosis of Alzheimer's disease were assigned as the cases (according to International Classification of Diseases, Ninth Revision, Clinical Modification, ICD-9 code 331.0). People aged≥65 years without any type of dementia were selected as the controls. In order to reduce the biassed results, people who had a cumulative period of hydroxychloroquine use<3 months were excluded from the study. Table 1 revealed that there was no statistical association between Alzheimer's disease and hydroxychloroquine use (crude OR=0.97, 95% CI=0.50 to 1.87, p=0.92), which was compatible with Fardet *et al*'s cohort study reporting no statistical association between hydroxychloroquine use and the risk of Alzheimer's disease. Due to only nine cases with Alzheimer's disease ever using hydroxychloroquine in our study, further research with a large case number is required to confirm our finding. Among quinoline-based antimalarial drugs, hydroxychloroquine does not have a good ability to penetrate the blood–brain barrier.6 It is not a rational hypothesis that hydroxychloroquine use can have an impact on the risk of Alzheimer's disease clinically. Those studies showing an association between hydroxychloroquine use and the risk of Alzheimer's disease should be interpreted with caution. We agree with the author's comments that there is no conclusive evidence linking hydroxychloroquine use and the risk of Alzheimer's disease,[4] regardless of the population studied. Randomised controlled trials are needed to explore the issue. In view of the above discussion, older people who are on long-time therapy of hydroxychloroquine do not need to worry about the risk of Alzheimer's disease because such a risk has not yet been confirmed. Finally, Fardet *et al*'s research has impressed the readers a lot and has drawn much attention from scholars specialising in this issue.

**Table 1** Association between Alzheimer's disease and hydroxychloroquine therapy in people aged≥65 years in 2005–2012

| | Alzheimer's disease (n=1131) | Non-dementia (n=92 063) | Crude OR (95% CI) | P value |
|---|---|---|---|---|
| Hydroxychloroquine use | 9 (0.80) | 758 (0.82) | 0.97 (0.50 to 1.87) | 0.920 |
| No use | 1122 (99.20) | 91 305 (99.18) | 1 | |

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# Response to: 'Chronic hydroxychloroquine exposure and the risk of Alzheimer's disease' by Lai *et al*

We thank Lai *et al* for their correspondence[1] in which they share their study findings that endorse our own study findings[2] reporting an absence of a link between hydroxychloroquine and Alzheimer's disease. More robust research on the topic emerging from randomised trials would of course be welcomed. However, it should be kept in mind that the effect of hydroxychloroquine on progression of dementia in early Alzheimer's disease has been investigated in 2001 in a randomised, placebo-controlled trial, and the results of the study showed no effect of treatment against placebo.[3] Taken all together, these results from two large national databases and a randomised controlled trial are reassuring. Therefore, we do agree with Lai *et al* that withdrawal of hydroxychloroquine in people on long-term therapy, especially in older people and those receiving the drug for an autoimmune disease, is not justified.

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# MS score in systemic juvenile idiopathic arthritis: suitable for routine use?

We read with great interest the article by Minoia *et al*[1] which reported a new scoring tool for the classification of macrophage activation syndrome (MAS), a potentially life-threatening complication requiring prompt treatment, in patients with systemic juvenile idiopathic arthritis (sJIA). Although the haemophagocytic lymphohistiocytosis diagnostic criteria were widely used, early diagnosis of MAS is still challenging.[2 3] The creation of the MAS/sJIA (MS) score provided a new tool for early detection of MAS in sJIA. However, a few points of concern raised when using the MS score in our centre.

First, the data of patients with MAS were recorded at the onset of the syndrome while the clinical features of patients with active sJIA without MAS were collected at the onset or flare of the disease. Since exacerbation could occur at any point. As a result, data that were not collected at a single point in time might cause selection bias. The comparison might be more reasonable if the clinical data of both groups were recorded at the same disease status.

Besides, the central nervous system (CNS) dysfunction, the most important clinical feature included in the scoring formula, was defined as 'the presence of lethargy, seizures, irritability, confusion, headache, mood changes or coma'. The percentage of CNS involvement was much higher in patients with active sJIA with MAS (35%) than those who did not have MAS in the article (1.8%, $p<0.0001$). The definition of headache and mood changes were not clarified. An ambiguous definition may cause variance in understanding these terms. For example, mood changes may refer to minor daily changes or to significant mood disturbances such as major depression or bipolar depression. And the mild headache is relatively common in feverish patients. We wonder if minor daily mood changes and mild headache were also counted as CNS dysfunction during MS score calculating.

What's more, took the presence of fever as a mandatory criterion for the diagnosis of MAS may lead to missed or delayed diagnosis. The body temperature might be under control by the initiation of glucocorticoids or other treatments such as interleukin-1 inhibitor, even if the occult MAS has already developed. Besides, they did not include sCD25 and natural killer cell activity during the score developing process because these tests were not routinely assessed in most paediatric rheumatology centres. However, both biomarkers are important objective indicators of increased T cell activation and impaired cytolytic function in the pathogenesis of MAS.[4] The exclusion of these two parameters may affect the sensitivity and specificity of MAS detection. The MS score should be further validated in centres capable of completing the measurement of sCD25 and natural killer cell activity.

Though the performance of the MS score is quite well in the validation group, the application of MS score for the diagnosis of MAS in clinical practice is questionable in its current state.

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# Response to: 'MS score in systemic juvenile idiopathic arthritis: suitable for routine use?' by Chi *et al*

We thank Chi *et al*[1] for their interest in our diagnostic score for macrophage activation syndrome (MAS) in systemic juvenile idiopathic arthritis (sJIA).[2]

Chi *et al* argue that the collection of MAS patient data at the onset of the syndrome and of sJIA patient data not only at the onset of the illness but also during a flare could have caused a selection bias. However, the primary purpose of our analysis was to scrutinise the ability of clinical and laboratory features to discriminate between MAS and active sJIA without MAS. Because the main sJIA manifestations at disease onset or at the time of a flare with ongoing systemic features are clinically similar, we thought that both time points were equally suitable as controls. Note that only 22% of MAS episodes observed in our series developed at onset of sJIA, whereas all the other instances occurred at various times during the underlying disease course.[3]

Regarding central nervous system dysfunction, we included in the definition the neuropsychiatric symptoms that are traditionally described as part of this organ involvement in MAS.[4–6] We recognise, however, that the definitions of headache and mood changes were not sufficiently detailed. In our view, headache can be related to MAS when it is severe, unrelenting, unresponsive to analgesics and persistent independently of fever. Mood changes can be defined as sudden mood alteration or unexplained major depression.

Another concern raised by Chi *et al* is that the presence of fever as mandatory criterion may lead to missed or delayed diagnosis of MAS, as fever may be suppressed by treatment with corticosteroids or IL-1 inhibitors. The choice of placing fever as a prerequisite for the diagnosis of MAS was based on the notion that it was the mostly highly ranked clinical feature of MAS in a Delphi survey conducted among international paediatric rheumatologists[7] and was considered a fundamental requirement for the classification of MAS by the expert panel that devised the 2016 classification criteria for MAS complicating sJIA.[8] In addition, fever was reported in 96.1% of 362 patients with sJIA-associated MAS collected in a multinational multicentre survey,[3] which confirms its key relevance in the clinical picture of MAS. However, a recent systematic literature review has shown that the 2016 MAS classification criteria may miss some episodes of MAS occurring in patients with sJIA under treatment with IL-1 and IL-6 blocking agents, owing to the substantial alterations in MAS features induced by these biologics, including lack of fever.[9] Due to the small number of patients under biological treatments in our database, we could not test the MS score in a subgroup of patients who had MAS under therapy with IL-1 and IL-6 inhibitors. More data from the real world of clinical practice are needed to establish whether the score should be refined to increase its power to pick up the instances of MAS occurring during treatment with biological medications.

As a final note, Chi *et al* contend that the exclusion of soluble CD25 and natural killer cell activity may affect the sensitivity and specificity of MAS detection. Although we agree that these biomarkers are important indicators of increased T cell activation and impaired cytolytic function and may help to detect subclinical instances of the syndrome, they are not readily available in most paediatric rheumatology centres, particularly in resource-limited areas. Furthermore, because these tests take time to complete they may not be suited to diagnose MAS timely at patient bedside. Nevertheless, the role of suggested immunological parameters, together with that of sCD163 IL-18, CXCL9 and others, in the routine diagnosis of MAS is worth being investigated in the future.

In conclusion, we are grateful to Chi *et al* because their comments led us to clarify some aspects of the MAS/sJIA (MS) score that may enhance its applicability. Further insights into the validity of the score will be obtained through its widespread use in clinical practice.

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# Correspondence to 'Time to change the primary outcome of lupus trials'

We note with interest and express our principle agreement with the views put forward by Professor Frederic Houssiau in the recent Editorial 'Time to change the primary outcome of lupus trials' published in *Annals of the Rheumatic Diseases*.[1] Professor Houssiau advocated using steroid reduction as a primary outcome measure in systemic lupus erythematosus (SLE) clinical trials, or as a minimum, incorporating a steroid reduction target into the primary endpoint. This proposal was prompted by the observation that, like many other recent phase 3 clinical trials conducted with drugs that have a sound biological mechanism for benefit in SLE, the recently published CHABLIS-SC phase 3 clinical trial of subcutaneous blisibimod in SLE[2] failed to meet its primary endpoint of a week 52-SLE Responder Index (SRI)-6. However, a significant steroid sparing effect was seen, with a modified endpoint of a week 52-SRI-6 combined with a reduction in steroid dose during weeks 40–52 compared with study entry, showing a trend to benefit for the blisibimod arm (23.3% of blisibimod-treated, compared with 14.3% of placebo-treatedpatients, p=0.056).

The harmful effects of long-term steroid use are well recognised, and there is specific evidence in SLE that steroids independently contribute to increased cardiovascular risk, osteoporotic fractures, avascular bone necrosis and diabetes mellitus.[3] In addition, a number of studies have found that steroid exposure in SLE is associated with increased damage accrual,[3 4] which is in turn associated with increased morbidity and mortality. Therefore, by extension, to be truly disease modifying, any SLE treatment should have a steroid sparing effect.

Last year, we published a systematic review and meta-analysis of steroid sparing effect of biological agents in phase 3 clinical trials in SLE that were published in the 10 years prior.[5] Twenty-eight studies were identified; 9 conducted in SLE, 5 in lupus nephritis and 14 *post hoc* analyses of the original phase 3 trials in SLE. Of the eight drugs trialled in these studies (rituximab, belimumab, tabalumab, epratuzumab, atacicept, ocrelizumab, abetimus sodium and abatacept), only the BLISS-52[6] and BLISS-76[7] (intravenous belimumab), BLISS-SC[8] (subcutaneous belimumab) and ILLUMINATE-2[9] (subcutaneous tabalumab) studies met their primary endpoints. However, effects on secondary endpoints including changes in serological markers were often seen, and a steroid reduction outcome measure was included in most, but not all, studies. As the steroid reduction endpoints reported in these studies were variable, to perform the meta-analysis of steroid sparing effect of these biological agents, we included the seven studies which reported a similar corticosteroid-reduction endpoint:

- ≤7.5 mg/day, and by ≥25% from baseline between weeks 40 and 52 (belimumab—BLISS-52,[6] BLISS-76[7] and BLISS-SC[8]);
- ≤7.5 mg/day, between weeks 24 and 52 for ≥3 consecutive months, without increase in antimalarials/immunosuppressants (tabalumab—ILLUMINATE-1[10] and ILLUMINATE-2[9]);
- ≤10 mg/day or ≤7.5 mg/day by week 24 (epratuzumab—ALLEVIATE-1 and ALLEVIATE-2, respectively[11]); or
- <10 mg/day between weeks 24 and 52, with a major clinical response (rituximab—EXPLORER[12]).

In this correspondence, we have updated our meta-analysis to include two additional phase 3 trials in SLE published since the systematic review—the CHABLIS-SC study,[2] and the BEL113750 trial of intravenous belimumab 10 mg/kg conducted in China, South Korea and Japan.[13] As noted above, the CHABLIS-SC study failed to meet its primary endpoint; however, the BEL113750 trial did meet its primary endpoint of a week 52-SRI-4 response (53.8% vs 40.1%, OR 1.99, p=0.0001). Similar to most other studies included in the meta-analysis (figure 1), both of these studies showed a reduction in steroids between weeks 40 and 52 to ≤7.5 mg/day, compared with placebo (17.2% vs 8.9%, p=0.019 for blisibimod, and 15.6% vs 10.9% for belimumab, p=0.0721).

All measures of SLE disease activity (including composite endpoints, such as the SRI-4 and BICLA (BILAG-based combined lupus assessment), which are commonly used as primary outcome measures in SLE clinical trials) have inherent limitations, and it is evident from studies such as CHABLIS-SC that the choice of endpoint can have significant implications for the outcome of a clinical trial. We have shown in our meta-analysis that many of the biological agents that failed to show benefit in phase 3 clinical trials using composite endpoints, showed a steroid sparing effect. Given the importance of steroids in contributing to morbidity in SLE, we agree with Professor Houssiau that it is time to give strong consideration to including steroid sparing effect (captured as a dose reduction or a specific dose reached) in composite endpoints in SLE clinical trials.

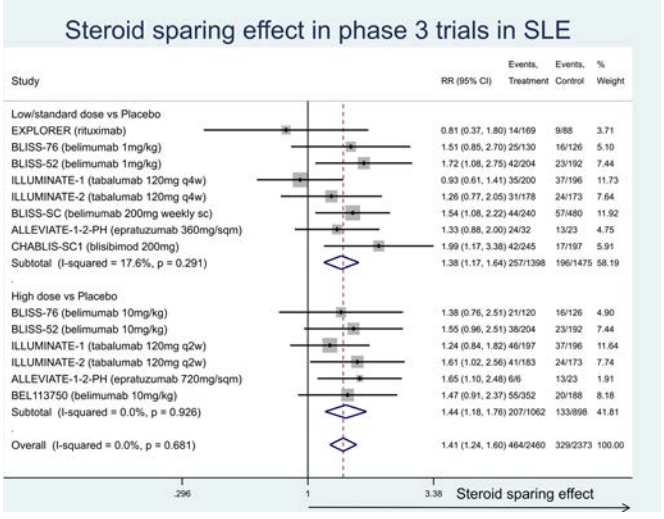


**Figure 1** Meta-analysis of corticosteroid-sparing effect (expressed as relative risk) in phase 3 clinical trials of biological agents in systemic lupus erythematosus.

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# Response to Correspondence to 'Time to change the primary outcome of lupus trials' by Oon *et al*

I read with interest the comment by Oon *et al* on my editorial '*Time to change the primary outcome of lupus trials*', published in a recent issue of the Annals of the Rheumatic Diseases.[1] The authors further fuel my viewpoint by updating their previous meta-analysis on glucocorticoid (GC) spare in phase III lupus trials.[2] They have now included two additional studies, that is, CHABLIS-SC[3]—which triggered the Editorial—and the Asian belimumab trial.[4] Quite interestingly, this extended meta-analysis confirmed that significantly more lupus patients receiving a targeted therapy within the frame of a phase III trial could successfully taper GC.

Achieving GC spare is well in line with EULAR recommendation *2.2.3* for the management of lupus: '*For chronic maintenance treatment, GC should be minimised to less than 7.5 mg/day (prednisone equivalent) and, when possible, withdrawn*'.[5] A similar statement was made—already *5* years ago—by an international task force advocating a treat-to-target approach in recommendation *8*: '*Lupus maintenance treatment should aim at the lowest GC dosage needed to control disease, and if possible, GC should be withdrawn completely*'.[6]

With such strong statements in mind, hopefully applied in clinical practice, why should GC taper not be included in lupus trials' primary outcome?

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# Ultrasonographic damages of major salivary glands are associated with cryoglobulinemic vasculitis and lymphoma in primary Sjogren's syndrome: are the ultrasonographic features of the salivary glands new prognostic markers in Sjogren's syndrome?

We read with great interest the new ultrasound scoring system of salivary glands for primary Sjogren's syndrome (pSS) developed by Outcome Measures in Rheumatology (OMERACT) and recently reported by Jousse-Joulin *et al*.[1] The salivary gland ultrasound (SGUS) is a simple, non-irradiating, non-expensive and accessible assessment tool. Several previous SGUS scoring systems have been proposed so far, but not all of them take into account every pathological features, especially in the parotid gland (such as hyperechoic bands) for the diagnosis of pSS. The lack of consensus between each of these SGUS scoring systems also had a negative impact on the reproducibility of the examination so far. By offering an updated consensual classification, the OMERACT initiative therefore constitutes an undeniable progress for the diagnosis of pSS.

Recently, in a multicentre prospective study of 97 patients referred for clinical sicca syndrome (39 pSS and 22 secondary Sjogren's syndrome (sSS) according to American-European Consensus Group (AECG) criteria in comparison with 36 controls), we reported good performances of various SGUS scoring systems for the diagnosis of pSS or sSS (area under the curve between 0.885–0.915 (pSS) and 0.808–0.851 (sSS) according to the used ultrasound scores, respectively).[2] After the diagnosis of SS, patients are considered at high risk of B lymphoma, a classical and severe complication of SS. The progressive continuum of glandular B lymphocyte hyperactivity, initially polyclonal and then oligoclonal, with the presence of abnormal germinative centres, production of rheumatoid factor, deposition of immune complexes characterised by the consumption of the complement and the presence of cryoglobulinemic vasculitis which may subsequently lead to monoclonal B lymphocyte expansion and low-grade marginal area B lymphoma, is now well accepted and requires specific monitoring of patients with pSS.[3]

In this correspondence, we would like to draw attention on the association between SGUS characteristics and systemic complications of pSS, with a focus on cryoglobulinemic vasculitis (a well-known risk factor for progression to B lymphoma)

**Table 1** Characteristics of the 97 patients with sicca syndrome included in the study

| | pSS, n (%) | sSS, n (%) | Controls, n (%) | P value* |
|---|---|---|---|---|
| n | 39 | 22 | 36 | |
| Age (±SD) | 59.1±13.4 | 55.3±15.0 | 55.8±11.9 | ns |
| Women | 36 (92.3) | 22 (100) | 34 (94.4) | ns |
| Non-specific autoimmune disease associated | | | | |
| None (n=62) | 39 (100) | 0 | 23 (63.9) | <0.05†‡§ |
| Rheumatoid arthritis (n=14) | 0 | 9 (41.0) | 5 (13.9) | <0.05† |
| Systemic lupus (n=16) | 0 | 9 (41.0) | 7 (19.4) | <0.05†‡ |
| Others¶ (n=7) | 0 | 4 (18.0) | 3 (12.0) | ns |
| Clinical sicca syndrome | | | | |
| Duration of sicca syndrome <5 years | 12 (30.8) | 6 (27.3) | 17 (47.2) | ns |
| Schirmer test (±SD) | 5.36±7.10 | 1.27±1.83 | 9.32±9.31 | 0.001‡§ |
| Schirmer test <5 mm | 28 (71.8) | 22 (100) | 14 (38.9) | <0.05‡ |
| Unstimulated salivary flow (±SD) | 1.3±1.26 | 1.2±0.96 | 2.1±1.50 | 0.02‡§ |
| Unstimulated salivary flow <0.5 mL/5 min | 14 (35.9) | 9 (40.9) | 0 | <0.05‡§ |
| Biology | | | | |
| ANA ≥1/320 | 31 (79.5) | 19 (86.4) | 11 (30.6) | <0.05‡§ |
| SSA antibodies | 30 (76.9) | 11 (50.0) | 9 (25.0) | <0.05†‡§ |
| Rheumatoid factor | 22 (56.4) | 13 (59.1) | 8 (22.2) | ns |
| Hypergammaglobulinemia >16 g/L | 19 (48.7) | 4 (18.2) | 4 (11.1) | <0.05†‡ |
| Salivary gland involvement | | | | |
| Focus score (±SD) | 2.57±4.42 | 1.91±1.53 | 0.72±1.82 | <0.05‡§ |
| Focus score=1/4 mm² | 34 (97.1) | 19 (90.5) | 5 (17.2) | <0.05‡§ |
| Pathological salivary glands in ultrasound | 24 (61.5) | 13 (33.3) | 2 (5.1) | <0.05‡§ |
| Systemic complications | | | | |
| Lymphoma | 3 (7.7) | 0 | 0 | ns |
| Cryoglobulinemic vasculitis | 3 (7.7) | 2 (9.0) | 1 (2.8) | ns |
| Severe systemic complications (composite index)** | 7 (17.9) | 8 (36.4) | 3 (8.3) | <0.05†‡ |

*Difference between two groups according to $\chi^2$ test adjusted by Bonferroni's method for qualitative variables or by ANOVA for quantitative variables. Univariate analyses, all preformed with SPSS.
†pSS versus sSS.
‡ pSS versus controls.
§sSS versus control.
¶Others non-specific autoimmune diseases associated: systemic sclerosis (n=4), mixed connective tissue disease (n =3).
**Composite index: interstitial lung disease, proliferative glomerulonephritis, central nervous system involvement, cryoglobulinemic vasculitis or lymphoma.
ANA, anti-nuclear antibodies; pSS, primary Sjogren's syndrome; sSS, secondary Sjogren's syndrome.

and lymphoma itself. The characteristics of our population are shown in table 1.[3] There were three B lymphomas in our population, all found in the pSS group (prevalence of 7.7% in the pSS group). Cryoglobulinemic vasculitis was present in 3 (7.7%), 2 (9.0%) and 1 (2.8%) of the pSS, sSS and control group, respectively. Factors associated with lymphomas were severe salivary dryness defined by an unstimulated salivary flow $<0.5$ g/5 min and pathological SGUS (regardless of the classification used) (observed in all patients with lymphoma, $p<0.01$). Pathological SGUS was also associated with the presence of cryoglobulinemic vasculitis (OR=44.0 (3.26–583), $p<0.001$). No lymphoma or cryoglobulinemic vasculitis was found in patients with normal SGUS. In all patient with lymphoma or cryoglobulinemic vasculitis, the salivary glands were all extremely pathological in ultrasound and characterised by a heterogeneous parenchyma containing either numerous cystic lesions with no healthy parenchyma left or significant fibrosis attested by the presence of numerous hyperechoic bands. All these cases fulfilled the definition of a stage 3 of the new OMERACT classification.[1]

These results confirm the data reported by Thender *et al*[4] on the association between morphological SGUS damages (particularly of the parotid glands), usual associated-risk factors for progression to lymphoma (CD4 lymphopenia, cryoglobulinemic vasculitis, germinative centre on accessory salivary gland biopsy) and the existence or occurrence of lymphoma. Nevertheless, our cross-sectional data do not allow to conclude on the prognostic value of SGUS for lymphoma occurrence in pSS. These results nonetheless support the use of this tool in daily practice and its systematic inclusion in the assessment of prospective cohorts of pSS, which would allow the evaluation of the relevance of SGUS features as predictive markers for lymphoma. In the end, the prospective evaluation of SGUS results will help to precise the place of SGUS examination in the global follow-up and management of patients with pSS, beyond diagnosis.

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# Could we use salivary gland ultrasonography as a prognostic marker in Sjogren's syndrome? Response to: 'Ultrasonographic damages of major salivary glands are associated with cryoglobulinemic vasculitis and lymphoma in primary Sjogren's syndrome: are the ultrasonographic features of the salivary glands new prognostic markers in Sjogren's syndrome?' by Coiffier *et al*

We thank Dr Coiffier and colleagues for their interesting letter in which they suggest that damages of major salivary glands (SG) might be associated with cryoglobulinemic vasculitis and lymphoma in primary Sjögren's syndrome. The authors raise the question whether the ultrasonographic (US) features of the salivary glands [our proposed novel ultrasound scoring system] could be used as the new prognostic markers for cryoglobulinemic vasculitis and lymphoma in patients with Sjögren's syndrome.[1]

In their recent study of different US scoring systems in 97 sicca syndrome patients, Coiffier *et al* found three B-cell lymphomas in the primary Sjogren's syndrome (pSS) group and three, two, and one patient with cryoglobulinemic vasculitis in the pSS, secondary Sjogren's syndrome (sSS) and control groups, respectively.[2] The authors concluded that the detection of B-cell lymphomas or cryoglobulinemic vasculitis was associated with pathological US findings regardless of the scoring used. The reported US pathological features of SG were either numerous cystic lesions without healthy parenchyma or fibrous glands scored as a grade 3 according to the new semi quantitative scoring system described by the SG sub group of the OMERACT US working group.[1] Although their findings may suggest specific ultrasound features as a risk factor for developing lymphoma or cryoglobulinemic vasculitis, we think at this point in time it is premature to draw such a conclusion.[3] Ultrasound may reveal predisposing factors, but these are not proven to be pathognomonic of lymphoma. Indeed, several predictors of lymphoma in pSS such as epidemiological, clinical (permanent swelling of the SG, palpable purpura, organomegaly), biological (cryoglobulinaemia, or low complement levels) and histopathological findings should also be taken into account.[4–9] Large sample and longitudinal studies assessing these clinical and biological predictors of lymphoma with US are currently ongoing and will probably shed more light on this challenging issue. Furthermore, pSS disease activity, for example, assessed by the EULAR SS index, can be used as a clinical predictor of lymphoma development with a dose effect.[10]

SG enlargement (eg, clinical aspects: unilateral, fixed and hard parotid glands) is regarded as the most dominant clinical symptom for lymphoma in patients with pSS.[6] SGUS performed by well-trained ultrasonographers can provide a precise structural assessment of the glands' surface compared with clinical examination.[11] In case of SGUS grade 3, that is, complete destruction of the gland, with numerous hypoechoic or hyper-echoic bands, the detection of abnormal lymph nodes should raise awareness of possible lymphoma development. Suspicion of abnormal lymph nodes can be confirmed during a long term monitoring of pSS patients especially those with high risk of lymphoma development. In addition, Doppler assessment of gland's vascularisation in pSS might be of help to detect at risk ultrasound lesions. To this end, the forthcoming results of a longitudinal study for development of consensual Doppler US scoring of gland's vascularisation in pSS can be quite helpful.

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# Anti-Ku syndrome with elevated CK: association with myocardial involvement in systemic sclerosis

We read with great interest the paper by Spielmann *et al* describing a large cohort of anti-Ku-positive connective tissue disease patients.[1] In their paper, the authors could identify two different subgroups of patients: 'anti-Ku with elevated CK patients', who are at risk of developing interstitial lung disease (ILD), and 'anti-Ku with anti-dsDNA patients', who are at risk of developing glomerulonephritis. Interestingly, the authors found an extremely low rate of myocarditis in both subgroups as only one patient in the C1 cluster ('elevated CK') was diagnosed with myocarditis. Of note, in our cohort of anti-Ku-positive systemic sclerosis (SSc) patients we found that the positivity for this rare antibody was invariably associated with myocardial inflammation. We indeed performed a retrospective review of anti-Ku-positive patients affected by SSc according to European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) 2013 criteria[2] followed-up at two referral centres for SSc. We routinely performed anti-Ku in all SSc patients without any detected positivity for other SSc-related antibodies, that is, antitopoisomerase I, anticentromere and anti-RNA polymerase III. All SSc patients with suspected myocardial inflammation (new onset cardiac signs and/or symptoms, raised troponin T and/or N-terminal pro-brain natriuretic peptide (NTproBNP)) routinely underwent cardiac magnetic resonance (CMR) and 24-hour-Holter-ECG tape. Myocarditis diagnosis was defined according to the Lake Louise Criteria[3] on CMR. We identified four anti-Ku-positive SSc patients. All patients were female, and had been diagnosed with limited cutaneous SSc; mean age at diagnosis was 52.5±19.74 years. In all cases, the anti-Ku positivity was confirmed by immunoblotting. Mean delay between myocarditis and SSc onset was 79.2±48.1 months. In three out of four patients (75%), ILD was also present. Myositis was diagnosed in all patients by creatine kinase (CK) increase, electromyography and skeletal MRI, and in all cases it preceded the diagnosis of myocarditis. None of the patients had scleroderma renal crisis or pulmonary arterial hypertension. Two patients had subclinical presentation, while the other two had signs of heart failure. At presentation, troponin T serum levels were increased in all patients (mean levels: 82±42.11 ng/L), and NTproBNP was slightly raised in three patients (mean levels 317.5±4.6 pg/mL). At 24-hour-Holter-ECG tape, three patients had also arrhythmic abnormalities, mainly mildly increased number of ventricular ectopic beats (VEBs) (mean 113±56/24 hours). CMR unequivocally showed late gadolinium enhancement abnormalities in all cases; Short TI Inversion Recovery (STIR) abnormalities with a non-ischaemic pattern, suggestive for myocardial oedema, were detectable in half of the patients. In two patients, moreover, pericardial effusion was also observed. Once myocarditis was diagnosed, three patients were started on mycophenolate mofetil (MMF) (2 g/day in all cases) and one patient was started on azathioprine 100 mg/day, subsequently reduced to 50 mg/day due to leucopoenia. Steroid pulses (1 g methylprendisolone for 3 days) and then oral steroid therapy were also started in one patient with concomitant myositis relapse. After a median follow-up time of 12 months (range 8–24) in two patients was achieved an optimal disease control. Unfortunately though, in two patients MMF therapy was not able to curb myocardial inflammation. One patient had indeed to stop MMF therapy due to gastrointestinal complains and she could only tolerate low-dose MMF therapy (500 mg/day). After 3 months, she developed arrhythmic complications with frequent VEBs (2370/24 hours) and she is being evaluated for implantable cardiac defibrillator (ICD) implantation. The other patient was also started or rituximab therapy due to poor myocardial, articular and muscular control with only partial benefit.

Our findings emphasised the reported association between skeletal myositis and myocarditis in SSc,[4 5] and the positivity for anti-Ku antibodies seems to strengthen this important clinical relationship. Once confirmed in larger cohorts, this notion could be of great clinical value, since myositis in frequently detected in SSc patients and myocarditis is unequivocally associated with a dismal prognosis.[4 5] Importantly though, myocarditis could be asymptomatic or clinically subtle, as in the two patients of our cohort; thus, it needs to be actively investigated, especially in high-risk patients. An early recognition of inflammatory myocardial involvement is indeed of cardinal importance to allow a prompt therapeutic intervention, thus improving patient's outcome.

Given the concomitant presence of elevated CK in all anti-Ku patients in our cohort, we suggest that not only ILD but also myocarditis, might be a specific feature of the anti-Ku with elevated CK subgroup and that all anti-Ku-positive SSc patients should be actively screened for potential myocardial involvement.

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# Response to: 'Anti-Ku syndrome with elevated CK: association with myocardial involvement in systemic sclerosis' by Campochiaro *et al*

We thank Campochiaro *et al* for their interesting comment[1] on our work in which we used hierarchical clustering on principal components to define clinically meaningful subgroups of patients with anti-Ku antibodies.[2]

Among a bi-centric cohort of patients with systemic sclerosis (SSc), Campochiaro *et al* identified four patients with anti-Ku and retrospectively reviewed these cases.

All patients had increased creatine kinase (CK), three (75%) of whom had interstitial lung disease (ILD). These findings support our observations according to which anti-Ku patients with elevated CK are at risk of ILD.

Of particular interest, Campochiaro *et al* proposed that myocarditis could further represent a specific feature of anti-Ku patients with elevated CK given that all of their four anti-Ku SSc patients had cardiac magnetic resonance (CMR) imaging established myocarditis according to Lake Louise criteria. Two (50%) had heart failure while the remaining two had subclinical presentation. By contrast, in our cohort, one anti-Ku patient had heart failure with positive CMR (2% of all anti-Ku patients and 7% of anti-Ku patients with elevated CK).

Comparability between the Campochiaro *et al*'s study and our study is limited however since: (1) Campochiaro *et al* studied patients with SSc and all of their anti-Ku patients were diagnosed with myositis. The association of these two conditions has been associated with a high risk of myocarditis per se.[3 4] By contrast, only two (5%) of our anti-Ku patients fulfilled the ACR/EULAR criteria for SSc and only one also fulfilled the EULAR/ACR criteria for myositis; (2) Campochiaro *et al* performed CMR in all patients with increased serum troponin T levels, an enzyme whose serum level is increased in myositis patients irrespectively of the presence of myocarditis.[5] By opposition, our patients underwent CMR only when clinical signs of myocarditis were present.

To further address the interesting point raised by Campochiaro *et al*, we conducted an extensive review of the literature. The inclusion criteria were original articles in English pertaining to anti-Ku in which cardiac manifestations were defined and prevalence was directly mentioned or easily calculated from the available data. Pubmed and Web of Science were searched using 'anti-Ku', 'auto-antibodies', 'myositis', 'systemic sclerosis' and 'myocarditis'. Reference lists of relevant papers were also reviewed. Results and ensuing meta-analysis are shown in table 1.

Nine articles were included, reporting the prevalence of cardiac involvement in a total of 198 anti-Ku patients with huge variations (0% to 100%). The meta-analysed prevalence of cardiac involvement in anti-Ku patients was 23% (95% CI 9% to 46%). A significant heterogeneity was also found ($p<0.001$), likely resulting from the heterogeneous screening and definition

**Table 1** Prevalence of cardiac involvement in patients with anti-Ku autoantibodies and controls

| First author, year of publication | Studied population | Patients assessed for heart involvement, n | Definition for heart involvement | Prevalence of heart involvement, n/total (%(95% CI)) | | Risk of heart involvement, OR (95% CI) |
|---|---|---|---|---|---|---|
| | | | | Anti-Ku patients | Control patients | |
| Parodi, 1989[7] | Any CTD | 3 | Abnormal ECG, echocardiogram, chest X-ray film, (depending on the patients) | 1/3 (33) | No control group | – |
| Hausmanova, 1997[8] | Myositis | 50 | Palpitation | 3/7 (43) | 18/43 (42) | 1.04 (0.21 to 5.24) |
| Rozman, 2007[9] | SSc | 52 | Palpitation or conduction block or abnormal diastolic function or reduced ventricular ejection fraction* | 3/14 (21) | 8/38 (21) | 1.02 (0.23 to 4.57) |
| Rodriguez-Reyna, 2011[10] | SSc | 60 | LVEF <45% or pericarditis by echocardiogram or CMR, or arrhythmia requiring treatment, or conduction defect | 3/6† (50) | 4/54‡ (7) | 12.50 (1.88 to 83.3) |
| Lakota, 2012[11] | Any CTD | 73 | Palpitations, conduction blocks, abnormal diastolic function | 14/73 (19) | No control group | – |
| Cruellas, 2013[12] | Myositis | 222 | Myocarditis or heart failure, as revealed by myocardial scintigraphy and echocardiogram examination | 0/9 (0) | 0/213 | 1.00 (0.00 to 21163) |
| Kaji, 2014[13] | SSc | 127 | Clinical evidence of symptomatic pericardial effusion, congestive heart failure, or an arrhythmia considered to be due to SSc requiring treatment | 8/40 (20) | 16/87 (18) | 1.11 (0.43 to 2.86) |
| Spielmann, 2019[2] | Any CTD | 42 | Clinical congestive heart failure and positive CMR | 1/42 (2) | No control group | |
| Campochiaro, 2019[1] | SSc | Not reported | New onset cardiac signs and/or symptoms, raised troponin T and/or NTproBNP and positive CMR | 4/4 (100) | No control group | – |
| *Meta-analysis*§<br>-All studies, test of heterogeneity: p<0.001 ($I^2$=81.8%, $\tau^2$=1.91, H=2.34)<br>Controlled studies, OR test of heterogeneity: p=0.226 ($I^2$=29.3%, $\tau^2$=0.29, H=1.19) | – | – | – | **22.7 (9.2 to 46.0)**<br>**22.4 (14.4 to 33.1)** | –<br>**8.5 (1.4 to 37.6)** | –<br>**1.60 (0.66 to 3.87)** |

Result of meta-analysis are in bold.
*For each definition, the highest prevalence reported was taken into account.
†Sample numbers are derived from the 10% prevalence in the whole cohort.
‡Sample numbers are derived from the 90% prevalence in the whole cohort).
§Result of the random effect (with a constant continuity correction of 0.5 for analysis of proportions and "treatment arm" continuity correction for pooling ORs).
CMR, cardiac magnetic resonance; CTD, connective tissue disoders; LVEF, left ventricular ejection fraction ;SSc, systemic sclerosis .

used for cardiac involvement; and/or from the heterogeneity of the studied populations.

Five studies were controlled (representing a total of 76 anti-Ku patients vs 435 anti-Ku negative patients). The meta-analysed risk of cardiac involvement was not significantly increased in anti-Ku patients vs anti-Ku negative patients (OR 1.60 (95% CI 0.66 to 3.87)).

The important comments of the Campochiaro *et al* study together with the above data highlight several crucial unmet needs for myocarditis in connective tissue diseases patients, namely:

- There is no widely accepted definition of cardiac involvement. Notably, the authors of the Lake Louise criteria warned that CMR criteria for myocarditis are based on expert consensus in light of the limited evidence of its performance compared with endomyocardial biopsy.[6]
- The screening strategies as well as definition for cardiac involvement are heterogeneous among centres.
- There is a need for identifying biomarker(s) of cardiac involvement of which auto-antibodies could be useful toward this aim.
- The prognosis of patients with subclinical CMR myocarditis is currently unknown and whether such patients benefit from increased immunomodulation (vs its potential risks for the patient) is unanswered.

Future research agendas should address these points.

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# Correspondence on 'Standardisation of myositis-specific antibodies: where are we today?'

We have read with great interest the recent article from Espinosa-Ortega *et al*[1] and the commenting letter by Mahler *et al*[2] on the reliability of line immunoassay (LIA) versus immunoprecipitation (IP) in the detection of myositis-specific autoantibodies (MSAs) for the diagnosis of idiopathic inflammatory myopathies (IIM). Even if the search for MSA by dot immunoassay (DIA) or LIA has been used for over a decade and represents a 'non-criteria' test to assist clinicians in IIM diagnosis, MSAs have been included in classification criteria only for a couple of years.[3] However, initial studies on the clinical utility of the DIA/LIA methods in diagnosing IIM were conducted on a restricted panel of MSA and mainly on selected IIM patients.[4]

Only recently have real-life studies on larger series of patients and using a larger panel of MSA-related antigens been published, highlighting on one side a great intra-method analytical variability of DIA/LIA in detecting MSA,[5] and on the other side their weak correlation with IP.[6] Considering the controversial data between Espinosa-Ortega's[1] and Cavazzana's[7 8] studies, it is emblematic that there is still no concordance among LIA and IP even for anti-Jo1, the most common MSA and the first discovered in this group of diseases. The low agreement of IP versus other methods, evidenced by recent studies,[2 6 7 9] raises the question of whether IP should still be considered the reference method for detecting MSA.

Another study that has opened Pandora's box is Vulsteke's[10] which compared three different DIA/LIA assays showing significant differences in diagnostic performance which, however, varied according to the MSA considered. This great variability clearly demonstrates the urgent need to harmonise methods, and that their clinical validation against the reference IP method remains an issue.[8 9] In addition, studies conducted so far are retrospective, including patients diagnosed using previous criteria,[11] and mostly performed on cohorts in which there was a very small number of some MSA, representing a further bias for comparative analysis.[12 13] Nevertheless, today some tools have emerged that may help to improve the specificity of MSA detection by DIA/LIA and to confirm the diagnosis of MSA-associated IIM (table 1). Among these tools, our group has observed the importance of the agreement between DIA/LIA results and a compatible HEp-2 IIF pattern, showing a concordance of around 50% in IIM patients.[14] Recently, Piette *et al* have confirmed these data, suggesting caution in interpreting the results in case of low-positive MSA signal intensity.[5] This may be due to a cut-off that is not well set in some cases, since combining different antigens in a single assay may produce suboptimal performance for each MSA. Creating MSA-related cut-off values could help to improve this issue. Moreover, since MSA are usually mutually exclusive, the simultaneous detection of two or more MSA might indicate possible false-positive results and the need for MSA positive findings to be confirmed by another method.

Even if DIA/LIA are promising MSA-detection technologies, their use in IIM diagnostics is still a challenge. Prospective and multicentre studies are needed to validate these new methods and clarify whether they can be reliably used instead of the reference IP method.

**Table 1** Tools to improve specificity of MSA detection by dot blot (DIA) or line immunoassay (LIA)

| | Study |
|---|---|
| HEp-2 IIF pattern compatible with myositis-specific antibodies detected by DIA/LIA | Picard *et al*[15]<br>Aggarwal *et al*[16]<br>Infantino *et al*[17]<br>Infantino *et al*[14] |
| High signal intensity of DIA/LIA measured by densitometric quantitation | Cavazzana *et al*[7]<br>Bundell *et al*[18]<br>Lecouffe-Desprets *et al*[19] |
| No coexisting MSAs (ie, isolate antibody reactivity) | Infantino *et al*[14]<br>Lega *et al*[20] |
| MSA positivity confirmed by another method (immunoenzymatic or fluoroimmunoenzymatic method, chemiluminescence, immunoprecipitation) | Cavazzana *et al*[8]<br>Damoiseaux *et al*[6] |

DIA, dot immunoassay; LIA, line immunoassay; MSA, myositis-specific autoantibody.

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# Response to: 'Comment on: standardisation of myositis-specific antibodies: where are we today?' by Infantino *et al*

We agree with the notions made by Infantino *et al*[1] in their reply to our previously published report[2] concerning the need for multicentre studies to obtain large enough number to validate new methods for detection of myositis-specific antibodies (MSA) and myositis-associated autoantibodies (MAA). We also agree with the suggestion to evaluate the possibility to individualise reference ranges (cut-off values) for the individual autoantibodies in multi-autoantibody assays like line immune assays (LIA). In relation to such proposals, we would like to stress our experience that the same LIA might yield very quantitatively divergent results in different laboratories, for example, due to differences in laboratory temperature[3] but certainly also other factors, and that these quantitative differences may result in qualitatively divergent results. One way to help standardisation of laboratory results might be to include quantitative internal controls for the individual autoantibodies included in the LIAs, as we have discussed before.[4] Multicentre studies on the evaluation of LIAs or other methods for detection of MSA and MAA should preferably be combined with use of such common internal controls in the participating laboratories.

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## Novel NLRP12 variant presenting with familial cold autoimmunity syndrome phenotype

We read with interest the paper by Ter Haar *et al* describing 187 patients with undefined autoinflammatory syndromes, some of whom had variants of unknown significance (VOUS) in the known genes.[1] Patients with genetic mutations had a higher frequency of family history of similar disorder, suggesting that these variants may have some role to play.

We report a 4-year-old girl who presented with familial cold autoinflammatory syndrome-2 (FCAS2) phenotype and a novel mutation in leucine-rich repeat (LRR) domain of the nucleotide oligomerization domain (NOD)-like receptor protein 12 (NLRP12).[2 3]

This child had episodic fever each lasting a fortnight and occurring once or twice a month. It was associated with recurrent, watery, non-infective diarrhoea since birth. Stools were occasionally admixed with blood. Colonoscopy was normal but biopsy showed cryptitis. She had several infections in childhood: one probable meningitis, one pneumonia and two episodes of subcutaneous abscesses. She had developed additive arthritis of large joints (knees, shoulders and elbows). Arthritis had a relapsing and remitting course. At 1.5 years of age, she was noticed to have sensorineural hearing loss (SNHL). She was borne out of a second-degree consanguineous marriage, her mother had a history of stillbirth in the previous pregnancy.

The child's height and weight were below the fifth centile. She had bilateral non-tender cervical lymphadenopathy and mild hepatosplenomegaly. Labs revealed neutrophilic leucocytosis, thrombocytosis and elevated acute phase reactants during febrile spells, with moderate microcytic hypochromic anaemia. Numerous cultures of blood, urine and stool were sterile.

The symptom complex of periodic fever, arthritis, sterile colitis and SNHL was consistent with cold associated autoinflammatory syndromes, although the attacks were not precipitated by cold in this child. Recurrent infections brought in the possibility of an associated underlying primary immunodeficiency (PID), which has been reported with NLRP12 defects.

Though workup for PID was negative (normal immunoglobulins, lymphocyte subsets, dihydrorhodamine assay for neutrophil oxidative burst, and baseline and lipopolysaccaride-stimulated production of tumour necrosis factor in whole blood cultures). Whole exome sequencing revealed a novel mutation in the exon 9 of the NLRP12 gene (c.54299276T>C:r.2935a>g:p.Ser979Gly) on chromosome 19. Sanger sequencing confirmed the presence of this homozygous variant in proband and parents were found to be heterozygous for this mutation (figure 1).

On follow-up, arthritis responded to naproxen but the fever persisted. Due to non-availability of interleukin-1 inhibitor, she was treated with prednisolone, with which she has good response. Meanwhile, her mother had another child for whom prenatal diagnosis showed no NLRP12 mutation.

The p.S979G mutation has not been reported so far. In silico prediction tools, MutationTaster and PolyPhen-2, suggested that this variant is probably damaging to the protein function. This missense variant alters a conserved residue in the protein function. In silico studies on the effect of this mutation to protein structure were inconclusive. This mutation in exon 9 possibly interferes with Pathogen Associated Molecular Pattern recognition leading to both autoimmunity and susceptibility to infection. Overall, p.F402L accounts for more than half of the cases of FCAS2.[4 5] p.H304Y has been reported in association with Common Variable Immunodeficiency. Most of the variants have been reported in the NOD domain.

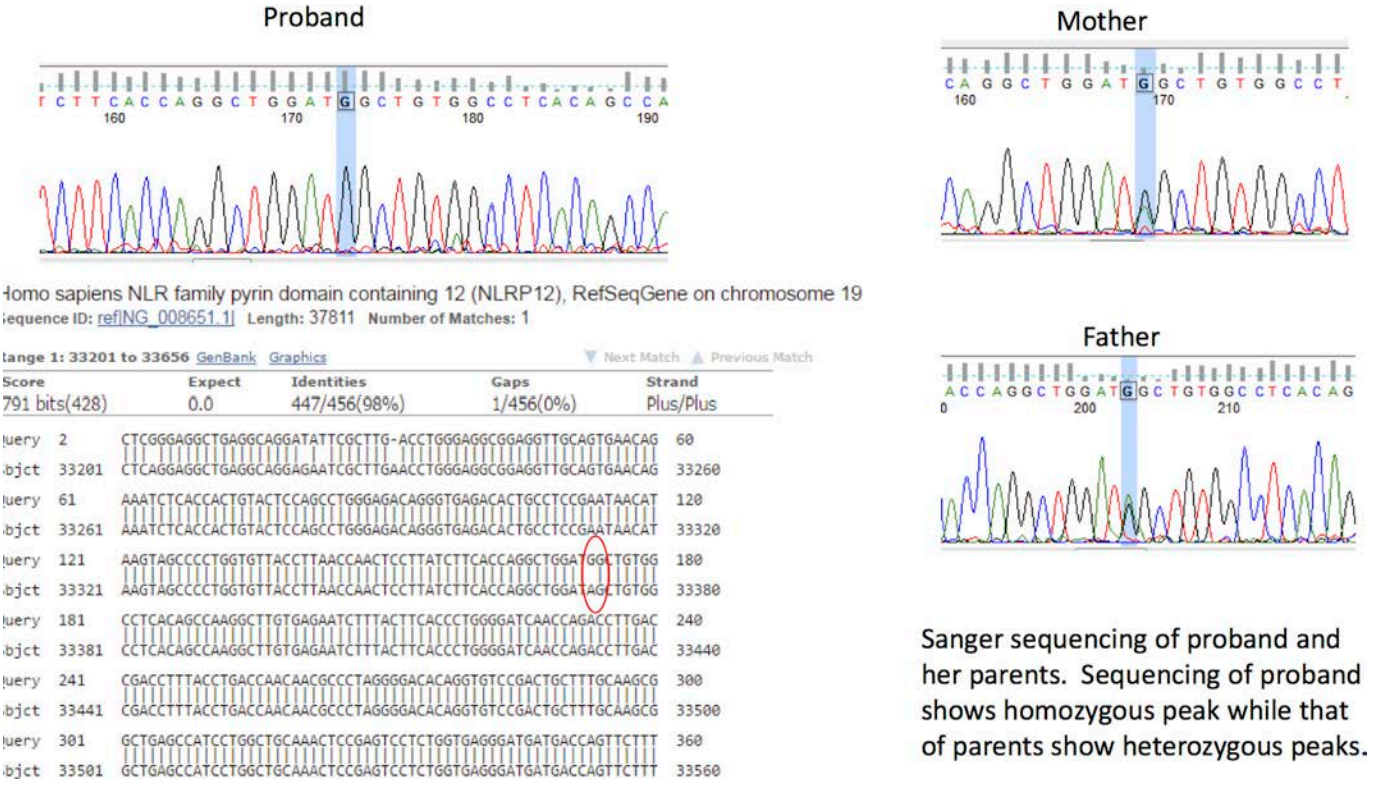


**Figure 1** Representative Sanger sequencing results of the patient compared with those of parents. NLR, nucleotide oligomerization domain-like receptor.

There are still some caveats, such as homozygous variants are not known in this disorder nor have a mutation in LRR region been described. In view of these facts, the variant was classified as VOUS. These variants need to be tested in vitro to know their pathogenic significance. Widespread availability of whole exome sequencing is likely to identify many more such variants and in patients with atypical autoinflammatory syndrome, they may have significance.

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# Response to: 'Novel NLRP12 variant presenting with familial cold autoimmunity syndrome phenotype' by Gupta *et al*

We read with great interest the letter by Aggarwal.[1] Her case is one of early-onset autoinflammatory disease.

Her case is not directly similar to any of the 187 cases we described in our paper on undefined autoinflammatory diseases.[2]

Given the finding of novel potentially pathogenic NLRP12-variants in homozygosity, we think the authors have most likely identified a hitherto unknown cause of this child's symptoms. As such, we recommend the case with all its details to be submitted for publication in a scientific journal.

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# How to use the Lupus Low Disease Activity State (LLDAS) in clinical trials

Recently, two different studies[1 2] applied the criteria of the Lupus Low Disease Activity State (LLDAS)[3] to the data sets of the BLISS-52[4] and BLISS-76[5] phase III trials of belimumab. The studies reported similar LLDAS attainment frequencies at key time points; however, not identical. We herein discuss possible explanations in order to guide future usage of the LLDAS.

In the study by Parodis *et al*,[1] LLDAS at week 52 was achieved by 10.0% of the patients in BLISS-52 and 7.1% in BLISS-76, with a greater percentage within patients who received belimumab 10 mg/kg compared with patients who received placebo in BLISS-52 (11.9% vs 6.2%; p=0.030), but not in BLISS-76 (8.3% vs 6.4%; p=0.473). In the study by Oon *et al*,[2] LLDAS at week 52 was attained by 12.5% and 14.4% in the belimumab 10 mg/kg arm versus 5.8% and 7.8% in the placebo arm in BLISS-52 (p=0.02) and BLISS-76 (p=0.04), respectively.

The small-scale differences in the two studies can be traced to the criteria used for the retrospective calculation of the LLDAS (table 1). Comparing the two setups, Parodis *et al* did not rely on British Isles Lupus Assessment Group (BILAG) evaluations claiming that the rationale for the development of LLDAS included the notion that the highly detailed BILAG index renders its use cumbersome in everyday practice.[3] In contrast, Oon *et al*[2] used BILAG information collected as a part of the BLISS studies. More specifically, Oon *et al* defined criterion 1 as (i) a Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) score≤4, (ii) zero score in the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA)-SLEDAI items seizures, psychosis, organic brain syndrome, visual disturbance, cranial nerve disorder, lupus headache, cerebrovascular accident, vasculitis, urinary casts, haematuria, proteinuria, pyuria, pleurisy, pericarditis and fever, and (iii) BILAG D or E only for the neurological, renal and cardiac systems, with no evidence of active haemolysis. The same study defined criterion 2 as (i) no new SELENA-SLEDAI items with score>0 and (ii) no BILAG items with new activity, irrespective of organ system, while Parodis *et al* defined criterion 2 as no new moderate or severe flare according to the SELENA-SLEDAI Flare Index (SFI).[6]

In fact, neither absence of new flares based on the SFI used by Parodis *et al* nor BILAG D/E scores used by Oon *et al* are validated interpretations of the LLDAS criterion 2. In retrospective settings, such assumptions may be inevitable, especially since the initial publication of the derivation of LLDAS[3] does not clarify what one should consider 'new features' of SLE disease activity. The discrepancies in the two studies underscore the importance of prospective evaluation of the LLDAS where possible. In prospective settings, no new lupus disease activity could be a clinician-assessed item in the case report form.

Despite the similar results in the two studies, the authors emphasised different points. Oon *et al* stressed the discriminatory performance of LLDAS when the belimumab and placebo arms were compared, while Parodis *et al* discussed the overall low LLDAS attainment rates. Indeed, a stringent outcome might be advantageous in trials of powerful therapies where the active treatment could generate higher rates of LLDAS achievers compared with the BLISS trials, but low attainment rates across all arms might impede approval by drug regulatory agencies despite adequate separation between the active substance and placebo arms.

The LLDAS was designed to reflect low SLE disease activity rather than changes in lupus activity; it can, therefore, be considered a more clinically relevant outcome in SLE studies compared with the SLE Responder Index (SRI)-4, and should preferably be assessed prospectively. Perhaps the time has come to put forth a standardised approach to the calculation the LLDAS components to ensure uniformity across both prospective and retrospective studies.

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**Table 1** Definitions of the LLDAS criteria used in the two *post hoc* studies

| Parodis *et al*[1] | Oon *et al*[2] |
|---|---|
| 1. SLEDAI-2K score≤4 with no activity in the renal descriptors (proteinuria, pyuria, haematuria and cellular casts), no pleurisy, no pericarditis and no fever. | 1. SLEDAI-2K score≤4 with no activity in major organ systems (renal, central nervous system, cardiopulmonary, vasculitis an fever) and no haemolytic anaemia or gastrointestinal activity. |
| 2. No new features of SLE activity, defined as no new moderate or severe flare according to the SELENA-SLEDAI Flare Index compared with baseline. | 2. No new features of lupus disease activity, defined as no new SELENA-SLEDAI item score>0 and no new BILAG activity compared with the previous assessment. |
| 3. SELENA-SLEDAI PGA score≤1 (scale: 0–3). | 3. SELENA-SLEDAI PGA score≤1 (scale: 0–3). |
| 4. Daily prednisone or prednisone equivalent dose≤7.5 mg. | 4. Current prednisone equivalent dose≤7.5 mg/day. |
| | 5. Standard maintenance dosages of immunosuppressive drugs and approved biologic agents. |

All criteria had to be met for attainment of LLDAS.
. BILAG, British Isles Lupus Assessment Group; LLDAS, Lupus Low Disease Activity State; PGA, physician's global assessment; SELENA, Safety of Estrogens in Lupus Erythematosus National Assessment; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000.

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# Drug-induced systemic lupus erythematosus: should immune checkpoint inhibitors be added to the evolving list?

Arnaud and colleagues used WHO's VigiBase, an international spontaneous reporting system, to compile an updated list (19 March 2018) of drugs suspected to be implicated in drug-induced systemic lupus erythematosus (SLE), an evolving clinical entity.[1] They analysed 12 166 reports of drug-induced SLE and identified 118 suspected drugs with pharmacovigilance signal, which were reported in 8163 cases, mainly occurring in women (81%, 49 years as median age), defined as serious (55%), with a median onset of 172 days. Of note, 76 drugs (64.4%) were already known to cause SLE in the literature, including anti-TNF agents (infliximab received the highest number of reports), procainamide and hydralazine (receiving the highest disproportional reporting).

Our attention was drawn to the lack of pharmacovigilance signal for immune checkpoint inhibitors (ICIs), emerging oncological drugs recently associated with a unique and distinct spectrum of side effects, the so-called immune-related adverse events (irAEs), virtually affecting any organ or tissue, with rheumatic manifestations including arthralgia/arthritis, myalgia/myositis, polymyalgia rheumatica, rheumatoid arthritis and Sjögren's syndrome.[2 3]

Therefore, we analysed the FDA Adverse Event Reporting System (FAERS) to verify whether SLE is reported with ICIs, and characterise relevant cases in terms of severity (eg, hospitalisation), mortality (death reported as outcome), onset time (in relation to ICI regimen), concomitant drugs known to cause SLE and coreported irAEs.[4] Among 4870 rheumatic events (arthralgia, n=711), SLE was reported in 18 cases (as of June 2018), plus 7 cases of cutaneous SLE (two recorded as subacute), 2 cases of lupus-like syndrome and 1 case each for lupus nephritis and central nervous system lupus. Among 18 cases of SLE, only inhibitors of programmed cell death 1 or its ligand (PD1/PDL1) were reported: nivolumab was the suspect ICI in 12 cases, followed by pembrolizumab (4 cases), avelumab and atezolizumab (one each). Mean age was 61 years, with female:male ratio of 1.6; hospitalisation was recorded in four cases, with only one death. The median onset time (calculated for eight cases with available information on event date and start of therapy) was 196 days. Notably, no anti-TNF drugs, procainamide or hydralazine were recorded among concomitant drugs; SLE was the only adverse event recorded in 10 cases, and arthralgia, arthritis and other rheumatic events co-occurred in only 2 cases.

These findings are partially in line with Arnaud and colleagues and open a question on whether ICIs should be added to the list of drug-induced SLE. We hypothesised that ICIs did not emerge with a pharmacovigilance signal from WHO's VigiBase because of potential drug-related and event-related competition bias; that is, the substantial over-reporting of SLE with hydralazine and procainamide, together with the large reporting of irAE with ICIs other than rheumatic events, might have masked the ability to detect disproportionality for events with low reporting rate.[5] The most intriguing and unexpected finding from FAERS is that SLE with ICIs does not appear to co-occur with other irAEs, especially rheumatic events, with very low fatality rate and delayed onset (more than 6 months).[6] Although ICI-related SLE appears rare, the increasing uptake of ICIs in clinical practice strengthens the importance of (1) real-time monitoring of pharmacovigilance databases, such as FAERS and WHO's VigiBase; (2) maintaining awareness and long-lasting vigilance by immunologists, rheumatologists and oncologists of this evolving drug-induced clinical entity. The awaited EULAR recommendations, together with accurate reporting of rheumatological irAEs with ICIs, will increase our understanding and relevant confidence of rheumatologists about mechanistic basis, drug-related and patient-related risk factors, as well as optimal management especially in patients with pre-existing autoimmune diseases.[7–10]

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# Aortic dilatation in a patient with Takayasu arteritis treated with tocilizumab

In recent years there has been growing interest in the use of tocilizumab for the treatment of large vessel vasculitis. Although the primary endpoint (time to relapse) was not met in the first randomised, placebo-controlled trial evaluating the efficacy and safety of tocilizumab in patients with refractory Takayasu arteritis, the results suggested favour for tocilizumab over placebo without new safety concerns.[1] In this journal, three cases of Takayasu arteritis progression during tocilizumab treatment have been described.[2 3] We report one additional patient with disease progression despite tocilizumab therapy.

A 25-year-old woman presented with constitutional symptoms, anaemia of chronic inflammation, elevated erythrocyte sedimentation rate and C reactive protein levels, and imaging evidence of large vessel vasculitis. CT angiography showed vessel wall thickening of the carotid arteries, thoracic descending and infrarenal abdominal aorta and dilatation of the ascending aorta (40 mm). The patient was treated with glucocorticoids (prednisone 1 mg/kg/day) and methotrexate (20 mg/week). While on methotrexate and low-dose prednisone, low-grade fever recurred and inflammatory markers increased. A whole-body fluorodeoxyglucose positron emission tomography (FDG-PET)/CT scanning showed increased FDG uptake of the thoracic and abdominal aorta, prompting a switch to tocilizumab (8 mg/kg/month). With this, symptoms remitted and inflammatory markers normalised. After 6 months, FDG uptake of the aorta normalised as well. The disease remained stable over the following 18 months, when a magnetic resonance angiography showed development of ascending aorta dilation (54 mm) and descending thoracic and infrarenal abdominal aorta stenosis (figure 1). She underwent ascending aorta and proximal hemiarch replacement. Pathology from the surgical specimen demonstrated adventitial fibrosis and mural thickening (figure 2A) with adventitial small vessel vasculitis (figure 2B), consistent with Takayasu aortitis. Infliximab with high-dose steroids was promptly started. One year after surgery, the patient remains in remission on infliximab and prednisone 5 mg daily.

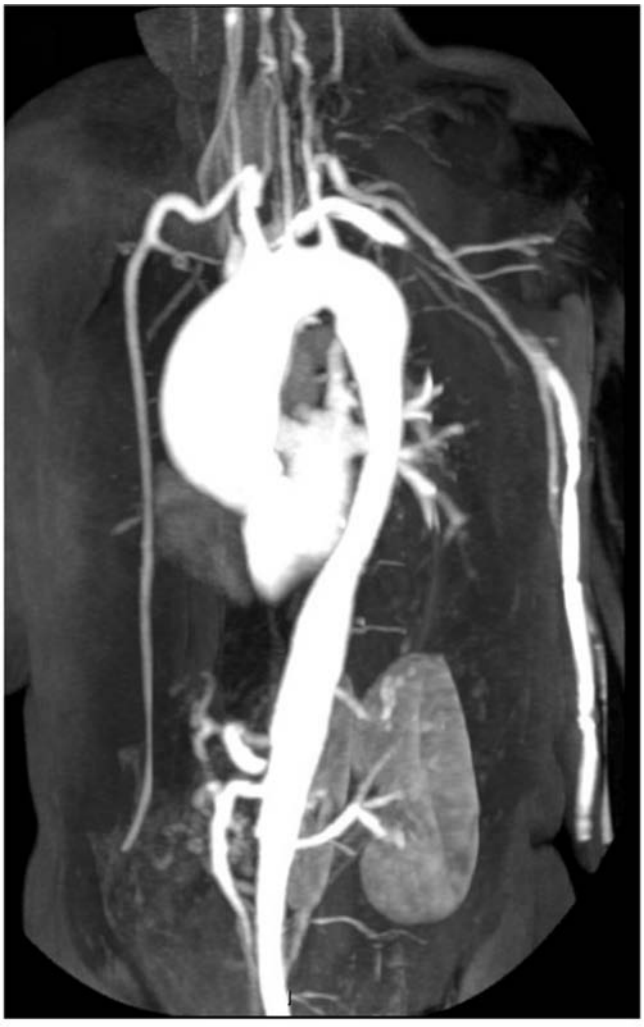

**Figure 1** MR angiography showing ascending aorta dilation and descending thoracic and infrarenal abdominal aorta stenosis.

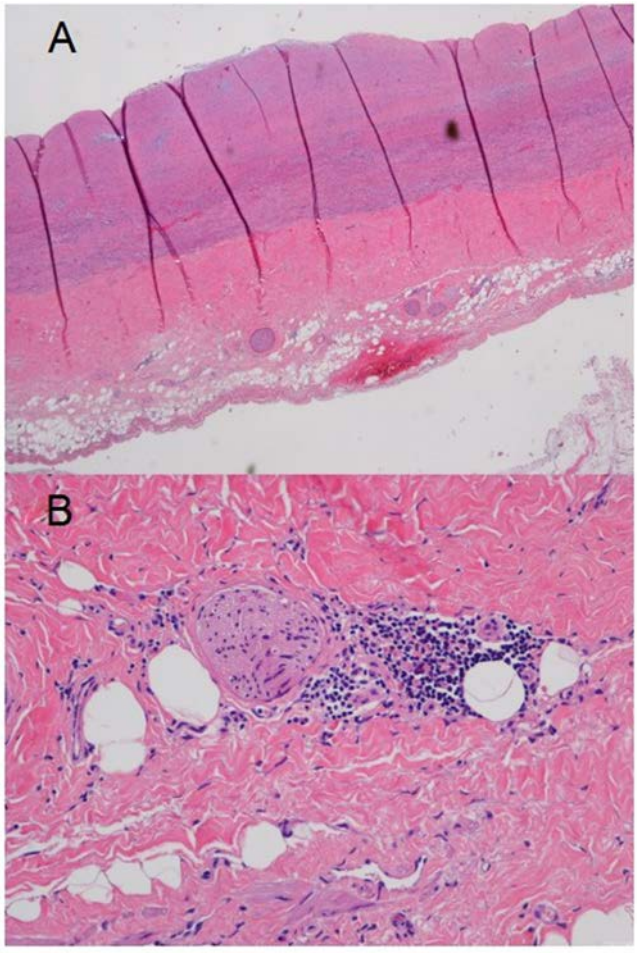


**Figure 2** Histologic features of the ascending aorta showing adventitial fibrosis and mural thickening (A) and adventitial small vessel vasculitis (B).

Assessment of disease activity in Takayasu arteritis is challenging as inflammatory markers often do not correlate with disease activity. Moreover tocilizumab suppresses serum inflammatory markers even in the absence of a clinical response. This case clearly demonstrates that TAK can progress despite normal inflammatory markers, absence of symptoms and FDG uptake at PET/CT scanning, and despite treatment with tocilizumab. Assessment of disease activity in

patients with Takayasu on tocilizumab should rely on a combination of clinical assessments and serial imaging studies.[4]

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