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Review

Pathogenic effects of anti-citrullinated protein antibodies in rheumatoid arthritis – role for glycosylation

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ABSTRACT

The identification in 1998 of the main antigenic substrate recognized by autoantibodies was a dramatic turning point in our understanding of rheumatoid arthritis (RA) biology. Now, two decades later, antibodies to citrullinated proteins are viewed no longer as mere biomarkers for RA, but also as major pathophysiological factors involved in the development of bone loss and joint pain. These pathogenic effects are ascribable to abnormal autoantibody glycosylation via a pathway involving the Th17T cells. In the future, abnormal autoantibody glycosylation may serve as a disease activity biomarker and suggest novel treatment strategies.

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1. Introduction

Two decades have elapsed since anti-citrullinated protein antibodies (ACPAs) were first identified in serum samples from patients with rheumatoid arthritis (RA). In 1998, two research groups demonstrated that the epitope recognized by the autoantibodies found in patients with RA was a peptide sequence that included a distinctive amino acid, citrulline [1,2]. Filaggrin, a protein involved in epidermal differentiation, was the first protein identified as a target of ACPAs [3]. Two years later, the first ACPAs ELISA kit was put on the market. In this first-generation kit, the antigen source was a synthetic peptide that was derived from filaggrin, citrullinized, and cyclized. By modifying the conformation of the peptide, cyclization improved the sensitivity of the assay. Second- then third-generation kits characterized by better diagnostic performance were then developed based on several synthetic cyclic citrullinated peptides that shared no homology with filaggrin and whose exact composition was unknown. The saga of serological markers for RA from their initial identification to their latest developments has been detailed by René-Louis Humbel in a review article published by the *Groupe d'Étude de l'Auto-Immunité* (http://www.geai-lesautoanticorps.fr/34-Revue-GEAI-L-Info).

Patients are now usually tested using a second- or third-generation kit to quantify the antibodies to cyclic citrullinated peptides (anti-CCPs) in serum samples. In France, over 20 different anti-CCP kits are widely used. This heterogeneity, combined with the uncertainty about the nature of the peptides used to detect the autoantibodies, translates into considerable variability in the results. In a nationwide quality-control study consisting in having 342 clinical laboratories in France test serum from an anti-CCP-positive patient, although anti-CCPs were consistently detected, their quantification produced widely variable values ranging from 69.5 to > 3000 U/mL (https://www.ansm.sante.fr/var/ansm_site/storage/original/application/98219eb69a0bb4f1a74da5ed5de6631d.pdf). This result clearly indicates a need for standardization.

After the introduction of second- and third-generation kits offering improved sensitivity and specificity, ACPAs proved to be the most specific markers of RA known to date. Patients with other conditions rarely produce ACPAs, notably in high titers [4]. However, ACPAs are not only useful as clinical tools. Several recent studies suggest that they may be strongly involved in the pathogenesis of at least two manifestations of RA, bone erosions and joint pain. The pathogenic potential of ACPAs has been ascribed to post-translational modifications. More specifically, glycosylation can generate highly pathogenic forms of ACPAs. These recent findings, which are reviewed here, may produce breakthroughs in our understanding of RA.

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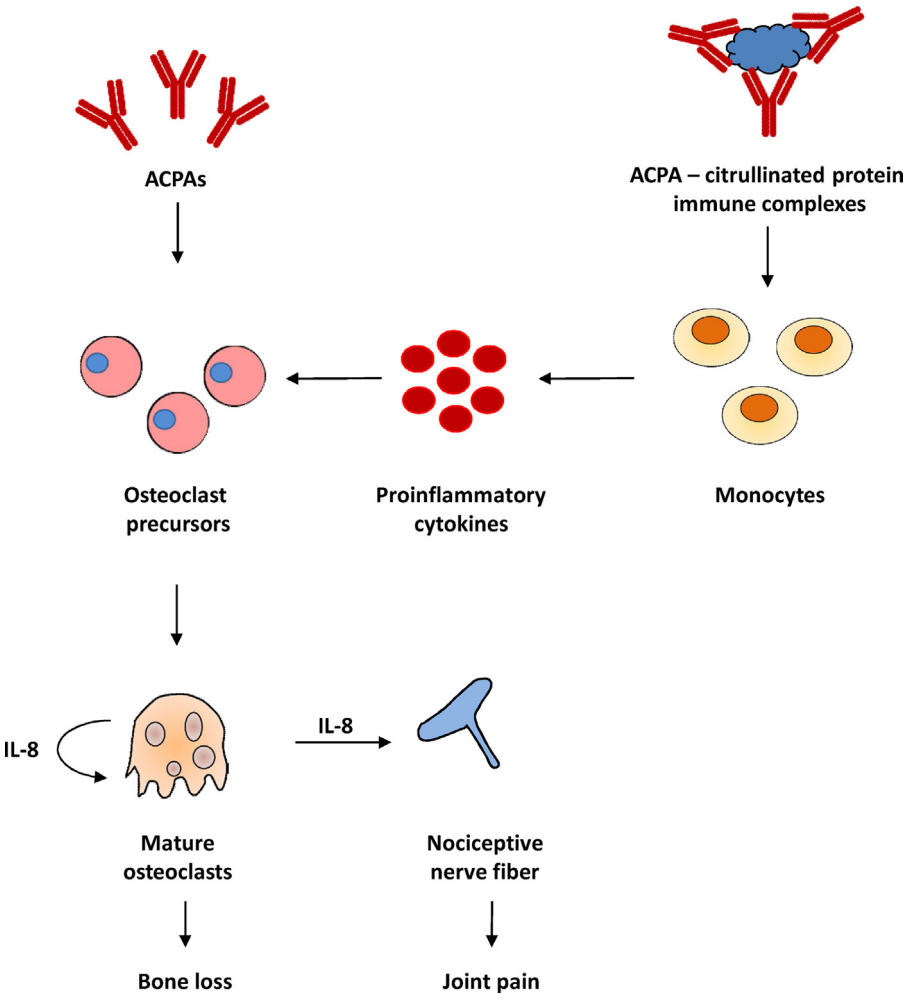

Fig. 1. Mechanisms by which ACPAs induce bone loss and pain ACPAs can induce osteoclastogenesis *via* direct and indirect mechanisms. By interacting directly with citrullinated proteins expressed on the surface of osteoclast precursors, ACPAs stimulate their differentiation into mature osteoclasts. In parallel, the detection by monocytes/macrophages of ACPAs/citrullinated protein immune complexes induces the production of pro-inflammatory mediators that promote osteoclastogenesis. Osteoclast activation by ACPAs involves IL-8, which acts in an autocrine loop to promote osteoclastogenesis and contributes to the development of joint pain by binding to its receptor at the surface of nociceptive nerve fibers.

2. ACPAs: more than biomarkers

ACPAs are highly valuable not only for diagnosing RA, but also for predicting whether undifferentiated arthritis will progress to RA. In addition, the presence of ACPAs is associated with an increased risk of developing bone erosions [5–7], suggesting a causal link between these two features. The first experimental data supporting this possibility were reported in 2012 by Harre et al., who found that purified serum ACPAs from patients with RA induced bone loss with increased osteoclast numbers along bone trabeculae in mice [8].

Two main mechanisms may explain the link between ACPAs and increased bone resorption. One is indirect and involves the ability of ACPAs to form immune complexes with the citrullinated proteins they target. Detection of these immune complexes by Fc receptors at the surface of macrophages induces the release of pro-inflammatory cytokines such as TNFα [9,10], which in turn trigger expression of the receptor activator of NK-κB ligand (RANKL) by mesenchymal cells, thereby promoting osteoclastogenesis [11]. The second possible mechanism is more direct and independent from inflammation. Differentiating osteoclasts express citrullinated proteins, some of which locate to the surface of precursor cells, where they can be targeted by ACPAs. In an in vitro model of osteoclast differentiation from human monocytes, adding ACPAs to the osteoclast precursors promoted the formation of osteoclasts characterized by a high level of functional activation [8].

Although the molecular mechanisms involved in osteoclast activation by ACPAs remain incompletely elucidated, they seem to be dependent on IL-8. IL-8 is not only a potent neutrophil chemoattractant, but is also released during normal osteoclast differentiation and acts in an autocrine loop to stimulate osteoclastogenesis. In vitro, adding ACPAs to differentiating osteoclasts increases the release of IL-8, which stimulates the development of fully functional osteoclasts [12]. This mechanism may operate also in vivo, at least in mice, since IL-8 antagonist treatment prevents the bone loss induced by the administration of ACPAs [12]. Importantly, mice given ACPAs exhibited no microscopic or macroscopic evidence of synovitis.

Thus, surprisingly, IL-8 may strongly influence osteoclast differentiation not only under normal circumstances, but also during stimulation by ACPAs. Bone loss may not be the only type of damage wrought by the malicious ACPAs/osteoclast/IL-8 trio. A study in mice supports a role for ACPAs in producing pain [13]. Mice given ACPAs exhibited decreased sensitivity thresholds to nociceptive, mechanical, and thermal stimuli. This effect was detectable as early as 24 hours after the ACPAs injection and persisted for 28 days. Importantly, no microscopic or macroscopic evidence of inflammation was detectable, demonstrating that the hyperalgesia was

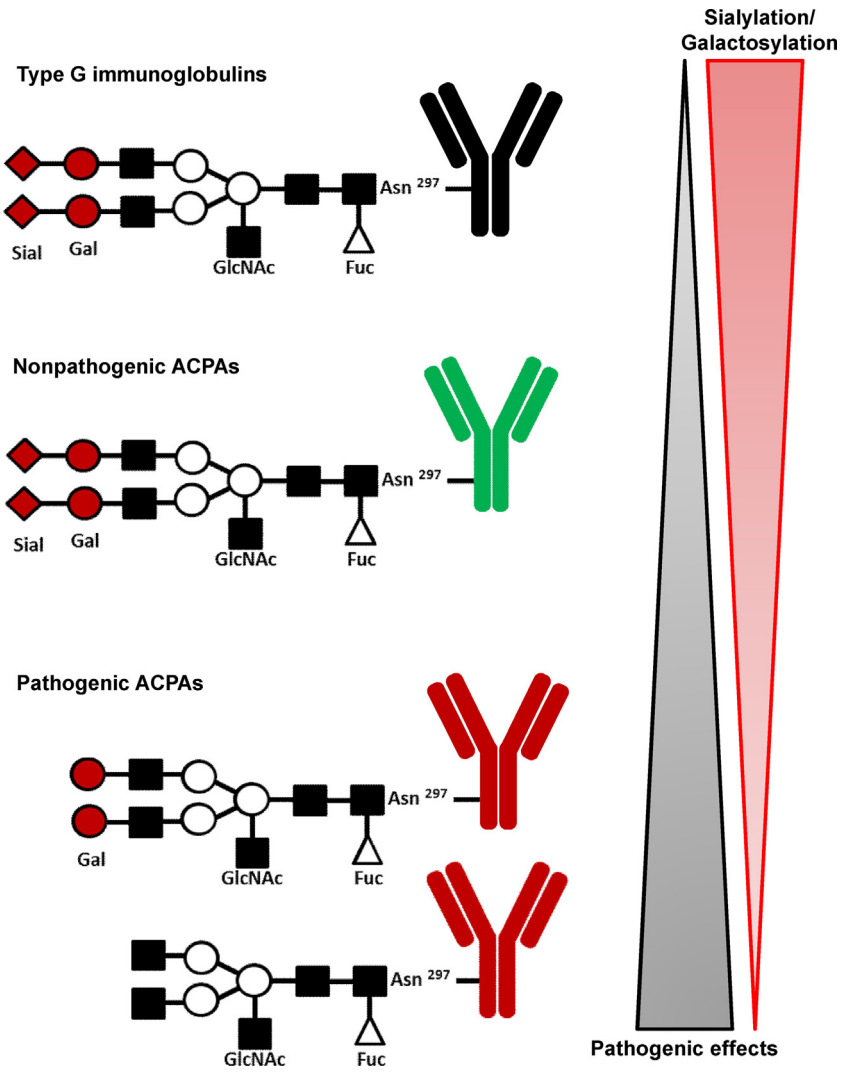

Fig. 2. Glycosylation and pathogenic potential of ACPAs Immunoglobulins have several glycosylation sites, which are located on both the variable and the constant domains. A sugar moiety covalently bound to the asparagine (Asn) at position 297 of the constant domain is composed of a constant heptasaccharide containing N-acetyl-glucosamine (GlcNAc) and mannose (Man), to which fucose (Fuc), galactose (Gal), and sialic acid (Sial) residues are attached. Adequately sialylated and galactosylated ACPAs have no pathogenic effects. In contrast, the presence of hyposialylated and/or hypogalactosylated ACPAs seems to predict progression from pre-clinical disease to chronic inflammation and to promote the formation of mature osteoclasts.

not mediated by ACPAs-induced inflammation. *In vitro,* incubation with ACPAs did not significantly increase the level of primary neuron activation, ruling out a direct effect of ACPAs on the mechanism observed *in vivo*. Whether the IL-8-mediated mechanisms linking ACPAs to osteoclastogenesis contributed to the hyperalgesia was then investigated [13]. In mice, IL-8 injection into the ankle joint induced pain in the ipsilateral paw, and IL-8-antagonist therapy abolished the hyperalgesia induced by ACPAs. These findings are probably ascribable to expression of the IL-8 receptor at the surface of nociceptive neurons.

These new and fascinating findings support a pathogenic effect of ACPAs, thereby suggesting a new pathophysiological model for RA (Fig. 1). In this model, joint pain is related to direct osteoclast activation by ACPAs, leading to the release of IL-8, which both promotes osteoclastogenesis and induces pain.

The body of work that uncovered the ACPAs-dependent pathophysiological pathway also consistently demonstrated the absence of any inflammatory response related to ACPAs. This point is important, as it indicates that ACPAs can exert pathogenic effects very early in the disease process, before the development of chronic inflammation. Joint pain and bone demineralization develop well before synovitis in ACPAs-positive patients. However, it is unclear why ACPAs become detectable 10 years on average before the onset of joint pain [14]. The answer to this conundrum might lie on the fact that only some ACPAs are pathogenic and that glycosylation of these autoantibodies dictates their pathogenicity.

3. Pathogenicity of ACPAs: follow the sugar trail

The post-translational modifications of the proteins targeted by ACPAs are now fairly well documented, and their decisive role in antigen recognition can result in loss of self tolerance; citrullination is the most important among them. Data about post-translational changes undergone by ACPAs, in contrast, are scarce. However, human immunoglobulins (Igs) are proteins that undergo modifications such as glycosylation, which exert a major influence on their biological activity. IgGs have several glycosylation sites in both their variable and their constant domains. In the constant domain, which is involved in effector antibody functions, the asparagine at position 297 of each chain of the fragment crystallizable region (Fc), carries a bi-antennary oligosaccharide composed of N-acetyl-glucosamine and mannose residues. Galactose and sialic acid residues can attach to this sugar moiety (Fig. 2). Several studies have established that glycosylation, and more specifically sialylation,

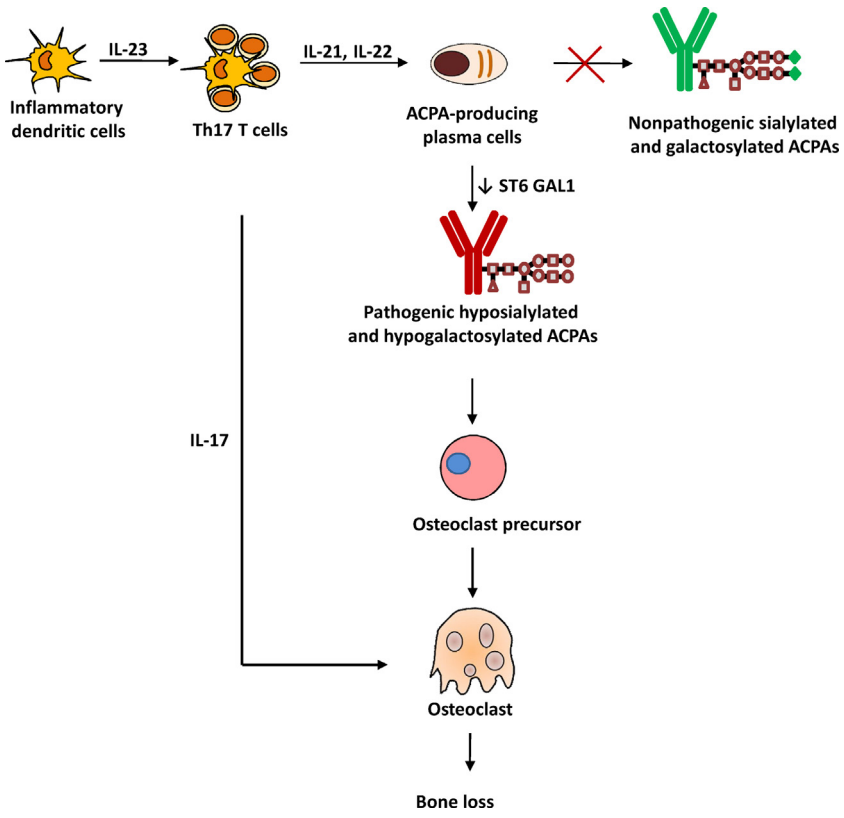

Fig. 3. Regulation of ACPAs pathogenicity by the IL-23/Th17/IL-21/IL-22 axis In RA, Th17 T cells are mainly induced by inflammatory dendritic cells via their ability to release IL-23. Th17 T cells release IL-17, which contributes to the destruction of joint and bone tissues. They also release IL-21 and IL-22, which suppress the expression by plasma cells of the enzyme ST6GAL1 involved in antibody sialylation. In contact with these cytokines, autoreactive plasma cells produce hyposialylated ACPAs capable of activating osteoclasts, thereby promoting bone loss.

modulates the pathogenic effects of some immunoglobulins. Thus, in granulomatosis with polyangiitis (formerly known as Wegener's granulomatosis), hyposialylation modulates the pathogenic effects of anti-PR3 antibodies [15]. However, hyposialylated anti-PR3 antibodies are also markers for disease activity, as they are present during flares, whereas during remissions the anti-PR3 antibodies are heavily sialylated [15].

These data led to formulate the hypothesis that abnormal glycosylation dictates the pathogenicty of ACPAs. This hypothesis was supported by data published in 2010, demonstrating that ACPAs exhibit a specific Fc-linked glycan profile that is distinct from that of other circulating IgGs, and characterized by hyposialylation [16]. Moreover, in several longitudinal studies of patients who had ACPAs but no evidence of RA at baseline, galactosylation and sialylation levels of ACPAs decreased shortly before symptom onset [17–19]. Thus, measuring the galactosylation and/or sialylation levels of ACPAs may predict the risk of progression from pre-clinical disease to chronic inflammatory disease.

Studies in animals also support a major role for altered antibody glycosylation in RA. In a model of RA induced in mice by the administration of antibodies to collagen type II, prior endoglycosidase treatment of the antibodies to remove the sugar moiety attached to asparagine 297 decreased the severity of the induced polyarthritis [20]. Finally, recently reported evidence indicates that the pathogenic effects of ACPAs on bone are related to the nature of the glycosylated chains present on these antibodies. Thus, the pro-osteoclastogenic effect of ACPAs was abolished by adding sialic acid residues to the sugar moiety [21].

This body of clinical and experimental data supports a pathogenic role of ACPAs and a major influence on this role of the nature of the glycosylated chains attached to these antibodies. It suggests the new and exciting concept that ACPAs may be pathogenic only if they are hyposialylated and/or hypogalactosylated. It also raises questions about the mechanisms involved in controlling ACPAs glycosylation. Very recent investigations into these mechanisms uncovered an unexpected link between the pathogenic potential of ACPAs and regulation by Th17 T cells.

4. Th17 T cells and ACPAs: an unexpected link

Ever since T cells were first identified, immunologists have striven to distinguish T-cell subsets based on the nature of the molecules present on the cell surface or released by the cell. These classification efforts provided valuable information on the defense strategies deployed by the adaptive immune system to fight infectious agents. Th1 $CD4^+$ T cells, which release IFNγ and IL-2, proved to be potent members of the armamentarium against intracellular microorganisms. Th2 T cells, which release IL-4, IL-5, and IL-13, demonstrated a pivotal role in controlling extracellular microorganisms. Two studies published in 2005 described a T-cell subset characterized by the ability to release IL-17, a cytokine identified 10 years earlier [22,23]. These cells were therefore designated Th17 $CD4^+$ T cells.

Since then, Th17 cells have been implicated in several mechanisms relevant to the induction and propagation of chronic inflammation in a variety of diseases. Their effects are partly ascribable to the release of large amounts of IL-17, whose harmful effects during inflammation have been well documented [24]. As with all T cells, the differentiation and activation of Th17 cells is controlled by dendritic cells (DCs), which orchestrate the adaptive immune

responses via their unique and fundamental antigen-presenting capabilities. DCs have also been divided into subsets based on phenotypic and functional characteristics. Th17 cell differentiation and activation in RA is chiefly induced by the inflammatory DC subset [25,26]. Inflammatory DCs develop from monocytes recruited to the synovial membrane and are found only under inflammatory conditions. One of their distinctive characteristics is the ability to release large amounts of cytokines, including IL-23, a key factor that orients T-cell differentiation toward the Th17 profile. In contact with IL-23-releasing inflammatory DCs, these cytokines induce the differentiation of Th17 cells and therefore the production of IL-17. By acting on multiple cell types including monocytes, synoviocytes, and osteoclasts, IL-17 induces the prolonged production of pro-inflammatory mediators, thereby contributing to progression toward chronic inflammation and to joint and bone tissue destruction [24].

IL-17 is not the only cytokine released by Th17 cells. Other Th17 cytokines include IL-21 and IL-22, which may be involved in the IL-23/Th17/IL-21/IL-22 axis recognized in 2017 as pathogenic in RA [19]. This axis connects three cell types – inflammatory dendritic cells, Th17 cells, and ACPA-secreting autoreactive plasma cells – in a three-phase process (Fig. 3). In the first phase, monocytes infiltrate the synovial membrane, where they respond to pro-inflammatory signals by differentiating into IL-23-secreting inflammatory DCs. The second phase consists in the differentiation of Th17 cells promoted by inflammatory DCs, which release not only IL-17, but also IL-21 and IL-22. Finally, in the third phase, IL-21 and IL-22 act on ACPA-producing autoreactive plasma cells, which respond by releasing ACPAs that lack sialic acid residues and are therefore pathogenic. Indeed, IL-21 and IL-22 suppress the expression by plasma cells of the enzyme sialyltransferase ST6GAL1 involved in sialylating the ACPA Fc. When these two cytokines are absent, plasma cells express an abundance of ST6GAL1 and consequently produce highly sialylated nonpathogenic ACPAs. By decreasing the expression of ST6GAL1, the IL-23/Th17/IL-21/IL-22 axis causes a shift from normally glycosylated non-pathogenic ACPAs toward inadequately glycosylated pathogenic ACPAs.

These studies unveiling a new pathogenic axis in RA were performed in various murine models. In human ACPAs-positive patients, circulating plasma cells have lower sialic acid contents compared to those from healthy individuals, indicating decreased sialyltransferase activity [19]. Among ACPA-positive individuals with no clinical evidence of RA at baseline, those who developed clinical RA during a 1-year follow-up had lower ACPAs sialylation levels compared to those in the participants who remained free of RA manifestations [19]. Although these preliminary findings need to be confirmed in larger cohorts, they suggest that hyposialylated ACPAs may predict progression from subclinical to clinical RA and perhaps also from remission to relapse.

5. Conclusion

Two decades after their identification, ACPAs should no longer be viewed merely as robust markers for RA. Clearly, ACPAs are not the only factor involved in the pathogenesis of RA. Nonetheless, recent studies highlighting their pathogenic potential have opened a new field of research focused on these autoantibodies. If future research confirms that inadequate glycosylation is highly predictive of progression from asymptomatic autoimmunity to chronic inflammation, tests for inadequately glycosylated serum ACPAs may be devised and used in clinical practice. Recognition of the IL-23/Th17/IL-21/IL-22 axis as strongly involved in modulating the pathogenic effects of ACPAs pave the way for the development of new therapeutic strategies. The development of drugs that affect this axis in order to directly or indirectly restore ST6GAL1 may hold promise. Existing drugs targeting this axis, but used at an earlier stage of the disease or for remission maintenance might also prove useful. The field of investigation of abnormal autoantibody glycosylation in RA is expanding steadily. The recent discovery of abnormal sugar moieties on Igs from ACPA-negative patients with RA suggests that new biomarkers of use for clinical rheumatology may emerge soon [27].

Disclosure of interest

The authors declare that they have no competing interest.

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