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Rheumatoid arthritis

Epitopes targeted by autoantibodies in presymptomatic individuals predict early rheumatoid arthritis

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ABSTRACT

Objectives: To determine anticitrullinated protein antibody (ACPA) responses to novel peptides predicting the clinical outcomes of treatment-naïve early rheumatoid arthritis (RA) in the presymptomatic stage.

Methods: We analysed monoclonal ACPAs derived from RA patients, including a characterised protective ACPA (clone E4), along with plasma samples collected from 520 presymptomatic individuals, of whom 244 were also sampled at diagnosis of RA, and 530 population controls in Sweden. The validation cohort (The Nordic Rheumatic Diseases Strategy Trials and Registries, NORD-STAR) consisted of 690 treatment-naïve early RA patients. Responses to citrullinated or native alpha-enolase (ENO1) or peptidylarginine deiminase 4 (PAD4) peptides were analysed by bead-based multiplex flow immunoassay. Clinical outcomes included C-reactive protein (CRP) and the 28-joint disease activity score (DAS28) with its components: tender joint count (TJC), swollen joint count (SJC), and erythrocyte sedimentation rate (ESR).

Results: Monoclonal ACPAs displayed distinct binding patterns to ENO1 and PAD4 peptides. A time-dependent increase of ACPA response to citrullinated peptides was observed in the presymptomatic stage towards onset. In the presymptomatic (0.2-5 years before onset) and early RA stage, ACPA responses to several ENO1 and PAD4 peptides were associated with less severe RA, assessed as lower levels of CRP and DAS28 and its components. In early RA, the association was more pronounced in rheumatoid factor (RF)-negative patients based on lower SJC. In presymptomatic individuals, ACPA responses widely predicted lower disease activity in early RA and were more pronounced in 5 selected peptides.

Conclusions: Antibody responses to certain citrullinated epitopes are associated with lower disease activity in treatment-naïve early RA and appear years before symptom onset of RA.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Promiscuous anticitrullinated protein antibodies (ACPAs) are common in rheumatoid arthritis (RA) and are specific for the citrulline side chain.
- A subset of ACPAs is protective in RA.
- ACPAs are associated with a lower number of swollen joints in early RA.

WHAT THIS STUDY ADDS

- ACPA responses to certain citrullinated epitopes of alpha-enolase (ENO1) and peptidylarginine deiminase 4 (PAD4) are associated with lower disease activity in early RA.
- In presymptomatic individuals, ACPA responses resembling the fine specificity of protective ACPAs predict disease activity at the early RA stage.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- A group of ACPA responses associated with a protective phenotype from the presymptomatic stage to early RA calls for an update on the clinical interpretation of the serological markers.
- Determination of ACPA fine specificities could supplement the clinical anti-second-generation cyclic citrullinated peptide (anti-CCP2) or anti-rheumatoid factor (RF) test to increase the precision of RA diagnosis and the accuracy of prediction of disease activity.

INTRODUCTION

Certain autoantibodies, such as anticitrullinated protein antibodies (ACPAs) and rheumatoid factors (RFs), are present in the circulation in individuals years before the onset of rheumatoid arthritis (RA) [1]. Their repertoire and concentration increase as the disease onset approaches [2]. The typical ACPA, denoted promiscuous ACPA, is highly specific for citrulline side chains exposed on a protein surface but has limitations in binding due to negative interactions of surrounding side chains within the epitopes [3,4]. This leads to a highly restricted detection of citrullinated epitopes *in vivo* but with a larger binding spectrum *in vitro*, especially for the cyclic peptide structure with more accessible citrulline in the centre for antibody binding, as in the anti-second-generation cyclic citrullinated peptide (anti-CCP2) assay [5,6].

The anti-CCP2 assay has shown that promiscuous ACPAs have high specificity (>98%) and sensitivity (>70%) for RA [7]. For a long time, it has, therefore, been generally assumed that these antibodies are pathogenic, and pathogenicity has been observed when tested using monoclonal or polyclonal ACPAs *in vitro* [8–11]. Lately, however, several monoclonal promiscuous ACPAs have been shown to protect against experimental arthritis [5,12,13]. In our recent study, an ACPA clone, denoted E4, was able to protect against arthritis by forming immune complexes with alpha-enolase (ENO1) and interacting with the downstream inhibitory receptor FCGR2B on macrophages, inducing a regulatory phenotype [5]. Shortly afterwards, it was shown that the presence of ACPAs, together with the absence of RF in early RA, was associated with fewer swollen joints and lower systemic inflammation [14]. Therefore, it is important to be able to utilise certain epitopes to detect a set of ACPA responses in connection with the protective function and to investigate any predictive value for clinical outcomes in the early or even presymptomatic stage of RA [15].

ENO1 is a glycolytic enzyme present in a large number of cell types and tissues. Its function depends on localisation; for example, it can serve as a tumour suppressor when present in the nucleus or a plasminogen receptor when located on the cell surface [16]. In addition, it has been shown that ENO1 protein is highly expressed in monocytes/macrophages and excessively present in RA synovial fluid [17]. Although the exact function of citrullinated ENO1 in RA is still unclear, its diagnostic and predictive potential in RA remains a topic of interest [18], especially with new evidence showing the formation of the immune

complex of protective ACPAs and ENO1 [5]. In RA, the epitope "peptide 1" of citrullinated ENO1 (CEP1) can be recognised by 37% to 63% of RA patients, making it one of the most detected epitopes [18]. However, the amino acid sequence of human ENO1 consists of 17 arginines, whereas CEP1 only encompasses the first 2 arginines from the N-terminus, replaced by citrulline.

Another key autoantigen in RA is peptidylarginine deiminase 4 (PAD4) [19], a crucial enzyme that citrullinates arginine side chains and plays an important role in many key physiological processes, including the formation of neutrophil extracellular traps (NETs) [20]. PAD4 can undergo autocitrullination, which may not necessarily modify its enzymatic function or activity [21,22] but may convert itself into a target of ACPAs. However, the extent and importance of this phenomenon in RA are not well understood.

The function of ACPAs depends on their specificity *in vivo*. Promiscuous ACPAs from the blood of RA patients have been shown to recognise a wide spectrum of citrullinated peptides *in vitro*, which may partly reflect their specificity *in vivo*. Since ACPAs can be detected in blood long before the clinical onset of RA, this raises the question of whether their peptide specificities are associated with clinical diagnosis and prediction in combination with the disease outcomes of RA. We have investigated not only the binding of various monoclonal ACPAs to a panel of citrullinated type II collagen (COL2) peptides but also other joint-related peptides in early RA cohorts using an established bead-based multiplex immunoassay [23,24]. Here, we developed a new panel of peptides covering different ENO1 and PAD4 epitopes and analysed the immunoglobulin (Ig) G responses to the panel in a Swedish cohort consisting of early RA patients.

METHODS

Subjects

A case-control study was performed within the Medical Biobank of northern Sweden and used as the discovery cohort in this study. The cohorts within the Medical Biobank are population-based, and all adult individuals residing in the county of Västerbotten are continuously invited to participate. Recruitment, blood sampling, and storage conditions (at −80°C) have been described previously [1]. The register of patients attending the Department of Rheumatology, Umeå, and fulfilling the 1987 American College of Rheumatology classification of RA [25] and with a known date for the onset of symptoms of joint disease was coanalysed with those of the Medical Biobank. Consequently, 520 individuals (148 men and 372 women, designated as "presymptomatic individuals") were identified as having donated blood samples before the onset of any symptoms of joint disease. Among the individuals identified as presymptomatic, 244 (75 males and 169 females) had also provided blood samples when attending the clinic at the time of being diagnosed with RA, thus creating paired samples. The median time (Q1-Q3) between the onset of symptoms and a diagnosis of RA was 0.6 (0.3-1.0) years. All cases were classified as either "nonsmokers" or "ever-smokers." A total of 530 control subjects (175 men and 355 women) were randomly selected from the same Biobank cohorts as the presymptomatic individuals and matched for sex, age, and area at the time of blood sampling ('sex' referes to biological classification of participants as male or female, as recorded in medical records). Baseline data and serum samples from patients with treatment-naïve early RA (N = 690) according to European Alliance of Associations for Rheumatology (EULAR)/American College of Rheumatology (ACR) classification criteria included in the randomised phase IV Nordic Rheumatic Diseases Strategy Trials and Registries (NORD-STAR) trial were analysed as a validation cohort at diagnosis before treatment initiation [26–29]. Briefly, NORD-STAR inclusion criteria were age 18 years and older with treatment-naïve RA, symptom duration less than 24 months, moderate to severe disease activity, and RF or ACPA positivity or increased C-reactive protein (CRP). The subjects are described in Table 1.

The Regional Ethics Committee at the University Hospital, Umeå, Sweden, approved this study (2013-347-31), and all participants gave their written informed consent when donating samples to the Medical Biobank and at the early arthritis clinic. The Swedish Ethical Review Authority approved the current NORD-STAR study (Dnr 2022-02326-02), with the inclusion of samples obtained from Sweden, Denmark, Norway, and the Netherlands for the current study. All participants gave their written informed consent before entering the study.

Monoclonal antibodies derived from RA patients

The acquisition and generation of all monoclonal antibodies in the present study (E4, "L" series, and 3F3WT) have been previously described [5,6]. Briefly, single B-cell clones based on

Table 1
Demographic data for study subjects

Characteristics of subjects	Discovery cohort			Validation cohort
	Presymptomatic individuals (N = 520) n/N (%)	Early RA patients (n = 244) n/N (%)	Population controls (N = 530) n/N (%)	Early RA patients (N = 690) n/N (%)
Females, (%)	71.5	69.3	67	69
Age (y), median (Q1-Q3)	55 (50-60)	61 (53-67)	51 (50-60)	56 (46-66)
Smoking ever	337/520 (65)	166/243 (68)	222/522 (42)	418/688 (61)
HLA-DR SE	390/511 (76)	190/240 (79)	165/299 (55)	NA
RF	189/513 (37)	188/244 (77)	33/251[a] (13)	512/687 (75)
Anti-CCP2	213/519 (41)	187/241 (78)	16/530 (3)	563/689 (82)
Anti-CCP2 + RF	146/513 (29)	168/244 (69)	2/251 (1)	466/685 (68)
Predating time (y), median (Q1-Q3)	4.5 (2.1-7.7)			

NA, not available; Q, quartile; RA, rheumatoid arthritis; RF, rheumatoid factor; CCP2, second-generation cyclic citrullinated peptide; HLA-DR SE, human leukocyte antigen-DR shared epitope.
[a] RF was analysed in 251 of the population controls.

CCP2 reactivity were isolated from RA patients in the Rheumatology Department of Leiden University Medical Center; the B cell receptors (BCRs) were sequenced and constructed with identical human IgG1 or mouse IgG2b constant domains. The E4NG is a variant of the E4 wild-type (E4WT) with the Fab glycosylation sites mutated, but the citrulline specificity is maintained. The E4m antibody is a mutant of the E4 antibody (W48M and S51A in paratope), lacking the citrulline specificity [5], and therefore, it is used as a negative control for E4. All antibodies were produced in Expi293F cells, purified by affinity chromatography, dialysed, and stored in phosphate buffered saline (PBS) at −20°C.

Multiplex bead-based flow immunoassay

The multiplex bead-based flow immunoassay was performed as described previously [15,23]. Cyclic citrullinated or native peptides derived from ENO1 and PAD4 (Vacara AB), as well as control peptides such as CCP4 and CEP1 (Vacara AB) (Supplementary Table S1), were coupled to magnetic beads. Samples (plasma or serum) were diluted 1:100 by Dulbecco's phosphate buffered saline (DPBS), randomised on assay plates in singlicates, preblocked, and incubated with peptide-coupled beads. Bound IgGs were detected with phycoerythrin-conjugated anti-human IgG Fcγ (Jackson ImmunoResearch Laboratories Inc) by measuring the median fluorescence intensity (MFI) using the Bioplex-200 instrument (Bio-Rad). Monoclonal antibodies diluted in different concentrations were assayed similarly, and in case they had the mouse IgG2b constant domain, phycoerythrin-conjugated anti-mouse IgG Fcγ (Jackson ImmunoResearch Laboratories Inc) was used for detection. Data integrity and/or result comparability between the plates was ensured by assay calibrators and control samples. In the validation cohort, 18 of the 708 assayed samples were excluded due to technical reasons, resulting in 690 samples entering data analyses.

Assays for anti-CCP2 antibodies and RF

In the discovery cohort, the detection of anti-CCP2 antibodies and RF in plasma were performed using enzyme-linked immunoassays according to the manufacturer's instructions (Euro-Diagnostica AB) as previously described [1]. The cutoff for anti-CCP2 positivity was set at 25 arbitrary units (AU)/mL and at 20 AU/mL for RF. Anti-CCP2 was considered low when >25 and <75 AU/mL and high when ≥75 AU/mL. In the validation cohort, anti-CCP2 and RF were analysed according to laboratory standards at each contributing hospital. Patients with ≥20 International Units (IU)/mL anti-CCP antibodies or RF in serum were considered anti-CCP2 or RF-positive, respectively.

Statistics

In the discovery cohort, we measured diagnostic accuracy using sensitivity and specificity with corresponding CIs to quantify uncertainty. Cutoffs for specifying antibody positivity/negativity were determined by maximising the Youden index for RA cases vs controls with respect to antibody concentration under the additional condition that specificity must be at least 0.95. For each RA manifestation, we compared antibody-positive/negative groups with respect to manifestation score as follows. First, we performed an overall sign test, where each antibody contributed a "+" if the mean antibody level was higher in the cases than in controls and a "−" if otherwise. This was followed up using the Mann−Whitney U-test for each individual antibody. For investigating trends between groups with an increasing number of positive antibodies, the Jonkheere-Terpstra trend test was used. When comparing proportions, we used large-sample tests based on a normal approximation unless otherwise stated. For the RA cases, sensitivity and specificity were compared between pairs of tests following a 2-step procedure outlined in Roldán-Nofuentes [30], where one first tests the joint equality of sensitivity and specificity. Should the joint test yield a significant result, sensitivity and specificity should be separately compared post hoc between tests. We used all available cases; however, a power calculation where we assume an exposure (antibody positivity) rate of 5% in controls and 15% in cases, assuming a .05 level of significance and 95% power, yields a sample size of 231 cases and controls, respectively. Thus, even the early RA patient sample comes with sufficient power.

For the validation cohort, we used the continuous values (MFI) for the antibodies and implemented the method described in Dinse et al [31] to accommodate our analysis values below the limit of detection (LoD). In short, in order to apply an existing statistical methodology that correctly incorporates censored values into statistical analyses (Cox regression), we had to transform the data from left-censored (ie, values below the LoD) to right-censored. The Cox regression is able to deal very well with right-censored data and produces unbiased estimates of the association between risk factors and outcome as a hazard ratio. For this, we first applied a scale reversal by calculating for each antibody individually the difference between an arbitrary constant value, M, that was chosen to exceed all antibody values (in this case, M = 13,000) and the respective antibody (ie, M-Antibody values). All original left-censored antibody values ≤ LoD were thus transformed into M-Antibody values and treated as right-censored when they exceeded M-LoD. Further, the transformed (M-Antibody) values were treated in the Cox model as the modelled outcome and the indicators of disease activity (tender joint count [TJC] 28, swollen joint count [SJC] 28 categories, etc) as the risk factor of interest, "implying a role reversal when the analyte is a putative cause of a health outcome," as in the study by Dinse et al [31]. Further, the association estimated from the Cox model can be interpreted as the odds ratio (OR), and ORs > 1 are indicative of a worse outcome (in our case, indicative of pathogenicity), and ORs < 1 are indicative of a better health outcome (in our case, indicative of protection). The effect on the 28-joint disease activity score (DAS28), erythrocyte sedimentation rate (ESR), and CRP was interpreted to be slightly/moderately/strongly indicative of protection/pathogenicity based on the OR and the p value. For SJC28 and TJC28, the patients were categorised into 3 groups (C1 = 0-3; C2 = 4-10; C3 ≥ 10 affected joints), and the effect was interpreted to be slightly/moderately/strongly indicative of protection/pathogenicity based on the number of significant differences when these groups were compared.

RESULTS

Reactivity of ACPAs to ENO1 and PAD4 epitopes

We analysed the reactivity of a series of earlier defined monoclonal antibodies derived from RA patients (E4, "L" clones, and 3F3WT) with cyclic citrullinated or unmodified peptides derived from ENO1 and PAD4, along with often used peptides, such as CCP4 and CEP1, as controls (Supplementary Table S1). The results from multiplex bead-based immunoassay show that in contrast to most of the used antibody clones that recognised

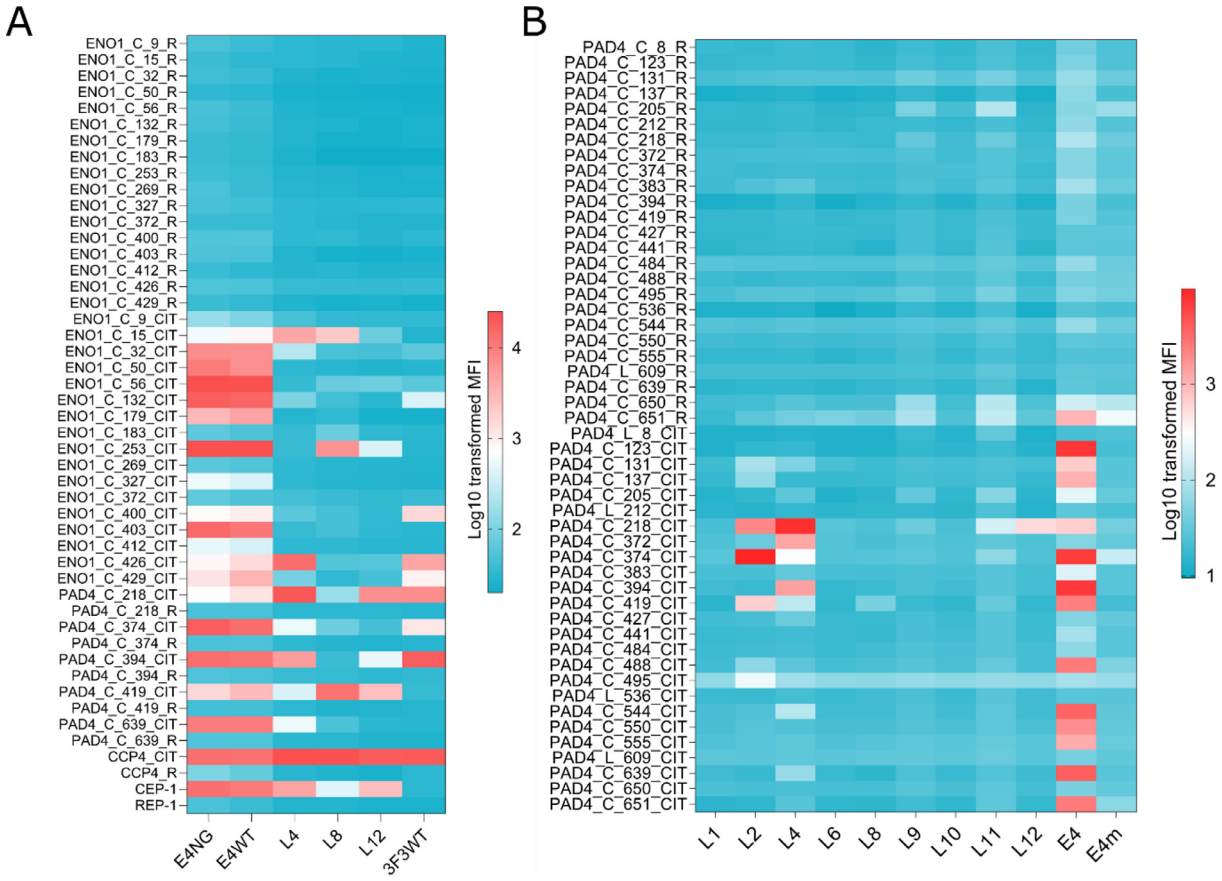

Figure 1. A, and B, Reactivity of monoclonal antibodies to citrullinated cyclic peptides derived from human alpha-enolase (ENO1) and peptidylarginine deiminase 4 (PAD4). The heatmaps represent the log10-transformed median fluorescence intensity (MFI) results at an antibody concentration of 1.0 μg/mL. Antibodies are denoted on the x-axis; antibodies used are (A) human immunoglobulin (Ig) G1 and (B) mouse IgG2b. E4, "L" series, and 3F3WT are monoclonal anticitrullinated protein antibodies derived from rheumatoid arthritis patients. Peptides are denoted on the y-axis. C, cyclic peptide; L, linear peptide; R, unmodified peptide with arginine; CIT, citrullinated peptide; E4m, mutated E4 lacking citrulline specificity; E4NG, non-Fab glycosylated E4; E4WT, wild-type E4 with Fab glycosylation; CCP4, fourth-generation cyclic citrullinated peptide; CEP1, citrullinated alpha-enolase peptide 1; REP1, alpha-enolase peptide 1.

only a very limited number of citrullinated epitopes on both ENO1 and PAD4, E4 bound to many citrullinated epitopes, indicating that E4-like protective ACPAs could have a distinct pattern for epitope recognition compared with other "nonprotective" ACPAs (eg, L2) (Fig 1).

Time-dependent increase of plasma IgG responses to citrullinated epitopes on ENO1 and PAD4 in presymptomatic individuals towards RA onset

The discovery cohort of presymptomatic individuals and early RA patients (patient characteristics are in Table 1) was analysed for plasma IgG responses with the 56-plex. Overall, the antibody responses to all citrullinated peptides were elevated in a time-dependent manner (Fig 2A,B, and Supplementary Tables S2 and S3). Upon the RA onset, the most frequent IgG response in this panel was against the CCP4 peptide, with a 75.4% sensitivity and 95.3% specificity. Among the ENO1 peptides, the most frequent one was ENO1_C_426_CIT (71.3% sensitivity and 94.5% specificity), followed by ENO1_C_9_CIT (68.3% sensitivity and 94.9% specificity) and ENO1_C_132_CIT (65.2% sensitivity and 94.7% specificity). For the selected PAD4 peptides (Fig 2A), the most frequent one was PAD4_C_394_CIT (72.5% sensitivity and 94.7% specificity), followed by PAD4_C_218_CIT (71.7% sensitivity and 94.7% specificity). In contrast, the responses to arginine peptides were at a minimal level, rarely above those in the control population, which is expected as background cross-reactivity.

When analysing the relationship between antibody responses, we found that most antibodies were positively correlated with each other both at the presymptomatic stage and in patients, whereas the correlations were clearly stronger in the presymptomatic samples (Fig 2C).

ACPA fine specificities are associated with lower SJC in early RF-negative RA

The association between ACPA responses to the peptides (positive or negative) and clinical outcomes in early RA (<1 year after onset, n = 244) was analysed based on different disease activity variables, including DAS28 and its components, such as the TJC, SJC, and ESR, as well as CRP (Fig 3). Without stratification for RF positivity (Fig 4A and Supplementary Table S4), several ACPA responses to the peptides, such as ENO1_C_9_CIT, ENO1_C_327_CIT, ENO1_C_426_CIT, and CCP4, were found to associate with a lower SJC or TJC, whereas another peptide, ENO1_C_269_CIT, was associated with a higher ESR. Notably, RF was not associated with any of these disease activity variables, and CCP2 positivity was associated only with a lower SJC in early RA. There were no differences between groups having low or high anti-CCP2 levels for any of the outcomes (Supplementary Table S4). By

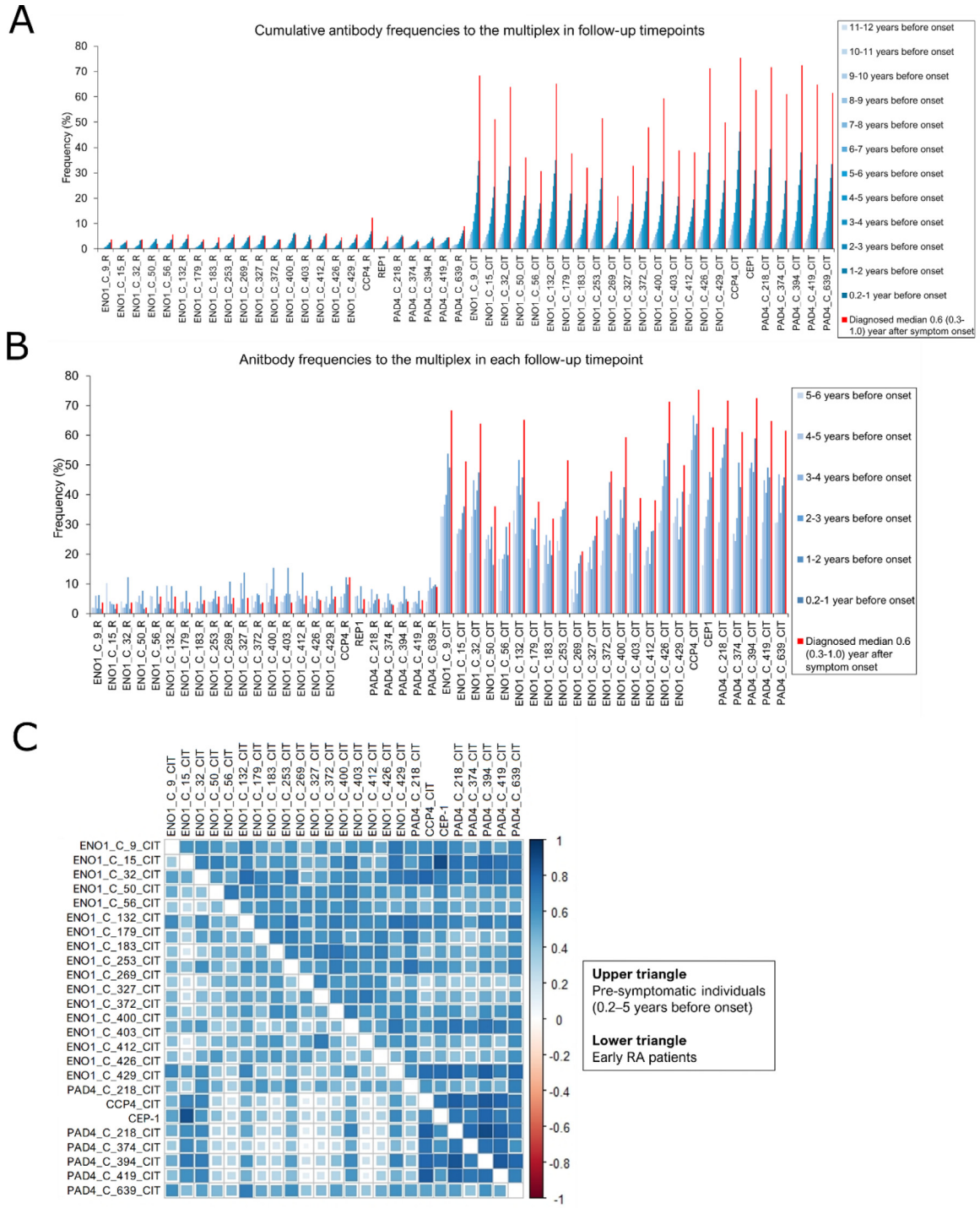

Figure 2. Immunoglobulin (Ig) G responses to citrullinated (CIT) or native/arginine (R) epitopes of alpha-enolase (ENO1) and peptidylarginine deiminase 4 (PAD4) in presymptomatic individuals and early rheumatoid arthritis (RA) patients in the discovery cohort. A, Cumulative frequency of antibody responses to ENO1 and PAD4 epitopes in plasma samples (N = 520) from the presymptomatic individuals during the predating time (from 12 to less than 0.2 years before onset). The frequency of patients with early RA (n = 244; diagnosed median [Q1-Q3] years after symptom onset, 0.6 [0.3-1.0]) stratified for time intervals is shown as a comparison. B, Individual frequency of antibody responses to ENO1 and PAD4 epitopes in plasma samples from the presymptomatic individuals during the predating time (from 6 to less than 0.2 years before symptom onset) (n = 335). The frequency of patients with early RA (n = 244; diagnosed median [Q1-Q3] years after symptom onset, 0.6 [0.3-1.0]) is shown as a comparison. The median fluorescence intensity (MFI) cutoff for positivity was determined as 95% specificity in population controls. The y-axis is the frequency of positivity (%). Response to CCP4, citrullinated alpha-enolase peptide 1 (CEP1), and their respective noncitrulline control peptides (CCP4_R and REP1) are shown for comparison. C, Correlation between different IgG responses to CIT epitopes based on MFI values in presymptomatic individuals (upper triangle, 0.2-5 years before onset) and early RA patients at disease onset (lower triangle). C, cyclic peptide. C, cyclic peptide; L, linear peptide; R, unmodified peptide with arginine; CIT, citrullinated peptide; CCP4, fourth-generation cyclic citrullinated peptide; CEP1, citrullinated alpha-enolase peptide 1.

stratifying these early RA patients with RF status (Fig 4A and Supplementary Table S5), we could observe an association with a lower SJC in RF-negative patients having ACPA fine specificities of 8 citrullinated peptides, aside from CCP2, compared with those not having those antibodies. The largest differences were found in PAD4_C_218_CIT, ENO1_C_13_CIT, ENO1_C_426_CIT, and PAD4_C_394_CIT (Fig 4A and Supplementary Table S5), and 3 of these peptides (3/8) were also found to have an association with a lower DAS28. In addition, the TJC, ESR, or CRP was not significantly different in the

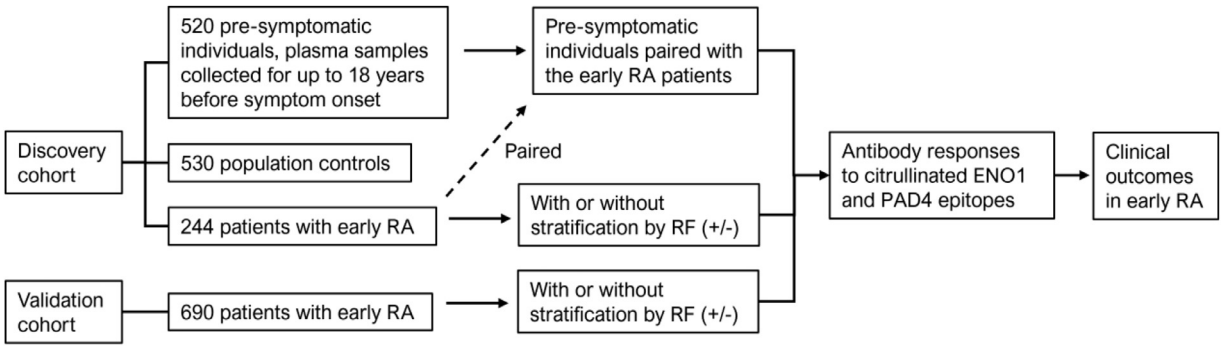

Figure 3. Flowchart of the analysis strategy for relationships (1) between antibody responses in presymptomatic individuals and their clinical outcomes after RA onset and (2) between antibody responses in early rheumatoid arthritis (RA) patients and their clinical outcomes in the discovery and validation cohorts. ENO1, alpha-enolase; PAD4, peptidylarginine deiminase 4; RF, rheumatoid factor.

RF-negative group with or without the measured ACPA fine specificities, although the tendency for a "protective" association was commonly observed (Fig 4A and Supplementary Table S5). In the RF-positive group, ACPA response to ENO1_C_179_CIT and ENO1_C_269_CIT was found to have an association with a higher DAS28 and ESR, respectively (Fig 4A and Supplementary Table S5). The results were confirmed in the treatment-naïve early RA validation cohort (Fig 4B and Supplementary Tables S6-S9). In short, the ACPAs in RF-negative RA patients were indicative of protection.

ACPA fine specificities in presymptomatic stage predict future onset outcomes

To investigate whether ACPAs in presymptomatic individuals could predict future RA severity, we looked at the discovery cohort's RA cases (n = 119) who had donated samples during the period of 0.2 to 5 years before disease onset. The paired samples from the presymptomatic phase were directly stratified by ACPA responses to the citrullinated peptides (positive or negative), and their association with the clinical outcomes in early RA were analysed (Fig 5 and Supplementary Table S10). Surprisingly, no ACPA responses could be found to show a "pathogenic" association with early RA clinical outcomes. In contrast, many were associated with a lower SJC, TJC, DAS28, ESR, or CRP at RA diagnosis. In fact, patients positive for responses to most citrullinated peptides within the panel in their presymptomatic stage showed overall tendencies of having lower disease severity at disease onset. Among these "protective" associations, several could be highlighted in more than 1 clinical variable, such as ENO1_C_15_CIT, ENO1_C_132_CIT, ENO1_C_426_CIT, CCP4, PAD4_C_419_CIT, and PAD4_C_639_CIT. Notably, ACPA responses to ENO1_C_426_CIT, PAD4_C_218_CIT, and PAD4_C_419_CIT in RF-negative early RA were also associated with a lower SJC as described above (Fig 4). Additionally, no associations between clinical manifestations and the anti-CCP2 response or RF status were found, neither between groups with high nor low anti-CCP2 responses (Fig 5 and Supplementary Table S10). Stratification of RF positivity was not achievable for presymptomatic individuals due to the small sample size at different time points and even lower RF positivity among the presymptomatic samples.

New biomarkers predict disease activity at RA onset

To evaluate whether our biomarkers could aid the clinical anti-CCP2 test in the prospect of providing a diagnostic/predictive value, we selected 5 candidate peptides with ACPA responses that were associated with less severe RA outcomes in the presymptomatic stage or early RA, including ENO1_C_132_CIT, ENO1_C_426_CIT, PAD4_C_218_CIT, PAD4_C_419_CIT, and PAD4_C_639_CIT, named "**Cit**rullinated Peptides for RA **Pre**diction and **Di**agnosis **5**-set (**CitPreDi5).**"

We first compared the frequencies of positive ACPA responses with these peptides in early RA patients, presymptomatic individuals, and population controls (Fig 6A and Table 2). In comparison with the anti-CCP2 test as a reference (77.6% sensitivity in early RA, 41% sensitivity in presymptomatic individuals, at 98% specificity), CitPreDi5 provided comparable sensitivities (77.0% in early RA and 42.9% in presymptomatic individuals) with a 94.7% specificity when 2 to 5 (≥2) ACPA responses to CitPreDi5 were detected (Table 2), extended by a frequency of 88.3% in RF-positive early RA patients, 39.3% in RF-negative early RA patients, and 53.7% in presymptomatic individuals with 0.2 to 5 years before onset (Fig 6A).

In anti−CCP2-negative cases, we could identify 31.5% of early RA patients and 29.1% (95% CI, 24%-34%) of presymptomatic individuals positive for at least 1 of the 5 ACPA responses (Table 3). Although the specificity was lower (87.0%), the CitPreDi5 test captured both early RA patients and presymptomatic individuals more often than the controls, as demonstrated by the nonoverlapping CIs (Table 3). In the case when samples were positive for 2 to 5 (≥2) ACPA responses to CitPreDi5, we could still detect 11.1% of early RA patients and 10.1% of presymptomatic individuals with a specificity of 96% (Table 3). There were significantly more presymptomatic individuals than controls who identified as testing positive with the CitPreDi5 test. Despite similar proportions (10%-11%) of test-positives in the early RA and presymptomatic groups, there was no statistical significance between early RA and controls due to the low patient number (n = 54) (Table 3).

Next, we investigated whether the CitPreDi5 test could demonstrate predictive value in presymptomatic individuals of their disease activity at diagnosis. The clinical outcomes of presymptomatic individuals (0.2-5 years before diagnosis) with ≥1, ≥2, ≥3, ≥4, or 5 positive ACPA responses to CitPreDi5 were compared with those of individuals with no response to CitPreDi5 (0). Those who were positive for ACPA responses to certain numbers of CitPreDi5 peptides showed significantly lower disease activity with a clear trend depending on the number of ACPA responses (Fig 6B). Analysis of the trend of having a positive antibody response for CitPreDi5 showed a significant decrease in DAS28, TSJ, SJC, ESR, and CRP ($p < .002$-$.030$, Jonckheere-Terpstra test). Notably, the individuals positive for

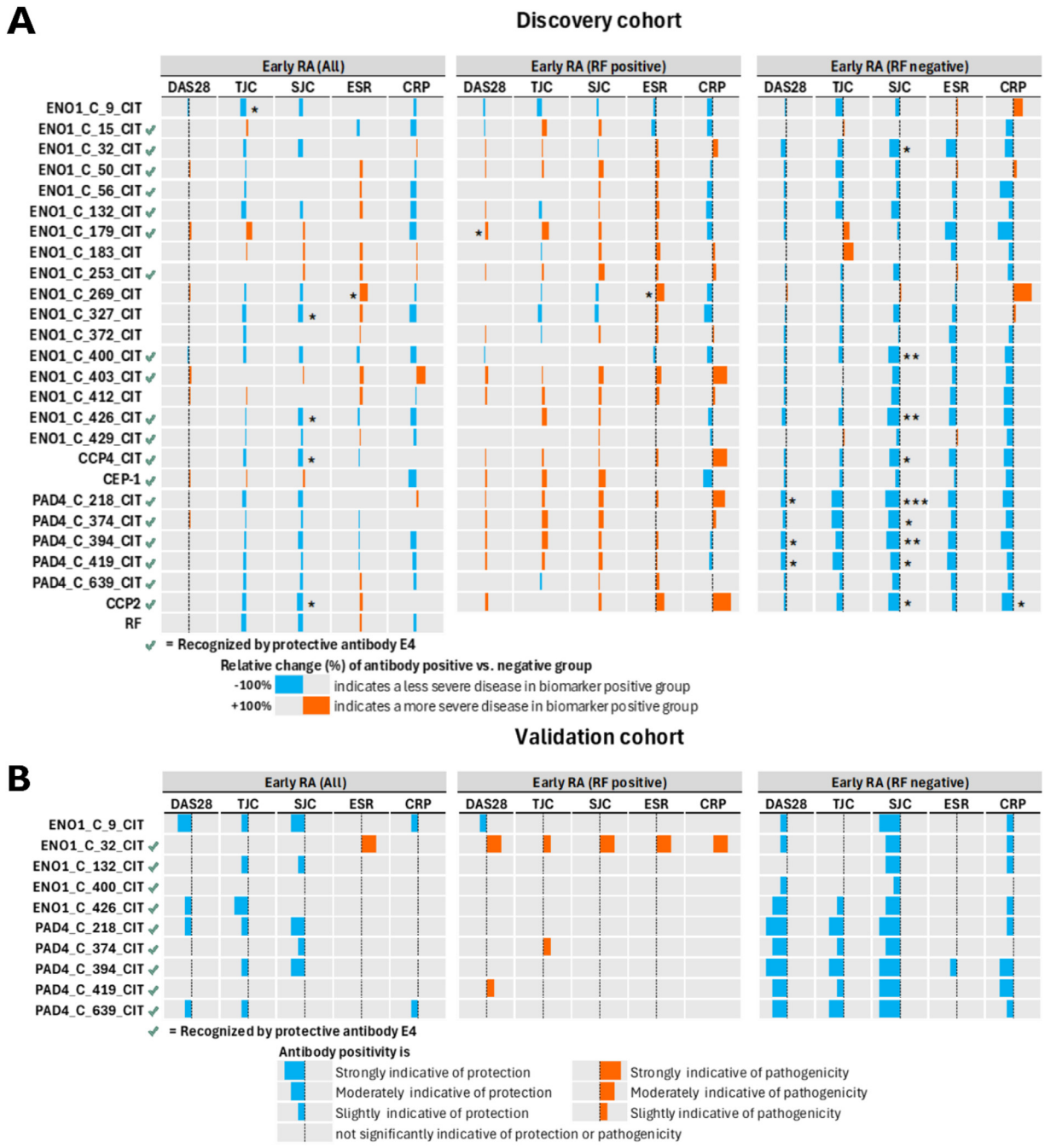

Figure 4. Associations of the serological biomarkers with clinical presentation (28-joint disease activity score [DAS28], tender joint count [TJC], swollen joint count [SJC], erythrocyte sedimentation rate [ESR], and C-reactive protein [CRP]) in early rheumatoid arthritis (RA) patients in the discovery (A) and validation (B) cohorts. A, Changes in clinical presentation in early RA patients are positive for the serological biomarkers compared with those who are negative. Biomarkers, including immunoglobulin Gs against the alpha-enolase (ENO1), peptidylarginine deiminase 4 (PAD4), and control peptides, as well as anti-second-generation cyclic citrullinated peptide (anti-CCP2) antibodies and rheumatoid factor (RF) in early RA patients (diagnosed median [Q1-Q3] years after symptom onset, 0.6 [0.3-1.0]), were determined, and each patient categorised as being positive or negative for each of the biomarkers. Results are shown as relative change (%), and the Mann–Whitney U-test (the overall differences were first validated by a sign test [results not shown]) was used when the means of the DAS28, TJC, SJC, ESR, or CRP data were compared between biomarker-positive and -negative groups. $*p < .05$, $**p < .01$, $***p < .001$. Early RA (All) n = 244; early RA (RF-positive) n = 188; early RA (RF-negative) n = 56, of which 22 were anti-CCP2 positive. B, The effect of selected antibodies in the early RA validation cohort. Early RA (All) N = 690; early RA (RF-positive) n = 512; early RA (RF-negative) n = 175; RF missing in n = 3. A decrease indicates a less severe disease in the biomarker-positive group. In addition, peptide epitopes recognised by the protective antibody E4 are indicated next to the peptide names. C, cyclic peptide; CIT, citrullinated peptide; R, unmodified peptide with arginine; CCP4, fourth-generation cyclic citrullinated peptide; CEP1, citrullinated alpha-enolase peptide 1.

≥4 or 5 ACPA responses in the CitPreDi5 test were found to have the lowest disease activity at diagnosis. In addition, despite the tendency, the anti-CCP2 test in the presymptomatic individuals did not show a similar association with any of the analysed clinical outcomes at RA onset (Figs 5 and 6B).

DISCUSSION

We have taken advantage of the epitope specificities of human monoclonal ACPAs with known protective and nonprotective functions to analyse plasma samples from presymptomatic

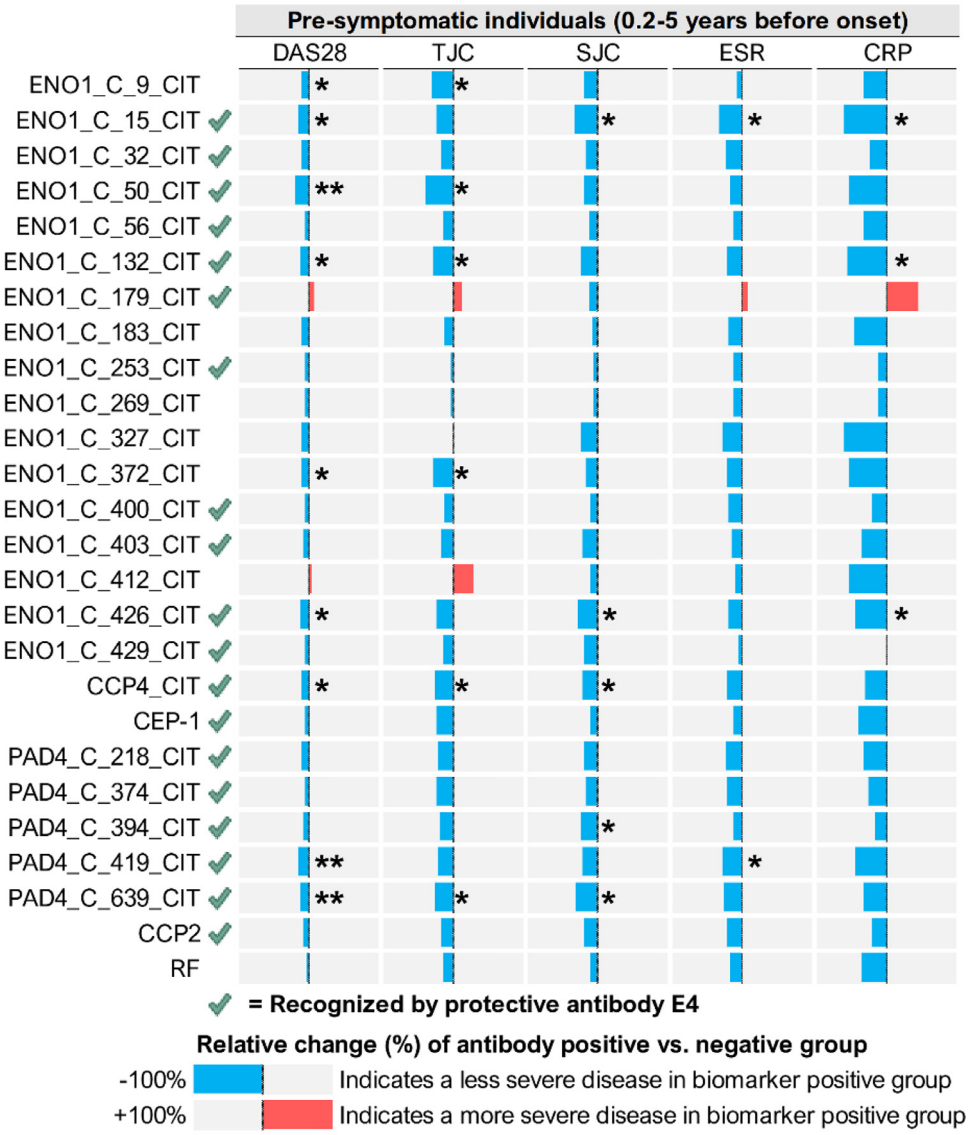

Figure 5. Change in the mean clinical presentation (28-joint disease activity score [DAS28], tender joint count [TJC], swollen joint count [SJC], erythrocyte sedimentation rate [ESR], and C-reactive protein [CRP]) at rheumatoid arthritis diagnosis of presymptomatic individuals positive for the serological biomarkers compared with those who were negative in the discovery cohort. Biomarkers, including immunoglobulin Gs against the alpha-enolase (ENO1), peptidylarginine deiminase 4 (PAD4), or control peptides, as well as anti-CCP2 antibodies and rheumatoid factor (RF) in presymptomatic individuals (0.2-5 years before the onset, n = 117 for DAS28 and ESR; n = 116 for SJC and TJC; and n = 82 for CRP), were determined, and each individual was categorised as being positive or negative for each of the biomarkers. Results are shown as relative change (%), and the Mann–Whitney U-test (the overall differences were first validated by a sign test [results are not shown]) was used when the means of the DAS28, TJC, SJC, ESR, or CRP data were compared between biomarker-positive and -negative groups. A decrease indicates a less severe disease in the biomarker-positive group, and an increase indicates a more severe disease in the biomarker-positive group. In addition, peptide epitopes recognised by the protective antibody E4 are indicated next to the peptide names. *$p < .05$, **$p < .01$. C, cyclic peptide; CEP-1, citrullinated ENO1; CIT, citrullinated peptide.

individuals and early RA patients. In line with a recent study [14], we confirmed that the association of ACPAs with a disease-protective response, such as a lower SJC, was the strongest in RF-negative early RA. Strikingly, without the need to stratify RF, we found that presymptomatic ACPA responses (0.2-5 years before the RA diagnosis) to a set of peptides, some of which are exclusively recognised by protective ACPAs (eg, clone E4), could predict a lower disease activity for the forthcoming RA onset. From these "protection-associated" peptides, we developed a new ACPA test encompassing 5 peptides (ENO1_C_132_CIT, ENO1_C_426_CIT, PAD4_C_218_CIT, PAD4_ C_419_CIT, and PAD4_C_639_CIT), named "CitPreDi5," which could predict the expected severity of disease.

According to current classification criteria, patients with RA can be divided into several subgroups depending on the presence of anti-CCP antibodies and/or RF. RF positivity has been shown to be associated with more destructive RA [32], whereas a recent study suggested that CCP2 positivity was associated with a low SJC and TJC in the absence of RF [14]. Initial studies on diagnostic peptides for ACPA detection focused on the citrullinated profilaggrin peptide (CCP1), whose epitope is not tissue-specific and widely present under physiological conditions [33], and later on synthetic CCP2, which is still the gold standard in the clinical diagnosis of RA. Diagnosis and initiation of appropriate treatment can be delayed in approximately 30% of patients who are negative for both RF and anti-CCP2, and nearly 20% of these patients still possess ACPA responses to a variety of citrullinated peptides [33]. Therefore, it is likely that more patient subgroups can be identified by deciphering the subsets of ACPAs and combining these findings with RF status [15], which may provide better implications for the timing and accuracy of diagnosis and treatment recommendations.

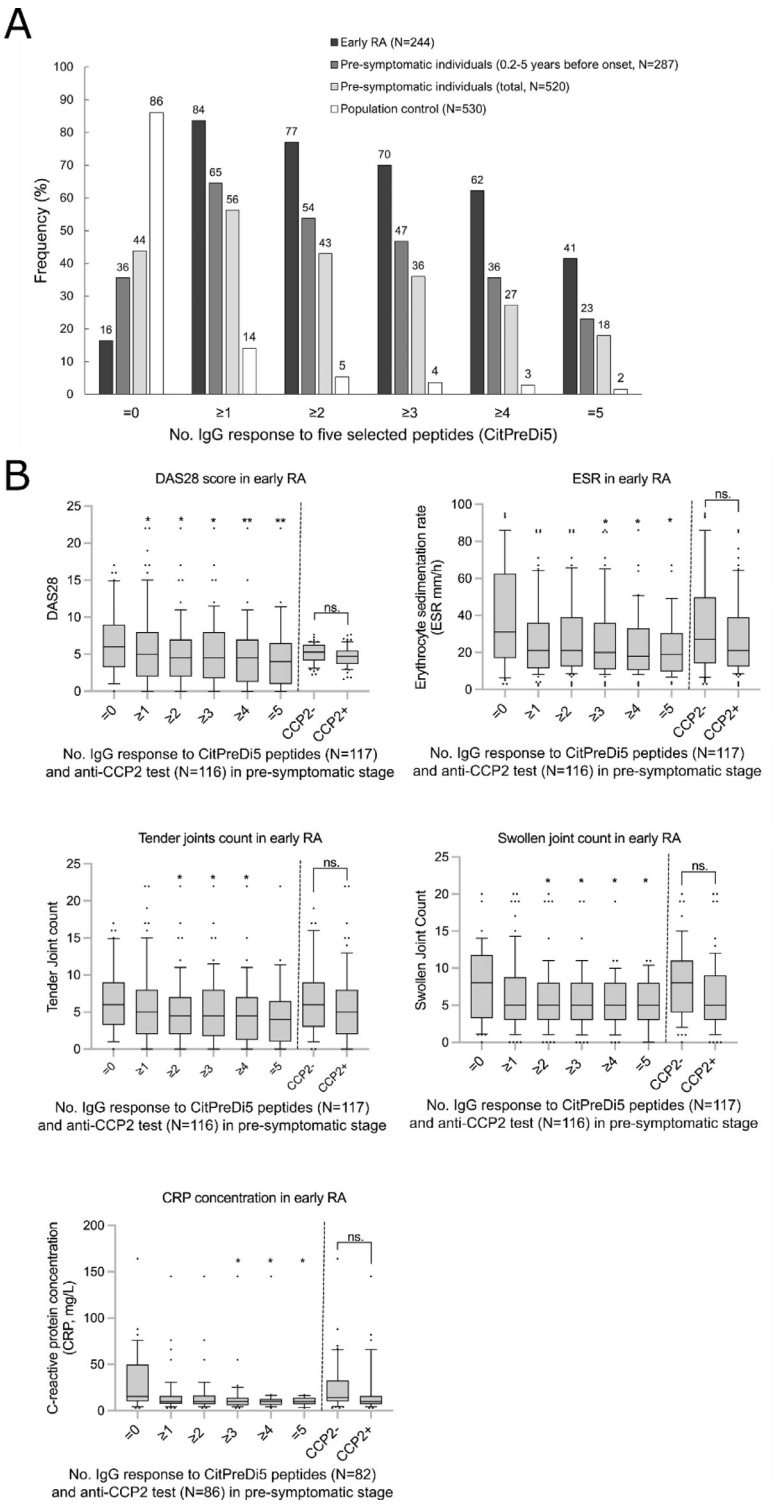

Figure 6. Diagnostic and predictive value of new biomarkers for anticitrullinated protein antibody (ACPA) responses. A, Frequencies of ACPA immunoglobulin (Ig) G responses to 5 peptides (Citrullinated Peptides for RA Prediction and Diagnosis 5-set [CitPreDi5]) in early rheumatoid arthritis (RA) patients, presymptomatic individuals, and population controls in the discovery cohort. B, Early RA clinical outcomes were associated with the number of ACPA responses from presymptomatic individuals (0.2-5 years before onset, n = 117 for the 28-joint disease activity score [DAS28] and its components: the tender joint count [TJC], swollen joint count [SJC], and erythrocyte sedimentation rate [ESR]; and n = 82 for C-reactive protein [CRP]), and similar data were included for the anti-second-generation cyclic citrullinated peptide (anti-CCP2) test (n = 116 for DAS28 and its components: the TJC, SJC, and ESR; and n = 86 for CRP) as a reference. For each clinical measure, the comparisons were separately made between the individuals with ≥1, ≥2, ≥3, ≥4, or 5 positive ACPA responses to the CitPreDi5 peptides vs the individuals with no response to the peptides (0); data were analysed with the Mann−Whitney U-test (2-tailed) and presented as median with 10th to 90th percentiles; outliers are shown as dots in groups. *$p < .05$, **$p < .01$, ns, not significant.

The CitPreDi5 peptides from our panel showed comparable specificity (95%) and sensitivity (77%) to the clinical anti-CCP2 test when the samples were positive for at least 2 of the CitPreDi5 peptides. In our analysis of presymptomatic individuals, the anti-CCP2 test was incapable of predicting future clinical outcomes, whereas the CitPreDi5 captured a subset of patients with lower disease activity at diagnosis. The functional role of ACPAs in RA is still an important unanswered question. Although the specificity of the citrulline side chain of ACPAs is highly conserved between mice and humans, the context is also important. Antibodies may play different roles if operating together; thus, complexes between promiscuous ACPAs and RF might add additional effects, and it is also possible that more specific ACPAs that cross-react with cartilaginous joints could have a pathogenic role. We hypothesise that our results reflect a subset of protective ACPAs that have a similar epitope fingerprint to the E4 antibody [5]. In fact, most human-derived ACPA clones, tested in different laboratories, have been found to exert certain levels of arthritis protection in animal models [5,12,13]. Here, ACPA responses to an epitope fingerprint associated with protection were present at least 10 years before RA diagnosis. Although their concentration could remain low in the presymptomatic stage, these antibodies correlated more strongly with each other than in early RA (Fig 2C), indicating that a wider B-cell repertoire that produces epitope-specific antibodies (eg, antibodies with more specific binding to cartilage), potentially

Table 2
Comparison of the anti-CCP2 test and the new ACPA test, where positivity for at least 2 of the CitPreDi5 peptides was considered a positive test result. Sensitivity and specificity are shown with 95% CIs

Discovery cohort	Early RA (n = 244)		Presymptomatic (N = 520)		Controls (N = 530)	
Test[a]	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)
RF	188/244 (77; 72, 82)	56/244 (23)	189/513 (37; 33, 41)	324/513 (63)	33/251 (13)	218/251 (87; 83, 91)
CCP2	189/244 (77; 72, 82)	55/244 (23)	213/519 (41; 37, 45)	306/519 (59)	16/530 (3)	514/530 (97; 96, 98)
RF+ and/or CCP2+	210/244 (86; 82, 90)	34/244 (14; 10, 18)	256/519 (49; 45, 54)	263/519 (51; 46, 55)	47/525 (9; 7, 11)	478/525 (91; 89, 93)
≥2 Ab+[b]	188/244 (77; 72, 82)	56/244 (23)	223/520 (43; 39, 47)	297/520 (57)	28/530 (5)	502/530 (95; 93, 97)
≥3 Ab+[c]	171/244 (70; 64, 76)	73/244 (30)	187/520 (36; 32, 40)	333/520 (64)	19/530 (4)	511/530 (96; 95, 98)

ACPA, anticitrullinated protein antibody; CitPreDi5, Citrullinated Peptides for RA Prediction and Diagnosis 5-set; RA, rheumatoid arthritis; RF, rheumatoid factor; CCP2, second-generation cyclic peptide; Ab, antibody.

[a] Ab+, positive ACPA response to CitPreDi5 peptides (ENO1_C_132_CIT, ENO1_C_426_CIT, PAD4_C_218_CIT, PAD4_C_419_CIT, and PAD4_C_639_CIT)

[b] The overall test p values are $p = .086$ (vs CCP2, no post hoc); $p = .015$ (vs RF+, post hoc: sensitivity $p = .88$, specificity $p = .0072$); $p = 1.7 * 10^{-6}$ (vs RF+ and/or CCP2+, post hoc: sensitivity $p = .000038$, specificity $p = .017$). Should a Bonferroni correction be applied to the 6 overall tests, the overall result for ≥2 Ab+ vs RF+ would be considered nonsignificant.

[c] The overall test p values are $p = .0019$ (vs CCP2, post hoc: sensitivity $p = .0013$, specificity $p = .66$); $p = .000062$ (vs RF+, post hoc: sensitivity $p = .034$, specificity $p = .00047$); $p = 2.3 * 10^{-13}$ (vs RF+ and/or CCP2+, post hoc: sensitivity $p = 6.8 * 10^{-9}$, specificity $p = .00010$).

pathogenic, arises shortly before onset, outweighing the protective antibodies and leading to clinical RA. This is consistent with the concept of epitope spreading before disease onset, where antibody reactivity broadens with enhanced specificities over time, and such dynamic change may explain why presymptomatic individuals exhibit more intercorrelated antibody responses, while early RA reflects a more individualised immune profile. It is intriguing to think that during the decades of the presymptomatic stage, the low level of protective ACPAs could act as a sentinel to eliminate abnormal citrullination but may not be potent enough to combat the dramatic disease development around the onset when hypercitrullination takes place and epitope-specific pathogenic antibodies become predominant [34]. Nevertheless, we were able to confirm that a subset of presymptomatic ACPAs is associated with less severe RA at diagnosis based on CRP, DAS28, and its components, the SJC, TJC, and ESR, which may have implications for future disease management.

When examining the fine specificities of a series of patient-derived monoclonal ACPAs on ENO1 and PAD4 epitopes, a distinct pattern exhibited by the protective ACPA E4 showed that E4 itself was sufficient to interact with most of the citrullinated epitopes on ENO1 and PAD4, covering those that were associated with less severe RA, whereas the others, such as the "nonprotective" ACPAs (ie, clone L2) [5], had more restricted access to most of the ENO1 or PAD4 epitopes. However, this difference in the binding pattern was reversed on citrullinated COL2 in our recent study, in which ACPAs, such as L2, could access more epitopes, whereas E4 became more selective [5]. By comparing the sequences of peptides, it is clear that the Cit-Gly motif within the epitope plays a critical role in such a pattern [23]. In our previous study [3], the used citrullinated COL2 peptides and CEP1 contained the Cit-Gly motif in their epitopes, and in crystallography, we observed additional polar contacts, ie, the hydrogen bonding between the Cit-Gly glycine and the carboxamide group, but the impact of such interaction on the E4 Fab-peptide complex is unknown. In this study, it is clear that E4 could strongly interact with many PAD4 or ENO1 epitopes that do not possess the Cit-Gly motif, unlike the L2 or L4 antibody. With this observation, we deemed that Cit-Gly is not required or has only a minimal impact on E4 for epitope binding. Nonetheless, we could conclude from the sequences of all tested peptides that a common subtype of promiscuous ACPAs (eg, clone L2 or L4) heavily relies on the Cit-Gly motif for interaction, whereas E4 does not require a glycine neighbouring citrulline for interaction, and such a difference in binding preference may have an impact on the function of the antibodies.

Seronegative RA patients experience a longer time from clinically documented joint swelling to clinical diagnosis of RA compared with seropositive patients [35]. For seronegative patients, the current practice of diagnosing RA relies on the other parameters in the classification criteria [25,26] and physicians' experience, which raises, eg, the SJC and TJC required to achieve diagnosis. Thus, seronegative patients may present more severe manifestations than the seropositive ones at early RA on a group level. This may influence the interpretation of our results when comparing our biomarkers' predictive/diagnostic value between seropositive and seronegative RA patients. The results of the RF-negative group are less affected by that. We envision that CitPreDi5 could improve the RA diagnosis/prediction of

Table 3
Performance of the anti-CitPreDi5 test in anti−CCP2-negative individuals. Positivity for at least 1 or 2 (≥1 or ≥2) of the 5 peptides was considered positive. Sensitivity and specificity are shown with 95% CIs

Discovery cohort	Early RA (n = 54)		Presymptomatic (n = 306)		Controls (n = 514)	
Test	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)	Test positive n/N (%; 95% CI)	Test negative n/N (%; 95% CI)
RF	20/54 (37; 24, 50)	34/54 (63; 50, 76)	43/303 (14; 10, 18)	260/303 (86; 82, 90)	31/243 (13; 9, 17)	212/243 (87; 83, 91)
≥1 Ab+	17/54 (31; 19, 44)[a]	37/54 (69)	89/306 (29; 24, 34)[a]	217/306 (71)	67/514 (13)	447/514 (87; 84, 89)
≥2 Ab+	6/54 (11; 3, 19)	48/54 (89)	31/306 (10; 7, 14)[a]	275/306 (90)	21/514 (4)	493/514 (96; 94, 98)

Ab+, positive anticitrullinated protein antibody response to CitPreDi5 peptides (ENO1_C_132_CIT, ENO1_C_426_CIT, PAD4_C_218_CIT, PAD4_C_419_CIT, and PAD4_C_639_CIT).

CitPreDi5, Citrullinated Peptides for RA Prediction and Diagnosis 5-set; RA, rheumatoid arthritis; RF, rheumatoid factor.

[a] The proportion positive is significantly different compared with the population control.

presymptomatic but at-risk individuals (eg, family history, predisposition of genetic and environmental risk factors, elevated inflammatory markers, or RF presence etc), as upon the appearance of symptoms, having ACPAs in circulation associated with a protective phenotype indicates a less severe early RA. This stratification provides an opportunity for rheumatologists and individuals to intervene more timely and efficiently through better monitoring, disease management, lifestyle adjustment, or even early treatment.

A strength of the study is the unique collection of samples from presymptomatic individuals. The lack of access to similar independent material is a limitation of the study, while the use of an independent early RA cohort for validation confirmed the results and provided evidence to establish generalisability. We also acknowledge that multiple test corrections may affect the significance of some biomarkers with borderline *p* values. However, the overall trend remains clear and evident, and several biomarkers continue to show significant associations.

To conclude, in early RA patients and presymptomatic individuals who subsequently developed RA, we identified ACPA responses to certain epitopes derived from ENO1and PAD4 that were associated with lower disease activity, expanding the notion that the majority of ACPA responses could be protective in RA, especially in the presymptomatic stage. To our knowledge, this is the first study showing the relationship between epitope-specific protective ACPA responses from either the presymptomatic or early stage of RA and clinical manifestations in early RA. The CitPreDi5 test could also contribute to the development of a more precise assay supplementing the anti-CCP2 test for RA diagnosis/prediction.

Competing interests

RH is a founder, YH and OS are employees, and EL is part of the steering group of Vacara AB. Vacara provided reagents for the multiplex flow immunoassay used in the study and has contracted with Karolinska Institutet and Sahlgrenska University Hospital for a collaboration project. Vacara has an approved US patent on protective antibodies to citrullinated proteins and has submitted a patent application for the invention of this study. DN has received consulting fees from BMS, Lilly, MSD, Novartis, Pfizer, and UCB. TU has received honoraria from Pfizer, Galapagos, and UCB. SR-D has a royalty agreement with Vacara. RH has received consulting fees from Lipum AB. For the remaining authors (LJ, MLA, LC, AL, MHL, BG, KH-P, MN, KL, JL, AR, GG, MØ, and IG), no competing interests were declared.

Ethics approval

For the discovery cohort, the Regional Ethics Committee at the University Hospital, Umeå, Sweden approved this study (2013-347-31), and all participants gave their written informed consent when donating samples to the Medical Biobank and at the early arthritis clinic. For the validation cohort, the Swedish Ethical Review Authority approved the current NORD-STAR study (Dnr 2022-02326-02), with the inclusion of samples obtained from Sweden, Denmark, Norway and The Netherlands for the current study. All participants gave their written informed consent before entering the study.

Provenance and peer review

Not commissioned, externally peer-reviewed.

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Contributors

YH: Conceptualisation, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualisation, Writing – original draft; OS: Conceptualisation, Data curation, Funding acquisition, Methodology, Validation, Visualisation, Writing – original draft; LJ: Formal analysis, Visualisation, Writing – review & editing; MLA: Data curation, Formal analysis, Writing – review & editing; LC: Investigation, Methodology, Writing – review & editing; AL: Formal analysis, Writing – review & editing; EL: Methodology, Writing – review & editing; TU: Resources; MLH: Resources; BG: Resources; KH-P: Resources; MN: Resources; KL: Resources; JL: Resources; DN: Resources; AR: Resources; GG: Resources; MØ: Resources; IG: Funding acquisition, Writing – original draft; SR-D: Data curation, Formal analysis, Funding acquisition, Resources, Supervision, Writing – original draft; RH: Conceptualisation, Funding acquisition, Investigation, Resources, Supervision, Writing – original draft.

TU, MLH, BG, KH-P, MN, KL, JL, DN, AR, GG, and MØ (contribution in the original NORD-STAR study): Conceptualisation, Funding acquisition, Investigation, and Writing. All authors revised the work critically for important intellectual content, provided final approval of the version to be published, and agreed 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.

YH and OS contributed equally to this paper. RH is responsible for the overall content as guarantor.

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Patient consent for publication

All participants gave their written informed consent.

Patient and public involvement

Besides providing samples, patients or the public were not involved in this study.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ard.2025.04.013.

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