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Clinical science

Anti-protein arginine deiminase antibodies are distinctly associated with joint and lung involvement in rheumatoid arthritis

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Abstract

Objectives: RA is a chronic inflammatory disease in which possible interstitial lung disease (ILD) is an extra-articular manifestation that carries significant morbidity and mortality. RF and ACPA are included in the RA classification criteria but prognostic and diagnostic biomarkers for disease endotyping and RA-ILD are lacking. Anti-protein arginine deiminase antibodies (anti-PAD) are a novel class of autoantibodies identified in RA. This study aimed to assess clinical features, ACPA and anti-PAD antibodies in RA patients with articular involvement and ILD.

Methods: We retrospectively collected joint erosions, space narrowing, clinical features and lung involvement of a cohort of 71 patients fulfilling the 2010 ACR/EULAR RA classification criteria. Serum samples from these patients were tested for ACPA IgG (QUANTA Flash CCP3), and anti-PAD3 and anti-PAD4 IgG, measured with novel assays based on a particle-based multi-analyte technology (PMAT).

Results: Anti-PAD4 antibodies were significantly associated with radiographic injury ($P=0.027$) and erosions ($P=0.02$). Similarly, ACPA levels were associated with erosive disease ($P=0.014$). Anti-PAD3/4 double-positive patients displayed more joint erosions than patients with anti-PAD4 antibodies only or negative for both ($P=0.014$ and $P=0.037$, respectively). RA-ILD (15.5%, 11/71 patients) was associated with older age ($P<0.001$), shorter disease duration ($P=0.045$) and less erosive disease ($P=0.0063$). ACPA were elevated in RA-ILD, while anti-PAD4 were negatively associated ($P=0.043$).

Conclusion: Anti-PAD4 and anti-PAD3 antibodies identify RA patients with higher radiographic injury and bone erosions. In our cohort, ILD is associated with lower radiographic and erosive damage, as well as low levels of anti-PAD4 antibodies.

Keywords: RA, interstitial lung disease, ACPA, anti-PAD antibodies, erosive arthritis, radiographic score

Rheumatology key messages

- Anti-PAD4 antibodies are associated with radiographic injury and disease activity in RA.
- Patients with both anti-PAD3 and anti-PAD4 antibodies have more joint erosions.
- The presence of anti-PAD4 antibodies is negatively correlated with RA-ILD.

Introduction

RA is a chronic, inflammatory autoimmune disease involving diarthrodial joints and periarticular structures, leading to a progressive bone erosion if untreated [1]. In addition, a large number of patients may also display systemic disease with extra-articular manifestations (EAMs), including lung involvement [2].

All the thoracic compartments, including pleura, lung parenchyma and the airways [3], may be affected, but the involvement of the interstitium is one of the most prevalent and serious thoracic manifestation. Interstitial lung disease in RA (RA-ILD) indeed represents a significant cause of morbidity and mortality [4–6] and the second major contributor to mortality, with a median survival of 2.6 years after the diagnosis [7]. Different from other autoimmune disorders, RA-ILD is more frequently associated with the histopathologic and radiographic pattern of usual interstitial pneumonia (UIP), followed by non-specific interstitial pneumonia (NSIP) [8–10].

Autoantibodies against citrullinated proteins (ACPA) are included together with RF in the RA classification criteria

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[11]. They are associated with a more aggressive course of RA, higher disease activity, increased erosions and mortality [12–16], as well as the development of ILD [17]. Several other autoantibodies have been associated and studied in patients with RA, such as anti-carbamylated protein antibodies (anti-CarP) and autoantibodies isotypes, including IgA, IgM and IgG ACPA and RF. A panel of novel autoantibodies recognizing the protein-arginine deiminase (PAD) antigens has recently been described [18]. The PAD proteins are a calcium-dependent group of enzymes that catalyse citrullination, a post-translational modification of arginine residues to citrulline. The human PAD enzymes family consists of five members (PAD1–4, and PAD6) with different tissue distribution. Beyond their activity in citrullination, PAD2, PAD3 and PAD4 have been identified as autoantigens, possibly involved in RA pathogenesis [18–20]. In particular, anti-PAD3, anti-PAD4 as well as anti-PAD3/4XR cross-reactive autoantibodies have been associated with RA disease activity, bone erosions and radiographic progression [21–24]. In contrast, data about a possible correlation with ILD are still scarce [25], and no aetiological association between smoking and anti-PAD autoantibodies has been found [26].

Our study aimed to explore clinical and serological features of a cohort of RA patients, and in particular the possible association of ACPA and anti-PAD antibodies with articular injury and erosions as well as with ILD.

Methods

Patients

Seventy-one consecutive patients from the Careggi University Hospital, Florence, Italy (Immunology and Cellular Therapy Unit and Immunoallergology Unit) were included in the study. All the study subjects were affected by RA according to the 2010 ACR and EULAR Classification Criteria [11]. Eleven out of 71 showed ILD. Serum samples were obtained from routine blood tests and stored at –20°C until the analysis. Demographic and clinical records, including current therapy, smoking history, disease onset, disease activity, symmetry of involved joints, disease duration and morning stiffness, were retrospectively collected. Due to the retrospective study design and the anonymization of the information, approval from the local ethics committee was not required.

Study design

The study design is cross-sectional and retrospective. Consecutive patients with a diagnosis of RA were recruited. The primary outcomes were anti-PAD4, anti-PAD3 antibodies and ACPA levels. The patients were grouped according to their radiographic damage, disease activity, presence of ILD and smoking history.

Antibody detection

Serum samples were assessed for ACPA, anti-PAD3 and anti-PAD4 IgG antibodies. All measurements were obtained with a single measurement in a single batch. ACPA were measured with the QUANTA Flash® CCP3 chemiluminescence immunoassay (CIA) (Werfen, Barcelona, Spain). Testing was performed in the chemiluminescent analyser BIO-FLASH® (Werfen, Barcelona, Spain) according to the manufacturers' instructions. The results were expressed in chemiluminescence units (CUs), and as recommended by the manufacturer, samples were considered as positive when ACPA levels were >20 CUs.

The anti-PAD antibodies were measured with novel assays based on a particle-based multi-analyte technology (PMAT) (research use only) (Werfen, Barcelona, Spain). In short, full-length human recombinant PAD3 and PAD4 proteins were coupled to paramagnetic beads with unique signatures. The testing reaction was performed on the Aptiva® instrument (Werfen, Barcelona, Spain). The results were expressed in median fluorescence intensity (MFI). Preliminary cut-offs in raw MFI units were used for research purpose analyses and determined as >1000 MFI.

Assessment of joint involvement

Joint involvement was retrospectively evaluated using the Simple Erosion Narrowing Score (SENS) [27] from the most recently available radiograph of hands, wrists and feet, not older than 2 years since blood sampling. SENS is the sum of Joint Erosion Score (JES) and Joint Space Narrowing Score (JSNS), which are respectively the number of joints with erosions and the number of joints with space narrowing, and ranges from 0 to 86, as defined by van der Heijde *et al.* [27]. The revision of radiographs and the calculation of the scores were carried out by a trained blinded rheumatologist.

Assessment of lung involvement

Lung involvement was evaluated by chest high-resolution CT (HRCT), acquired within the last year since serum collection. All the images were read by the multidisciplinary team dedicated to ILD, which included two experienced thoracic radiologists [28].

Statistical analysis

χ^2 test with Yates's correction was used for discrete random variables, replaced, if necessary, by Fisher's exact test. Data were compared by Wilcoxon Rank sums test for nonparametric continuous variables. Spearman's Rank Test was performed to assess the strength of the relationship between two nonparametric variables. Results were expressed as mean (S.D.). unless otherwise stated. *P*-values <0.05 were considered as significant. Statistical analysis was carried out using R Studio version 1.1.463.

Results

Prevalence and associations of ACPA and anti-PAD autoantibodies

Anti-PAD4, anti-PAD3 and ACPA were found in 23.9% ($n = 17$), 8% ($n = 6$) and 67% ($n = 48$) of our RA patient cohort, respectively. A significant overlap in positivity was observed: all patients with positive anti-PAD3 were also positive for anti-PAD4 and 20% ($n = 14$) of patients positive for ACPA were also positive for anti-PAD4. Among ACPA-negative patients 4.2% ($n = 3$) were positive for anti-PAD antibodies (Fig. 1A). The level of anti-PAD4 and anti-PAD3 antibodies was statistically correlated ($R = 0.74$, $P < 0.0001$) (Fig. 1B). No significant correlation was found between anti-PAD4 and ACPA ($R = 0.19$, $P =$ ns) (Fig. 1C) and anti-PAD3 and ACPA ($R = 0.07$, $P =$ ns) (Fig. 1D).

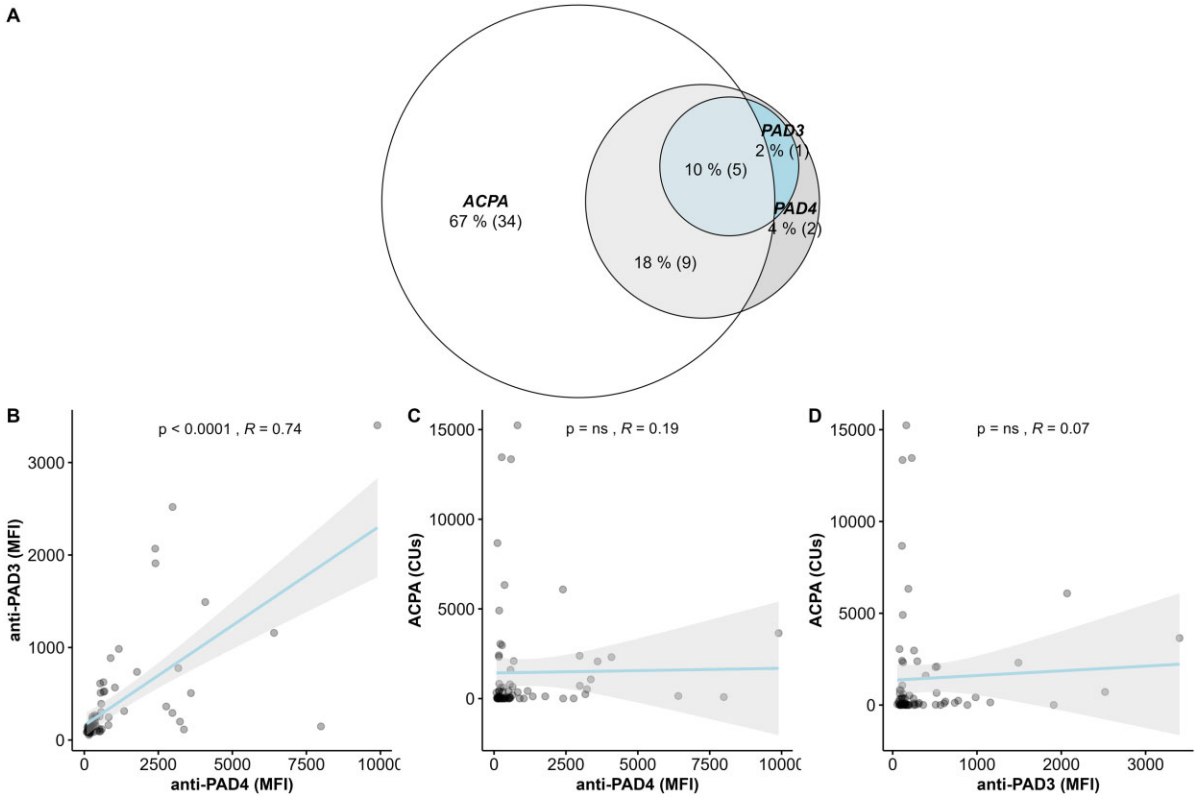

Figure 1. Venn diagram of the prevalence of RA-related autoantibodies and their correlation. Levels of anti-PAD4, anti-PAD3 and ACPA were assessed by PMAT (anti-PADs) and QUANTA Flash (ACPA), as specified in the Methods. The correlation between levels of each antibody was calculated by Spearman's Rank Test. The lines and grey areas indicate regression line and CI, respectively. Statistically relevant correlations were observed between anti-PAD4 antibodies and anti-PAD3 antibodies. The remaining comparisons did not show statistically relevant associations. PMAT: particle-based multi-analyte technology; anti-PAD4: anti-protein arginine deiminase type 4 antibodies; anti-PAD3: anti-protein arginine deiminase type 3 antibodies; MFI: median fluorescence intensity; CUs: chemiluminescence units; ns: not significant

Levels of anti-PAD4 antibodies are associated with radiographic injury and disease activity

Seventy-one patients were included in the study [62 females, mean age at recruitment 63.3 (±12.4) years, age range 29–90 years]. Supplementary Table S1, available at *Rheumatology* online, shows demographic and clinical characteristics of the patients. Table 1 shows the serological features of the studied cohort.

We first assessed whether RA-related antibodies were associated with radiographic injury. As shown in Fig. 2A, levels of anti-PAD4 antibodies were significantly associated with SENS, which combines JES and JSNS ($P = 0.027$). Surprisingly, we did not find any association with ACPA or anti-PAD3 antibody levels (Fig. 2B and C). We were indeed able to demonstrate that levels of both anti-PAD4 antibodies and ACPA were significantly associated with JES ($P = 0.014$ and $P = 0.02$, respectively) (Supplementary Fig. S1A and C, available at *Rheumatology* online), whereas no significant association with JSNS was found for all the three antibodies tested (Supplementary Fig. S1D–F, available at *Rheumatology* online).

Disease activity was evaluated by disease activity score DAS28-ESR at the time of blood sample collection. Levels of both anti-PAD3 and anti-PAD4 antibodies were significantly associated with DAS28-ESR (both $P < 0.05$), whereas no association was found with ACPA levels (Fig. 2D–F).

Table 1. Serological features of the studied cohort

Variable	All patients
Anti-PAD4, mean (S.D.), MFI	1108 (1812)
Anti-PAD3, mean (S.D.), MFI	397 (596)
ACPA, mean (S.D.), CUs	1457 (3135)
Anti-PAD4$^+$, *N* (%)	17 (23.9)
Anti-PAD3$^+$, *N* (%)	6 (8.5)
ACPA$^+$, *N* (%)	48 (67.6)
Anti-PAD4$^+$/3$^+$, *N* (%)	6 (8.5)
Anti-PAD4$^+$/3$^-$, *N* (%)	11 (15.5)
Anti-PAD4$^-$/3$^-$, *N* (%)	54 (76.1)

Serum samples from the 71 RA patients of the cohort were assessed for anti-PAD4, anti-PAD3 and ACPA antibodies, as described in the Methods. Antibody levels are expressed as MFI for anti-PAD and CUs for ACPA. Patients were assigned as anti-PAD4, anti-PAD3 or ACPA positive when levels of circulating antibodies were >1.000 MFI for anti-PAD4 and anti-PAD3 or 20 CUs for ACPA accordingly to the manufacturer's recommendation as specified in the Methods. Positivity for the single antibody and the combination between anti-PAD4 and anti-PAD3 antibodies are shown. Anti-PAD4: anti-protein arginine deiminase type 4 antibodies; anti-PAD3: anti-protein arginine deiminase type 3 antibodies; MFI: median fluorescence intensity; CUs: chemiluminescence units.

Patients carrying both anti-PAD4 and anti-PAD3 antibodies exhibit more joint erosions

In order to further investigate the association between RA-related antibodies positivity and radiographic injury, patients of our cohort were stratified into three different groups according to their anti-PAD antibodies status as:

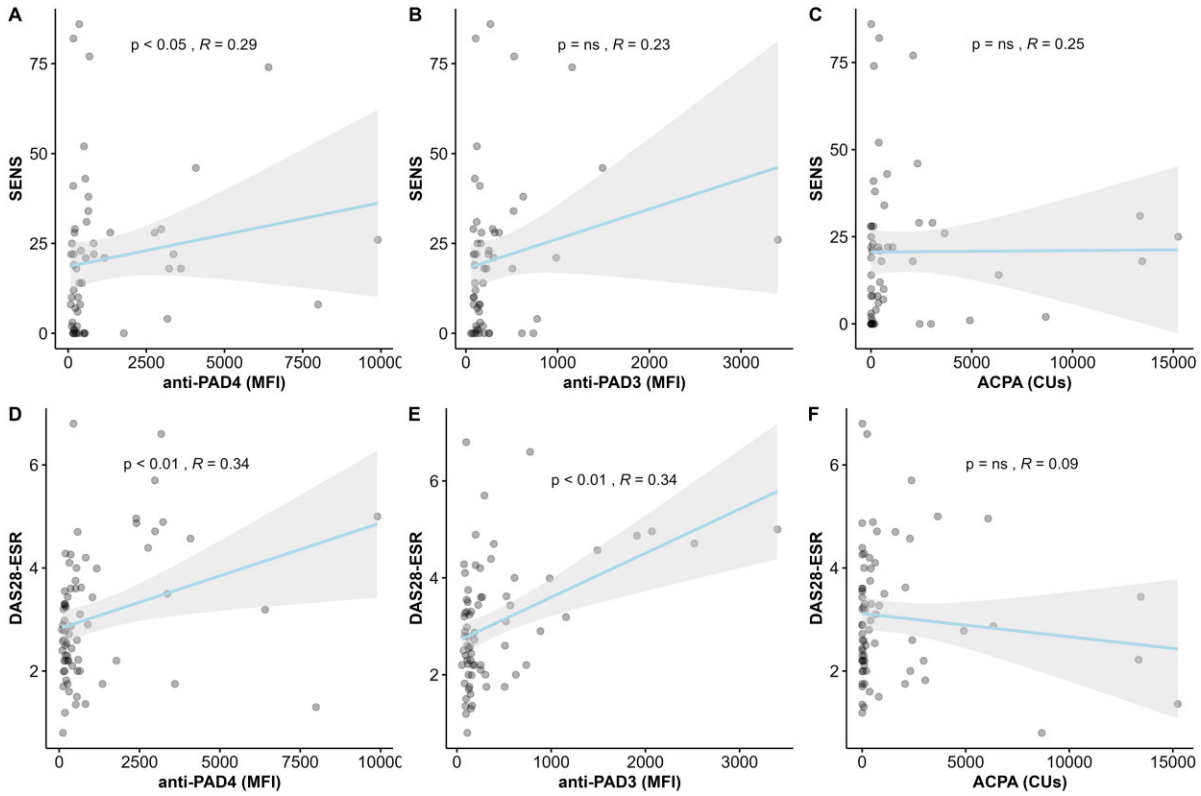

Figure 2. Association between RA-related antibodies, radiologic injury and disease activity. Joint involvement and disease activity were evaluated in the cohort of RA patients by SENS and DAS28-ESR, respectively. Levels of anti-PAD4, anti-PAD3 and ACPA were assessed by PMAT (anti-PADs) and QUANTA Flash (ACPA), as specified in the Methods. The correlation between levels of each antibody and the radiologic and laboratory parameters was calculated by Spearman's Rank Test. The lines and grey areas indicate regression line and CI, respectively. Statistically relevant correlations were observed between anti-PAD4 antibodies and both joint damage and disease activity as well as between anti-PAD3 and disease activity. The remaining comparisons did not show statistically relevant associations. PMAT: particle-based multi-analyte technology; anti-PAD4: anti-protein arginine deiminase type 4 antibodies; anti-PAD3: anti-protein arginine deiminase type 3 antibodies; SENS: Simple Erosion Narrowing Score; MFI: median fluorescence intensity; CUs: chemiluminescence units; ns: not significant

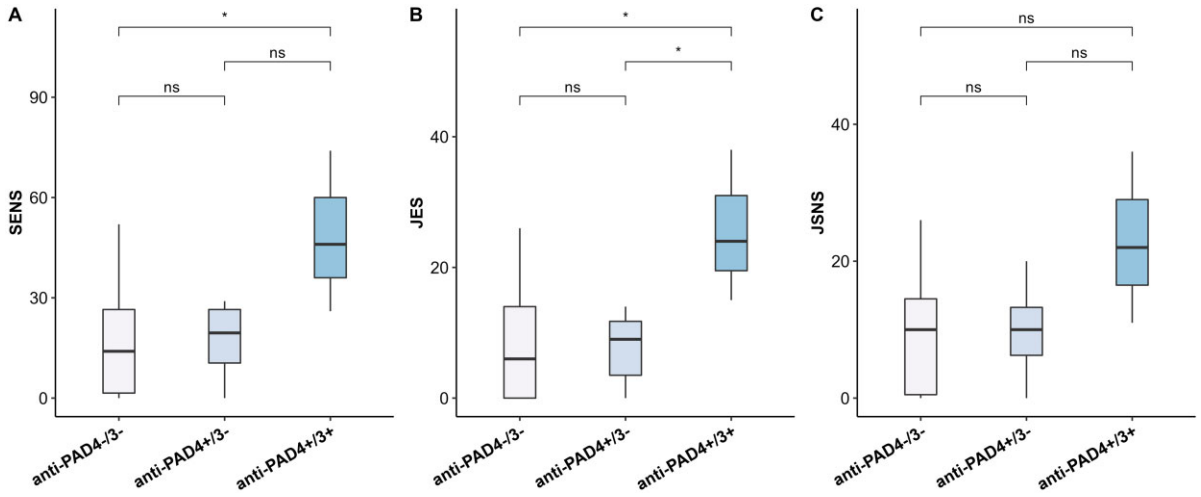

Figure 3. Anti-PAD antibodies and radiologic scores. RA patients of the cohort were categorized based on their positivity to both anti-PAD4 and anti-PAD3 antibodies (anti-PAD4$^+$/3$^+$), anti-PAD4 antibodies alone (anti-PAD4$^+$/3$^-$) or negative for both (anti-PAD4$^-$/3$^-$). Correlation between joint involvement evaluated by SENS, JES and JSNS (expressed as median, represented by the boxes) and the anti-PAD antibodies status was calculated by means of Wilcoxon Rank Sum Test and *P*-values <0.05 were considered as significant. Anti-PAD4: anti-protein arginine deiminase type 4 antibodies; anti-PAD3: anti-protein arginine deiminase type 3 antibodies; SENS: Simple Erosion Narrowing Score; JES: Joint Erosion Score; JSNS: Joint Space Narrowing Score; ns: not significant

(i) anti-PAD4 and anti-PAD3 double positive (anti-PAD4$^+$/3$^+$) ($n = 6$); (ii) anti-PAD4 positive but anti-PAD3 negative (anti-PAD4$^+$/3$^-$) ($n = 11$); and (iii) anti-PAD4 and anti-PAD3 double negative (anti-PAD4$^-$/3$^-$) ($n = 54$) patients (Table 1). None of the patients resulted as negative for anti-PAD4 but positive for anti-PAD3 antibodies.

Fig. 2A shows that SENS was significantly higher in the anti-PAD4$^+$/3$^+$ patients when compared with the anti-PAD4$^-$/3$^-$ group [48.7 (24.1), median 24, *vs* 19.4 (22.1), median 14, respectively; $P = 0.04$], whereas the comparison with the anti-PAD4$^+$/3$^-$ [17.6 (10.4), median 19.5] did not reach statistical significance ($P = 0.051$). On the basis of the previously demonstrated association between anti-PAD4 levels and bone erosions [21, 29, 30], we investigated how patterns of antibody positivity correlated to JES. Actually, anti-PAD4$^+$/3$^+$ patients exhibited a significantly higher JES value [25.7 (11.6), median 24] when compared with both anti-PAD4$^+$/3$^-$ [8 (5), median 9; $P = 0.014$] or anti-PAD4$^-$/3$^-$ [9.3 (11.5), median 6; $P = 0.037$] patients (Fig. 3B). Neither JSNS (Fig. 3C) nor disease activity measured by DAS28-ESR correlated with the antibody positivity status (data not shown).

Anti-PAD3 antibodies are negatively associated with smoking history

Next we analysed whether anti-PAD antibodies were associated with smoking. Consequently, patients were divided according to their status in ever-smokers (current or history of smoking) ($n = 27$) and non-smokers ($n = 44$), and the relationship with levels of RA-autoantibodies levels was investigated. Interestingly, anti-PAD3 levels were significantly lower in ever-smokers when compared with non-smokers [198 (200), median 120, *vs* 520 (717), median 520, respectively; $P = 0.0042$], whereas no relationship was found for anti-PAD4 antibodies or ACPA (Supplementary Fig. S2, available at *Rheumatology* online).

Anti-PAD4 antibodies are negatively correlated with RA-ILD

Lastly, to evaluate how anti-PAD antibodies might correlate with lung disease, patients were divided in two groups according to the presence (RA-ILD$^+$, $n = 11$) or absence (RA-ILD$^-$, $n = 60$) of interstitial lung involvement. Eleven out of 71 patients (15.5%) belonged to the RA-ILD$^+$ group; of the 11 (15.5%) patients with ILD, 5 (45.5%) presented with UIP, 3 with NSIP (27.3%) and 3 (27.3%) with an undetermined pattern. When compared with RA-ILD$^-$, RA-ILD$^+$ patients were significantly older [74.4 (9.6) *vs* 60.8 (11.8) years, $P < 0.001$] and presented a shorter disease duration [130.3 (116.7) *vs* 225.9 (131.6) months, $P = 0.045$] (Supplementary Table S2, available at *Rheumatology* online).

As shown in Fig. 4 and Table 2, RA-ILD$^+$ patients displayed significantly lower levels of anti-PAD4 antibodies when compared with RA-ILD$^-$ [337 (315), median 185, *vs* 1249 (1936), median 427, respectively; $P = 0.043$]. In contrast, we could not demonstrate any association between the levels of ACPA or anti-PAD3 antibodies and the presence of ILD.

Regarding the clinical course of the articular disease, RA-ILD$^+$ patients exhibited significantly lower values of both SENS [8.8 (9.9) *vs* 23.6 (22.5), respectively; $P = 0.016$] and JES [2.9 (3.8) *vs* 11.7 (11.7), respectively; $P = 0.006$] than patients with RA alone (Table 2).

Discussion

This retrospective cross-sectional study explored the usefulness and behaviour of anti-PAD4, anti-PAD3 antibodies and ACPA in a cohort of patients affected by RA. We considered the association with disease activity, radiographical progression and lung involvement.

Anti-PAD4 autoantibodies were found in almost one out of four (23.9%) RA patients in our cohort, including 4.2% of patients that were negative for ACPA. Therefore, they are a promising biomarker to improve diagnosis by closing serological gap of 'seronegative' RA. In our hands, the levels of anti-PAD4 antibodies were significantly associated with the radiographic index of disease progression expressed by the composite SENS [27]. When this overall score was split into its two components represented by the JES and the JSNS, anti-PAD4 antibodies uniquely correlated with JES. These results are in keeping with previous papers where a different method to evaluate joint involvement such as the van der Heijde-modified Sharp erosion score (SvdH) was used [29, 30]. The simplified version of the radiologic scores used here has been validated in previous studies [31, 32] and their validity is further confirmed in our study. Regarding the other RA-related autoantibodies, we were not able to find any association between anti-PAD3 levels and joint damage, at variance from what has previously been reported by others [23, 33], despite the similar cross-sectional approach. The small number of anti-PAD3-positive patients in our cohort (<10%) combined with the small cohort size might have affected our results in terms of statistical significance, as suggested by the slope of the curve, with a similar trend as anti-PAD4 antibodies. A second possibility, albeit less likely, might be related to the type of the studied population, with a long disease history and/or prolonged therapeutic interventions which might have negatively (even though selectively) influenced antibody production. Levels of ACPA, positive in about two-thirds of our patients, were significantly associated with radiographic erosions assessed by JES. This confirms previously published observations [34, 35]. Similarly, the absence of significant association between ACPA and JSNS was previously reported for SvdH-JSN [36]. Additionally, we evaluated the disease activity using DAS28-ESR [37]. The relation between anti-PAD4 antibodies and DAS28 has already been reported, but ESR was not included into the evaluation [21, 38]. In a recent paper by Lamacchia *et al.* analysing serum samples from a large number of patients of the prospective Swiss Clinical Quality Management registry, a significantly higher DAS28-ESR score was found at baseline in anti-PAD3-positive rather than -negative patients [33]. However, no association between levels of anti-PAD3 antibodies and DAS28-ESR was considered and anti-PAD4 autoantibodies were not measured. Our study finds for the first time a significant association between DAS28-ESR and anti-PAD4 antibodies in patients with RA.

It was suggested that anti-PAD4 antibodies may play a pathogenetic role in joint damage. A close association between synovial tissue inflammation and PAD4 protein expression was demonstrated and associated to a dysregulated PAD activity [39]. Actually, Darrah *et al.* [24] showed that a subset of anti-PAD4 antibodies that cross-react with PAD3 (anti-PAD3/4XR antibodies) increases PAD4 catalytic efficiency by reducing calcium request with the consequence of supporting protein citrullination. Hence, patients with anti-PAD3/4XR antibodies have a higher radiographic score at baseline and higher radiographic progression in comparison with PAD-negative individuals [24]. Similar findings on disease activity have been obtained in other studies with anti-PAD4 antibodies but not cross reacting with PAD3 [24, 26, 40]. Although

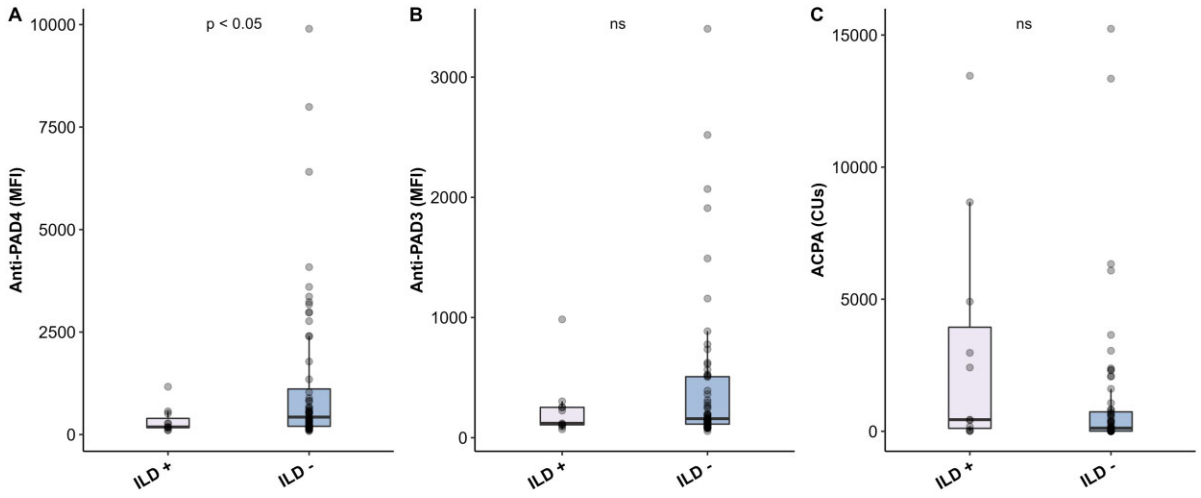

Figure 4. Association between RA-related autoantibodies and ILD. Patients were categorized on the basis of the presence (ILD^+) ($n = 11$) or absence (ILD^-) ($n = 60$) of the interstitial lung involvement and anti-PAD4, anti-PAD3 and ACPA were assessed by PMAT (anti-PADs) and QUANTA Flash (ACPA) as specified in the Methods. Results were expressed in MFI and CUs, respectively. Boxes express median levels. The correlation between ILD and RA-related antibodies was evaluated by Wilcoxon Rank Sum Test and *P*-values <0.05 were considered as significant. Anti-PAD4 antibodies were significantly lower in patients with ILD *vs* the counterpart without ILD. No association was found for anti-PAD3 or ACPA. Anti-PAD4: anti-protein arginine deiminase type 4 antibodies; anti-PAD3: anti-protein arginine deiminase type 3 antibodies; ILD: interstitial lung disease; MFI: median fluorescence intensity; CUs: chemiluminescence units; ns: not significant

Table 2. Serological and radiographic features of RA patients with ILD

Variable	RA lung involvement		*P*-value
	ILD^+ ($n = 11$)	ILD^- ($n = 60$)	
Serological features			
Anti-PAD4, mean (S.D.), MFI	337 (315)	1249 (1936)	0.043
Anti-PAD3, mean (S.D.), MFI	239 (260)	426 (636)	0.42
ACPA, mean (S.D.), CUs	3048 (4396)	1165 (2797)	0.061
Radiographic features			
SENS, mean (S.D.)	8.8 (9.9)	23.6 (22.5)	0.016
JES, mean (S.D.)	2.9 (3.8)	11.7 (11.7)	0.006
JSNS, mean (S.D.)	5.9 (6.6)	11.9 (11.3)	0.093

Anti-PAD4, anti-PAD3 and ACPA antibodies were assessed by PMAT and QUANTA Flash and levels expressed as MFI and CUs, respectively. Patients were categorized on the presence (ILD^+) or absence (ILD^-) of interstitial lung disease by high-resolution CT. SENS, JES and JSNS were evaluated as described. Values were analysed by Wilcoxon Rank Sum Test and *P*-values <0.05 considered as significant. Anti-PAD4: anti-protein arginine deiminase type 4 antibodies; anti-PAD3: anti-protein arginine deiminase type 3 antibodies; SENS: Simple Erosion Narrowing Score; JES: Joint Erosion Score; JSNS: Joint Space Narrowing Score; ILD: interstitial lung disease; MFI: median fluorescence intensity; CUs: chemiluminescence units.

we did not perform immunoprecipitation or competition studies, we presumed that double-positive patients for anti-PAD3 and anti-PAD4 antibodies carry anti-PAD3/4XR antibodies. In our cohort anti-$PAD4^+/3^+$ patients displayed a more erosive disease as expressed by significantly higher values of SENS and JES than anti-$PAD4^+/3^-$ or anti-$PAD4^-/3^-$ patients. Further prospective studies are needed to assess the ability of anti-PAD antibodies to predict a more erosive course of the disease.

Cigarette smoking is a robust risk factor for the development of RA [41] but the relationship between smoking and anti-PAD autoantibodies, however, is not clear [26]. In our hands, an inverse correlation between anti-PAD3 antibodies and tobacco smoke was found, as anti-PAD3 antibody levels resulted significantly lower in smokers rather than non-smokers. Similar findings have been recently described by Lamacchia *et al.* [33]. This counterintuitive negative correlation might point to the existence of disease subgroups driven by different environmental factors.

The second aim of our study was to examine the behaviour of anti-PAD autoantibodies with respect to extra-articular disease with focus on ILD. The prevalence of ILD in cohorts of RA patients ranges between 5 and 70% depending on inclusion criteria and studied population [42]. In any case, there is a consensus about the severity of this manifestation [4] and the urgent need for reliable markers, including autoantibodies, for an early identification and risk prediction. In our population, 11 patients (15.5%) presented ILD at HRCT scans. Half of them showed an UIP pattern, with a minority of NSIP. The prevalence of ILD in our cohort agrees with the literature data, as well as the predominance of the UIP pattern, which distinguishes RA from the other autoimmune diseases where the NSIP pattern largely prevails [7, 8, 43]. Similar to other studies, patients with ILD were significantly older [44, 45] without any link with smoking habits. Smoking has indeed been conflictingly associated with RA-ILD [46]. Except for a lower disease duration, no statistical differences were noted in terms of diseases activity as expressed by DAS28-ESR or type of articular disease at the onset between RA-ILD in comparison with patients with RA alone. An inverse correlation between anti-PAD4 antibodies and ILD was found. In our cohort patients with RA-ILD had significantly lower titres of anti-PAD4 antibodies compared with patients with RA alone. This observation contrasts the previous report by Giles *et al.* [25] that found anti-PAD3/4XR antibodies and smoking to be associated to RA-ILD. In their cohort however, male–female ratio was almost close to one, thus diverging from what has been previously reported for RA. Moreover, cardiac multi-detector row CT not including all the lung parenchyma was used, rather than HRCT. Finally, anti-PAD autoantibodies were measured using a different methodology. An alternative explanation is selection bias caused by the shorter life expectancy of patients with ILD. The average disease duration in our cohort was 222.5 months for patients without ILD and 130.3 months for patients with ILD. In fact, the shorter disease

duration could also imply that the patients with ILD simply did not have enough time to develop the radiographic damage. Further prospective and larger studies aimed at this issue are needed to clarify if this observation is caused by different disease endotypes or by a survivorship bias.

No significant association between ACPA levels and ILD could be found in our study population. The relation between ACPA and the risk of developing ILD has been repeatedly reported in the literature [17, 25] and ACPA production in other tissues than joints might indeed occur [17, 25, 47]. It is possible that the small number of RA-ILD patients in our cohort was responsible for the lack of significance, in as much as the RA-ILD group displayed a 3-fold higher ACPA value *vs* RA non-ILD patients. Given that anti-PAD4 antibodies are associated with erosive disease, we hypothesize that patients with ILD might represent a distinct population with fewer bone erosions and less joint damage, although fully respecting classification criteria of RA, and, possibly, more elevated levels of ACPA [48]. In our cohort, these patients are characterized by older age, lack of correlation with smoke, shorter disease duration and low disease activity as expressed by DAS28-ESR.

This research has strengths and limitations. We assessed novel autoantibodies such as anti-PAD4 and anti-PAD3 in a real-life cohort fulfilling RA classification criteria. Furthermore, the patients with ILD were well characterized, studied by HRCT scans and interpreted by expert thoracic radiologists as part of a multidisciplinary team on lung fibrosis. Finally, the erosivity was evaluated with a radiographic score (SENS) which helped us to stratify joint involvement. The main limitations are the small size of the cohort and the cross-sectional design of our study. Additional confounding factors could be the time difference between the blood sampling and the joint involvement assessment and the treatment heterogeneity.

In conclusion, we assessed anti-PAD4, anti-PAD3 antibodies and ACPA in a cohort of RA patients with and without ILD, in combination with radiologic scores considering bone erosions and joint narrowing. This real-life study confirms that anti-PAD4 and anti-PAD3 antibodies are helpful biomarkers of radiographic injury. Furthermore, patients positive for both anti-PAD4 and anti-PAD3 exhibited higher radiographic injury than single anti-PAD4-positive, and anti-PAD4 and anti-PAD3 double-negative patients. Finally, we found lower levels of anti-PAD4 antibodies in patients with RA-ILD. We hypothesize that low anti-PAD4 antibodies might identify a subgroup of patients with non-erosive RA and prevalent ILD. Future prospective studies on larger RA-ILD cohorts are needed to confirm this association and their clinical and serological features. The recognition of serological signatures that cluster specific disease phenotypes could contribute to early diagnosis and to tailoring of personalized treatment strategies. Our data further validate the association of anti-PAD3/4 antibodies with more erosive disease, but also provide contradictory data on association with ILD that warrants further research. The difference between the findings might be related to the patient cohorts, or potentially based on methodological differences. As recently demonstrated, the isotype of anti-PAD antibodies has a major effect on clinical associations. Consequently, it is feasible that different anti-PAD assays, depending on the anti-human IgG conjugate used, have varying preference of IgG subclasses which can therefore drive clinical associations.

Supplementary data

Supplementary data are available at *Rheumatology* online.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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