Citrullination in extra-articular manifestations of rheumatoid arthritis

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Background. Anti-citrullinated protein antibodies have been detected with high specificity in serum of patients with rheumatoid arthritis (RA), and citrullination of proteins may play a key role in the pathogenesis of RA. We therefore investigated the presence of citrullination in two extra-articular manifestations of RA, interstitial pneumonia (IP) and rheumatoid nodules.

Methods. Open-lung biopsy specimens from patients with RA-associated IP (n = 18), idiopathic IP (n = 20) and controls (n = 10), as well as specimens of rheumatoid nodules from 26 patients, were examined. All sections were incubated with an anti-modified citrulline antibody. Masked scoring of stained sections and analysis of results by stratification according to demographic and clinical characteristics was performed.

Results. Presence of citrulline could be detected in eight lung specimens of patients with RA-associated IP (44%) and nine patients with idiopathic IP (46%). Conversely, lung tissue from control patients showed weak extracellular citrullination in only two cases (20%). Citrullination did not show any significant associations with demographic or clinical characteristics such as age, gender, smoking habits, disease severity, histological subtype, degree of inflammation or steroid use. Rheumatoid nodules were citrulline positive in a majority of cases (70%).

Conclusion. Citrullination is present in extra-articular manifestations of RA such as IP and nodules. In contrast to the high specificity of anti-citrulline antibodies in RA, citrullination is not only restricted to RA but can also be observed in idiopathic IP. Whether citrullination significantly contributes to the initiation or perpetuation of autoimmunity or merely reflects ongoing inflammation remains to be clarified.

KEY WORDS: Citrullination, Interstitial pneumonia, Rheumatoid arthritis, Extra-articular disease, Rheumatoid nodules.

Introduction

Anti-citrullinated protein antibodies (ACPA) have been detected with high specificity in serum and synovial fluid of patients with rheumatoid arthritis (RA) [1, 2]. Several observations have indicated that ACPA-positive patients with RA may develop a more severe disease than those without ACPA [3]. The presence of their targets in the inflamed synovium, some of which appear to be RA-specific, suggests a key role for citrullinated proteins in the pathogenesis of RA [4–6]. Indeed, synovial citrullinated proteins may be one of the major autoantigens driving the local immune response as suggested by the local production of ACPA in the joint [7] and the direct association between RA-specific synovial citrullinated proteins and local and systemic ACPA levels [8]. While extra-articular disease, including interstitial lung disease, is a major contributor to the morbidity and premature mortality of patients with RA [9], it remains unknown if citrullinated proteins are present at extra-articular sites of RA and if they could contribute to the local disease process by a citrullinated protein–ACPA immune mediated process.

Therefore, we undertook an investigation to determine whether citrullination can be detected in lung tissue from patients affected by RA-associated interstitial pneumonia (IP). We further examined whether citrullination is specific for RA-associated IP by comparing our findings to idiopathic IP and normal lung tissue. In order to clarify whether citrullination is restricted to RA lung tissue, we also evaluated rheumatoid nodules as another form of extra-articular disease for evidence of citrullination.

Methods

Selection of tissue samples

Paraffin-embedded open-lung haematoxylin and eosin (H&E)-stained biopsy specimens from patients with RA-associated IP, idiopathic IP and control lung were reviewed by a participating pathologist (J.L.M) blinded to the clinical diagnoses in order to assign a histological subclassification according to the international consensus statement of the American Thoracic Society (ATS) and the European Respiratory Society (ERS) [10]. Paraffin-embedded specimens of subcutaneous rheumatoid nodules from 26 patients with RA were also included in our analysis. All patients classified as having RA met the 1987 American College of Rheumatology criteria for diagnosis of RA [11].

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The medical records from all patients and controls were reviewed for demographic information, disease duration, pulmonary function testing, smoking habits and medications at the time of biopsy.

Lung specimens were also examined from DBA1 male mice \((n=6)\) with severe collagen-induced arthritis (arthritis score 8–10) induced by immunization with 100 \(\mu\)g collagen in complete Freund’s adjuvant, followed by a second boost at day 21 in incomplete Freund’s adjuvant and killed at day 120. Four DBA1-positive naïve male mice served as controls.

The study was approved by the Mayo Clinic Institutional Review Board.

Immunohistochemical staining

Citrullination was detected using an anti-modified citrulline antibody (Upstate, New York, NY, USA) that allows the detection of all citrulline residues after chemical modification independently of their amino acid context. After post-fixation in formaldehyde/glutaraldehyde and antigen retrieval in sodium citrate, the citrulline residues were derived as previously described [4, 5]. Sections were then incubated overnight with the anti-modified citrulline antibody, followed by a two-step peroxidase staining.

Non-derived sections were used as negative control and normal human skin as positive control. The sections were blinded and scored by three independent observers. In order to evaluate a potential association of the presence of inflammatory cells and citrullination, we performed a quantitative analysis of CD4, CD3- and CD20-positive cells in our tissue samples. Immunohistochemical (IHC) staining of these cells was performed as reported previously [12]. The following mouse monoclonal antibodies were used: anti-CD20 (clone L26, dilution 1/60, DAKO Cytomation), anti-CD1a (clone O10 dilution 1/50 DAKO Cytomation) and anti-CD3 (clone PS1 dilution 1/100 DAKO Cytomation). The sections were counterstained with Modified Schmidt's Haematoxylin. Each slide was scanned using the Bacus Laboratories Inc. Slide Scanner (BLISS; Bacus Laboratories, Inc.). Computer-assisted analysis was performed by one of the investigators (S.R.A) who was blinded to the diagnosis, using the IHCScore software (Bacus Laboratories, Inc.).

This software allows the measurement of the IHC staining area, quantified as the IHC percentage area (%IHC, referenced to the total tissue area).

As shown previously, comparison of stained tissue areas reflect the ratios calculated on the basis of actual lymphocyte counts [12].

Analysis and data display

Proportions of samples with a positive anti-citrulline staining were calculated for RA-associated IP, idiopathic IP and control tissue, and were compared between disease groups using Fisher’s exact test.

To examine for potential associations between patient characteristics and citrulline-positivity, results were stratified according to demographic characteristics such as age, gender, smoking status, and steroid use, and according to the disease characteristics such as forced vital capacity (FVC) as a measure of disease severity [13], histological subtype and degree of inflammatory infiltration (quantified as percentage of tissue area stained for CD3, CD20 or CD1a, referenced to the total tissue area). In each disease group, these data were compared between the citrulline-positive and the citrulline-negative group using Fisher’s exact test or Mann–Whitney \(U\)-test as appropriate.

In addition, staining for inflammatory cell markers in RA-associated IP vs idiopathic IP was compared in the citrulline-positive and citrulline-negative groups separately. A \(P\)-value <0.05 was regarded as statistically significant. All calculations were performed using StatsDirect statistical Software (www.statsdirect.com).

Results

Patient Characteristics

Lung tissue specimens from 18 patients diagnosed with RA-associated IP could be retrieved from our archive, classified as either non-specific IP (NSIP) type \((n=10)\) or usual IP (UIP) type \((n=8)\). This group of patients with RA consisted of 10 women and eight men with a mean (±S.D.) age of 62.5 ± 10.0 yrs and a mean disease duration of 8.3 (±7.8) yrs.

There were 20 patients with idiopathic IP (eight with UIP; 12 with NSIP). The mean age of these 10 men and 10 women was 58.6 (±11.4) yrs at the time of lung biopsy. Control biopsies were from 10 patients (six men and four women) with lung carcinoma, who had a mean age of 56.8 (±15.9) yrs; tissue specimens used for analysis were from uninvolved tissue areas as confirmed by histological assessment.

Citrullination in RA-associated IP

Staining with the anti-modified citrulline antibody, which recognizes citrulline after chemical modification independently of the surrounding amino acid context, indicated the presence of citrulline in the lung tissue of eight patients with RA-associated IP. As illustrated in Figure 1, citrullination was located intracellularly in mononuclear cells in the vast majority of cases. Citrulline-positive cells were predominantly found in the subpleural tissue, with occasional staining of interalveolar tissues. The staining was more pronounced in areas of inflammatory infiltrates. No clear spatial relation to areas of fibrosis could be detected.

Specificity of citrullination for RA-associated interstitial pneumonia

In order to assess if this citrullination was specific for RA-associated IP, we compared this with lung tissue from patients with idiopathic IP and control patients. Whereas anti-citrulline staining was observed in 44% of the RA lung samples, lung tissue from normal controls showed presence of weak extracellular citrullination in only two cases (20%). Conversely, pulmonary citrullination was detected in nine of 20 patients with idiopathic IP (45%) and was not significantly different from patients with RA-associated IP \((P=0.99)\). Further characterization of the staining pattern showed that intracellular staining was seen in 39% of the RA samples vs 35% in idiopathic IP. Extracellular staining accompanied intracellular staining in two patients with RA-associated pneumonia (11%) and one patient with idiopathic IP (5%). Exclusive extracellular staining was observed in one patient with RA-associated and two patients with idiopathic IP (Table 1). Thus, citrullination occurs commonly in RA as well as in idiopathic IP.

In order to investigate if diseased lung tissue is the only extra-articular site of RA characterized by citrullination, we also stained rheumatoid nodules from 26 patients (Figure 2). As shown in Table 1, intracellular staining was seen in five of these specimens (19%), and was accompanied by extracellular staining in three patients (12%). Exclusive extracellular staining, which occurred mostly in necrotic areas (Figure 2) was seen in 13 patients (50%).

Association of pulmonary citrullination with demographic characteristics

Considering the presence of citrullination in some but not all lung samples of both RA-associated and idiopathic IP, we next analysed if important demographic features may influence this process. As shown in Table 2, there were no significant differences in age and gender between citrulline-positive and citrulline-negative patients in both the RA and the control group.
FIG. 1. Lung biopsy specimens of RA-associated interstitial pneumonia (IP) and idiopathic IP, stained using an anti-modified citrulline antibody. RA-associated UIP (A); Idiopathic UIP (B); RA-associated NSIP with positive staining in the area of inflammatory infiltrates (C); Idiopathic NSIP (D).

**TABLE 1.** Citrulline staining pattern in RA-associated interstitial pneumonia (IP), idiopathic IP, control lungs and rheumatoid nodules

<table>
<thead>
<tr>
<th></th>
<th>RA-associated IP (n = 18)</th>
<th>Idiopathic IP (n = 20)</th>
<th>Control lungs (n = 10)</th>
<th>Rheumatoid nodules (n = 26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Only extracellular</td>
<td>1 (5%)</td>
<td>2 (10%)</td>
<td>2 (20%)</td>
<td>13 (50%)</td>
</tr>
<tr>
<td>Only intracellular</td>
<td>5 (28%)</td>
<td>6 (30%)</td>
<td>0 (0%)</td>
<td>2 (8%)</td>
</tr>
<tr>
<td>Intra- and extracellular</td>
<td>2 (11%)</td>
<td>1 (5%)</td>
<td>0 (0%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>Overall citrulline</td>
<td>8 (44%)</td>
<td>9 (45%)</td>
<td>2 (20%)</td>
<td>18 (70%)</td>
</tr>
<tr>
<td>vs control lung, $P^a = 0.25$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vs idiopathic IP, $P^b = 0.99$</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

RA, rheumatoid arthritis; IP, interstitial pneumonia.

$^a$Fisher’s exact test.

FIG. 2. Rheumatoid nodule stained using an anti-modified citrulline antibody (A); skin control (B).
Moreover, although positive staining was essentially seen in areas relation to the presence or absence of citrullination were noted. Disease severity
Steroid use
Histology subtype
Smoking status
Gender
Age at diagnosis

Citrull, citrullination; RA, rheumatoid arthritis; IP, interstitial pneumonia; FVC, forced vital capacity; UIP, usual IP; NSIP, non-specific IP.

Association of pulmonary citrullination with disease characteristics
To further assess the relationship between citrullination and the type or degree of local inflammation of the lung, we assessed the lung disease severity (FVC), the histological subtype and the degree of inflammatory infiltration in citrulline-positive vs citrulline-negative patients in both RA-associated and idiopathic IP. As shown in Table 2, severity of pulmonary disease as assessed by the FVC was not different between citrulline-positive and citrulline-negative patients. No differences in NSIP vs UIP in relation to the presence or absence of citrullination were noted. Moreover, although positive staining was essentially seen in areas with a high degree of inflammatory infiltration, the global degree of inflammation as assessed by the infiltration with CD3+ T-lymphocytes, CD20+ B-lymphocytes and CD1a+ antigen-presenting cells was not significantly different between citrulline-positive and citrulline-negative patients in RA as well as idiopathic IP (Table 3).

However, in patients with positive citrulline staining, pulmonary infiltrates of CD3+ T-cells (P < 0.01), CD20+ B-cells (P = 0.03) and CD1a+ cells (P < 0.01) were significantly more prominent in RA-associated IP as compared with idiopathic IP. This statistically significant difference in cellularity between RA-associated and idiopathic disease was not observed in citrulline-negative patients.

Pulmonary citrullination in collagen-induced arthritis
To further investigate whether there was an association between peripheral arthritis and subclinical pulmonary inflammation with or without local citrullination, we next investigated lung tissue in the most commonly used mouse model of RA: collagen-induced arthritis in DBA1 mice. In the lung tissues of non-immunized control mice (n = 4) without arthritis, there were neither histological alterations on H&E staining nor positive staining with the

Table 2. Citrullination in RA-associated IP, idiopathic IP and normal lungs, stratified by demographic and clinical characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RA-associated IP</th>
<th>Idiopathic IP</th>
<th>Control lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citrull+</td>
<td>Citrull−</td>
<td>P*</td>
</tr>
<tr>
<td>Total samples</td>
<td>8 (44%)</td>
<td>10 (56%)</td>
<td>–</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>5 (63%)</td>
<td>3 (37%)</td>
<td>0.34</td>
</tr>
<tr>
<td>Women</td>
<td>3 (30%)</td>
<td>7 (70%)</td>
<td>–</td>
</tr>
<tr>
<td>Age at diagnosisb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
<td>0.66</td>
</tr>
<tr>
<td>&gt;65</td>
<td>3 (50%)</td>
<td>3 (50%)</td>
<td>–</td>
</tr>
<tr>
<td>Smoking statusb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-smokers</td>
<td>3 (60%)</td>
<td>2 (40%)</td>
<td>0.99</td>
</tr>
<tr>
<td>Ever smokers</td>
<td>5 (45%)</td>
<td>6 (55%)</td>
<td>–</td>
</tr>
<tr>
<td>Steroid useb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (38%)</td>
<td>5 (62%)</td>
<td>0.62</td>
</tr>
<tr>
<td>No</td>
<td>5 (63%)</td>
<td>3 (37%)</td>
<td>–</td>
</tr>
<tr>
<td>Disease severityb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FVC &gt; 70</td>
<td>2 (40%)</td>
<td>3 (60%)</td>
<td>0.99</td>
</tr>
<tr>
<td>FVC &lt; 70</td>
<td>5 (50%)</td>
<td>5 (50%)</td>
<td>–</td>
</tr>
<tr>
<td>Histology subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UIP</td>
<td>4 (50%)</td>
<td>4 (50%)</td>
<td>0.99</td>
</tr>
<tr>
<td>NSIP</td>
<td>4 (40%)</td>
<td>6 (60%)</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 3. Quantitative comparison of inflammatory cells between citrulline-positive and citrulline-negative lung tissue (quantification as stained tissue area:%IHC)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>RA-associated IP</th>
<th>Idiopathic IP</th>
<th>Control lungs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Citrull+</td>
<td>Citrull−</td>
<td>P*</td>
</tr>
<tr>
<td>CD1a%IHC</td>
<td>1.17 (0.77;1.62)</td>
<td>1.15 (0.33;2.38)</td>
<td>0.95</td>
</tr>
<tr>
<td>(IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD20%IHC</td>
<td>3.23 (0.92;6.47)</td>
<td>1.90 (0.83;4.68)</td>
<td>0.99</td>
</tr>
<tr>
<td>(IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3% IHC</td>
<td>0.047 (0.005;0.104)</td>
<td>0.013 (0.004;0.021)</td>
<td>0.11</td>
</tr>
<tr>
<td>(IQR)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Citrull, citrullination; RA, rheumatoid arthritis; IP, interstitial pneumonia; IQR, interquartile range.

Comparison of citrulline-positive vs citrulline-negative (Mann–Whitney U-test).
anti-citrulline antibody. Similarly, the DBA1 mice immunized with collagen and developing severe arthritis \((n = 6)\) displayed no signs of histological inflammation or citrullination in their lungs.

**Conclusions**

Protein modification by citrullination may represent an important step in bypassing immunotolerance [14] or enhancing autoimmune reactivity [15]. In RA, the high specificity of ACPA [1, 2] and the presence of distinctive citrullinated proteins in the synovial tissue [6] raised the hypothesis that the immune response to citrullinated autoantigens may play a specific role in the pathogenesis of RA. Considering this hypothesis and the fact that extra-articular disease is also associated with increased RA severity, we investigated the presence of citrullination in RA-associated IP and a potential contribution of citrullination to the local disease process.

According to the results of our study, citrullination is not only present in synovial tissue of patients with RA but can also occur in extra-articular tissue affected by the disease. Almost half of our patients with RA-associated IP showed evidence of pulmonary citrullination, which is similar to the proportion of RA patients with synovial citrullination observed in previous studies [6]. It is, however, unclear if indeed only a subset of the patients display citrullination in their affected lungs or if this process occurs more commonly but is variable over time in an individual patient. Besides evidence of sporadic extracellular positive staining, citrullination in both RA-associated as well as idiopathic IP (but not control tissue) was especially located inside mononuclear cells. This intracellular location appears especially interesting in the light of recent insights in the role of cellular location of antigens and the positive and negative selection of autoreactive B-cells; while extracellular presence of self-antigens usually leads to B-cell tolerance, intracellular location may lead to exaggerated positive B-cell selection and predispose for autoantibody production [16]. In this context, our findings provide a solid basis for proceeding with biochemical characterization of citrullinated proteins from fresh RA lung tissue and compare these with the citrullinated proteins present in synovium. Independent of the exact biochemical nature of these proteins, the demonstration of citrullination in RA-associated IP raises the question if this process is related to the disease pathogenesis. Although ACPA are highly specific for RA, citrullination was not restricted to RA-associated IP and could also be detected in idiopathic IP. These findings are consistent with previous observations that citrullination of proteins such as fibrin is apparent in different types of synovial tissue inflammation and is not specific for RA [4, 17]. Along the same line, citrullination was not only found in RA-associated IP but also at other sites of extra-articular disease such as rheumatoid nodules.

Because we used an antibody that recognizes all citrullinated proteins after chemical modification independently of their amino acid context, our observation does not exclude the presence of distinctive citrullinated epitopes, which are specific for RA-associated IP. In fact, Baeten et al. [6] detected RA-specific synovial citrullinated proteins using a polyclonal and a monoclonal anti-citrulline antibody, which recognizes not only the citrulline residue but also the surrounding amino acids. Unfortunately, assay with these antibodies, which have only been successfully used on frozen tissue sections, is not technically possible using paraffin-embedded lung tissue specimens. Taken together, our data confirm that citrullination appears to occur in a variety of pathological conditions, but the question remains open if citrullination of some well-defined proteins is specific for RA and which of the citrullinated proteins are really physiologically relevant for the humoral autoimmune response [4, 7].

In order to evaluate whether certain clinical characteristics predispose individual patients with RA-associated or idiopathic IP to citrullination in the lung tissue, we performed a stratified analysis based on several demographic and clinical variables. Although the power of our analysis was limited, there was no strong association between the presence of citrullination and age, gender or use of steroids in both patient cohorts. Moreover, there was no association with smoking status in the RA-associated and idiopathic IP and normal lung tissue obtained from smokers did not show intracellular citrullination.

Thus, these data do not confirm the recent data that intracellular citrullination in the lungs may be induced by smoking, as suggested by the positive immunostaining for citrullinated proteins in bronchoalveolar lavage cells from smokers but not from non-smokers [18]. However, this issue certainly deserves further confirmatory studies considering the major gene-environment interaction between smoking and HLA-DR shared epitope genes in ACPA-positive RA patients.

We have previously shown a significant increase of infiltrating T-cells [12] and B-cells [19] in RA-associated IP as compared with idiopathic IP. In the present staining, a statistically significant increase of infiltrating cells in RA-associated IP vs idiopathic IP was only observed when comparing citrulline-positive lung tissue. This suggests that although citrullination may occur only in a subset of RA-associated IP, it could play an important role in dysregulated immune responses and lymphocyte proliferation. Although a recent study indicated a small increase of the immunogenicity of collagen after citrullination in collagen-induced arthritis [15], the exact role of citrullination in synovial as well as extra-articular inflammation is unclear. Interestingly, intracellular citrullination in our tissue samples was spatially associated with areas of inflammatory infiltrates, independent of the histological subtype of IP. This could simply be due to the higher cellularity in these areas, but also may be a sign of an enhanced immunostimulation in areas with citrullination of potential antigens. However, the quantification of CD1a-, CD3- and C20-positive cells in citrulline-positive vs citrulline-negative tissue specimens revealed no statistically significant differences.

It is as yet uncertain why citrullination takes place in only some inflamed tissues independently of the amount of infiltrating cells. Still, when present, citrullination appears to be spatially associated with areas of inflammation. It is also unclear if this citrullination may enhance the local disease process with clinically detectable consequence, since we could not detect any difference in FVC between patients with and without citrullination in the lungs. Because it is unlikely that this question can be fully addressed in human RA and since inflammation-related citrullination has been demonstrated in synovium in experimental arthritis models [20], we also assessed pulmonary citrullination in collagen-induced arthritis. Although subclinical interstitial lung disease may be present in a majority of patients with early RA [21], we did not detect any abnormalities including presence of citrullinated proteins in the lung tissues of mice with collagen-induced arthritis. Apparently, this mouse model differs significantly from human disease, and these mice do not develop pulmonary disease as an extra-articular manifestation of RA.

Due to the retrospective nature of our study, we were unable to evaluate the presence of ACPA in our cohort. Therefore, we determined ACPA titres in a separate, consecutive sample of 31 patients recently diagnosed with idiopathic IP (20 UIP, 10 NSIP, 1 desquamative IP). None of these patients was ACPA-positive. Conversely, in a sample of 10 patients with RA-associated IP, all but one individuals showed high titres of anti-CCP antibodies (unpublished data).

This observation makes it unlikely that pulmonary citrullination as such is sufficient to drive the autoimmune response but does not exclude the possibility that it contributes to the local pathology in ACPA-positive RA patients.

In conclusion, our study indicated clearly that citrullination occurs in RA-associated IP but also demonstrated similar findings in idiopathic IP. This strengthens the concept that citrullination...
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itself is not specific for RA but rather is associated with the inflammatory process in certain patients. However, the detection of citrullination independently of the amino acid context cannot exclude a specificity of distinctive citrullinated proteins for RA-associated disease as previously indicated in synovium. The absence of relation with features such as smoking status and with severity of pulmonary disease leaves the question open as to what factors lead to pulmonary citrullination and whether citrullination of pulmonary proteins contributes to the initiation, perpetuation or acceleration of autoimmunity or merely reflects ongoing inflammation without a significant pathophysiological role in the disease process.

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