

# Potential protein targets of the peptidylarginine deiminase 2 and peptidylarginine deiminase 4 enzymes in rheumatoid synovial tissue and its possible meaning

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## Abstract

**Objective:** The molecular mechanism of citrullination involves the calcium-dependent peptidylarginine deiminase (PAD) family of enzymes. These enzymes induce a stereochemical modification of normal proteins and transform them into autoantigens, which in rheumatoid arthritis trigger a complex cascade of joint inflammatory events followed by chronic synovitis, pannus formation, and finally, cartilage destruction. By hypothesizing that PAD2 and PAD4 enzymes produce autoantigens, we investigated five possible synovial protein targets of PAD enzymes.

**Material and Methods:** We measured PAD2, PAD4, and citrullinated proteins in 10 rheumatoid and 10 osteoarthritis synovial biopsies and then assessed the post-translational modifications of fibrinogen, cytokeratin, tubulin, IgG, and vimentin proteins using a double-fluorescence assay with specific antibodies and an affinity-purified anti-citrullinated peptide (CCP) antibody. The degree of co-localization was analyzed, and statistical significance was determined by ANOVA, Fisher's exact test, and regression analysis.

**Results:** The principal results of this study demonstrated that citrullinated proteins, such as fibrinogen, IgG, and other probed proteins, were targets of PAD2 and PAD4 activity in rheumatoid synovial biopsies, whereas osteoarthritis biopsies were negative for this enzyme ( $p < 0.0001$ ). An analysis of citrullination sites using the UniProtKB/Swiss-Prot data bank predicts that the secondary structure of the analyzed proteins displays most of the sites for citrullination; a discussion regarding its possible meaning in terms of pathogenesis is made.

**Conclusion:** Our results support the conclusion that the synovial citrullination of proteins is PAD2 and PAD4 dependent. Furthermore, there is a collection of candidate proteins that can be citrullinated.

**Keywords:** Peptidylarginine deiminase 2, peptidylarginine deiminase 4, rheumatoid arthritis, synovial membrane

## Introduction

Peptidylarginine deiminase (PAD) belongs to a family of calcium-dependent enzymes that convert arginine residues of proteins into citrulline. These enzymes are expressed in most mammalian tissues, with the most common PAD2 enzyme widely distributed throughout human and murine tissues (1). This isoform is responsible for the majority of PAD activity, and the importance of PAD2 has been demonstrated by the observation that all PAD activity is abrogated in the PAD2 knockout mouse (2). Protein citrullination is an important pathophysiological mechanism in rheumatoid arthritis (RA), which is supported by the evidence that patients with RA produce antibodies against modified cellular components known as antiperinuclear factor (APF). These autoantibodies are specific to RA, and their immunochemical specificity is directed against filaggrin. The APF antibody was identified using normal epithelial cells of the oral mucosa as the antigenic source and rheumatoid serum (3-5). The anti-citrullinated peptide (CCP) antibodies were identified many years later and are considered as early markers of RA (6-10). Therefore, the importance of citrullinated epitopes in rheumatoid pathogenesis has continued to increase, and the molecular significance of citrullination has been tested *in vitro* using various approaches. Because of these studies, it is known that fibrinogen, vimentin, enolase, collagen, and other proteins are post-translationally modified (11-14) through their arginine residues being deiminated and transformed into citrulline. This steric change modifies the electrostatic properties of peptides, resulting in an increase in immunogenicity and changes that intensify the interaction of target proteins with certain pockets of Class II major histocompatibility complex (MHC) molecules (DR4 0401). By this mechanism, a modified protein increases its probability of being presented in a restricted peptide repertoire as an autoantigen (15, 16).



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Submitted: 07.07.2015

Accepted: 05.09.2015

Available Online Date: 01.02.2016

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Considering the fact that PAD2 and PAD4 isoforms are overexpressed in rheumatoid synovial tissue (17-20), it appears reasonable to infer that an antigenic stimulation may arise at a synovial level. Therefore, this study aimed to assess potential protein targets of PAD2 and PAD4 enzymes in rheumatoid synovial tissue.

## Material and Methods

### Rheumatoid arthritis and control synovial tissue

Tissue samples were obtained during joint replacement for prosthesis surgery, and 10 biopsies from patients with a mean age of  $41.2 \pm 13.1$  years and who met the American College of Rheumatology (ACR) criteria for RA classification (21) were analyzed. There were six females and four males with 10 years of evolution (range, 3–13 years), three with functional class (FC) III, five with II, one with I, and one was not defined by the ACR classification of functional status in RA (22). All patients were positive for rheumatoid factor and anti-CCP, were under treatment with a low dose of  $\leq 5$  mg prednisone, and received methotrexate with a mean dose of 12.5 mg (range, 7.5–17.5 mg). Additionally, 10 biopsies were obtained from patients with osteoarthritis (mean age,  $54.2 \pm 7.1$  years), wherein six patients were females and four males. They were included as controls, none of them had anti-CCP antibodies, and one was positive for rheumatoid factor at an irrelevant concentration; furthermore, these patients underwent knee surgery for osteoarthritis, all of them received analgesics and/or NSAIDs, and obesity was the accelerating factor for knee osteoarthritis (23, 24). In all cases, signed informed consent was obtained, and the bioethics committee at our institution approved this protocol.

### Purification and labeling of affinity-purified anti-CCP antibodies

An anti-CCP serum sample obtained from a patient with RA and who displayed high anti-CCP antibodies according to ELISA (25) was used to purify high-affinity antibodies in the following manner: serum was incubated in a commercial ELISA microwell plate (Axis-Shield Diagnostics Ltd.; Dundee, Scotland), and the anti-CCP-bound antibodies were then eluted from the polystyrene CCP-coated plates after a 2-h incubation with 0.2 M glycine-HCl at pH 2.8. The eluted antibodies were then neutralized with 1 M Tris at pH 9.5. The recovered antibodies were concentrated using a Centricon® centrifugal device (Merck Millipore Co.; Billerica, MA, USA). One fraction of the high-affinity-purified anti-CCP antibodies was labeled with horseradish peroxidase (HRP) (Sigma; St. Louis, MO, USA) using the method described by Abrameas with our own modifications. The molar ratio of the high-affinity-purified antibodies to peroxi-

dase was 1:10 (26, 27). Another fraction of the affinity-purified anti-CCP antibody was used to perform the double-fluorescence assays.

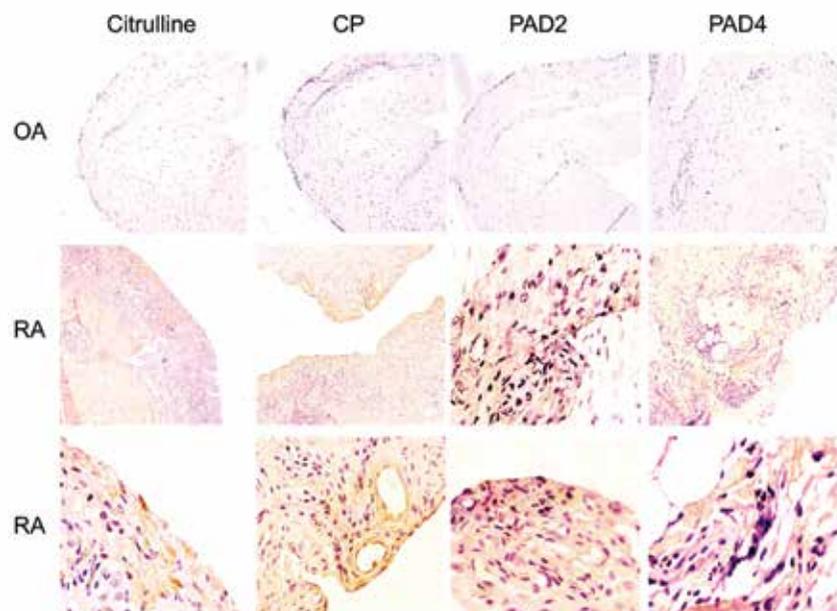
### Immunohistochemistry

Slides containing 4- $\mu$ m sections of the synovial tissue were dewaxed, permeabilized, and washed three times with PBS. Endogenous peroxidase was then blocked with horse serum that had been inactivated at 56°C. The tissues were incubated with purified human IgG (precipitated from normal human sera with ammonium sulfate and IgG was purified using HiTrap protein G HP columns) to neutralize the presence of possible rheumatoid factor activity in the synovial tissues. After three washes, the tissues were incubated for 1 h with the monoclonal antibody or affinity-purified human autoantibodies diluted in 10% fetal calf serum/PBS (anti-citrulline antibody, cat. 231246, Calbiochem, Darmstadt, Germany; 1:100 dilution), anti-PAD2 antibody (PA5-19474, Pierce Antibody products, Thermo Fisher Scientific Inc.; Rockford, IL, USA; 1:30 dilution), and anti-PAD4 (Pierce PA5-12236; 1:30 dilution). After

multiple washes with PBS, the slides were then incubated for 1 h with HRP-labeled goat anti-human IgG. After additional washes, a color reaction was induced using 3,3'-diaminobenzidine–0.06% H<sub>2</sub>O<sub>2</sub> (Sigma; San Louis, MO, USA), and the reaction was stopped with 2 N sulfuric acid. All assays were performed in duplicates. In the case of the HRP-labeled anti-CCP, the immunohistochemistry was direct and did not rely on the use of a secondary antibody.

### Double-fluorescence labeling assays

To assess the presence of citrullinated protein in the synovial tissue, we co-localized the possible target proteins using the following steps. Citrullinated proteins were tagged as green through a 120-min incubation with an unlabeled and affinity-purified anti-CCP antibody, followed by a 120-min incubation with a FITC-labeled secondary antibody (ab4220 or ab68881, Abcam; Cambridge, MA, USA). After each incubation, the slides were washed three times with PBS. The target protein was tagged as red by overnight incubation with specific antibodies against fibrinogen (Abcam ab666),



**Figure 1.** Detection of peptidylarginine deiminase (PAD) expression in the synovial tissue by immunohistochemistry. In the coronal panels, biopsies of osteoarthritis (OA; controls) are negative. The middle panels show rheumatoid arthritis (RA) biopsies at 10 $\times$  magnification. The basal panels show RA biopsies at 20 $\times$  magnification

**Table 1.** PAD enzymes and citrullinated protein expression in the synovial tissues of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) as controls

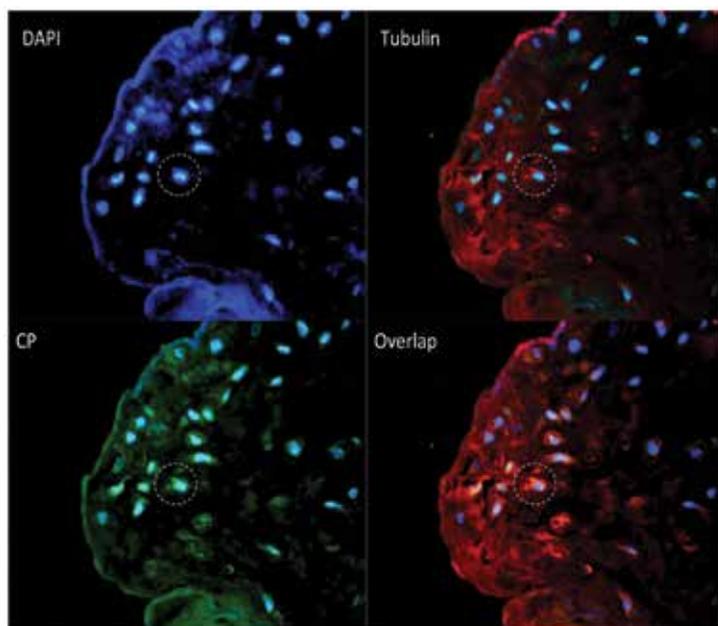
Biopsies	PAD2 positive	PAD4 positive	CP positive	Citrulline positive
RA (n=10)	10 (100%)	7 (70%)	10 (100%)	10 (100%)
OA (n=10)	0	0	0	1 (10%)
p	<0.0001	<0.003	<0.0001	<0.002

CP: citrullinated proteins; PAD: peptidylarginine deiminase; RA: rheumatoid arthritis; OA: osteoarthritis  
Fisher's exact test (two tailed).

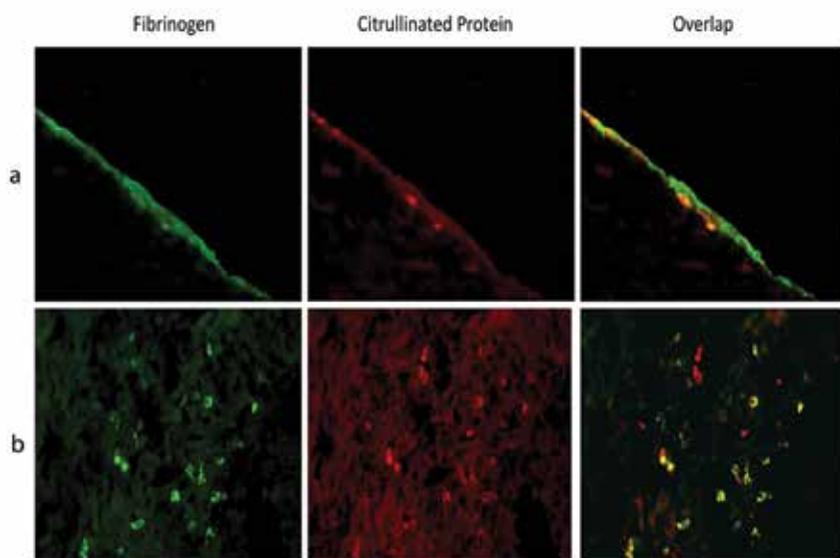
**Table 2.** Localization of PAD2 and PAD4 enzymes and citrullinated proteins (CP) in synovial of rheumatoid arthritis (RA) patients

RA biopsies (n=10)			
Localization	In villi	In fibroblast-like cells	In inflammatory infiltrates
PAD2	8 (80%)	2 (20%)	6 (60%)
PAD4	8 (80%)	2 (20%)	6 (60%)
CP	8 (80%)	5 (50%)	3 (30%)

CP: citrullinated proteins; PAD: peptidylarginine deiminase; RA: rheumatoid arthritis



**Figure 2.** Double-fluorescence assays show the co-localization of tubulin and citrullinated protein (CP) in the synovial tissue of rheumatoid arthritis (RA). Circle of overlap cell figure in yellow that probably represents citrullinated tubulin. Note that tubulin is stained in red and CP in green; nuclei were counterstained by DAPI in blue



**Figure 3.** Double fluorescence of rheumatoid synovial tissue (a), the axial panel represents the synovial edge, the green immunofluorescence corresponds to the synovial edge, the red fluorescence corresponds to the staining by an antibody reactive against citrullinated proteins, and the overlap picture corresponds to the merged image where the red and green fluorescence has a significant correlation ( $r=0.846$ ) (b), the panel represents a field with an inflammatory focus of the same biopsy

cytokeratin (Sigma K8.13, Prod. C6909), IgG, vimentin (Cell Signaling D2H3), and  $\beta$ -tubulin (Abcam ab21057), followed by a 120-min incubation with a rabbit anti-human Texas Red-labeled antibody (IgG-TR: sc-3920, Santa Cruz Biotechnology; Paso Robles, CA, USA). Finally, the slides were counterstained with DAPI, mounted, and evaluated under a fluorescence microscope using the appropriate filters for the dyes.

### Statistical analyses

Data were processed using the GraphPad Prism software (GraphPad Software Inc.; CA, USA) for the non-parametric assays, such as ANOVA and Fisher's exact test. Regression analyses were performed using Pearson's test. A p value  $<0.05$  was considered statistically significant.

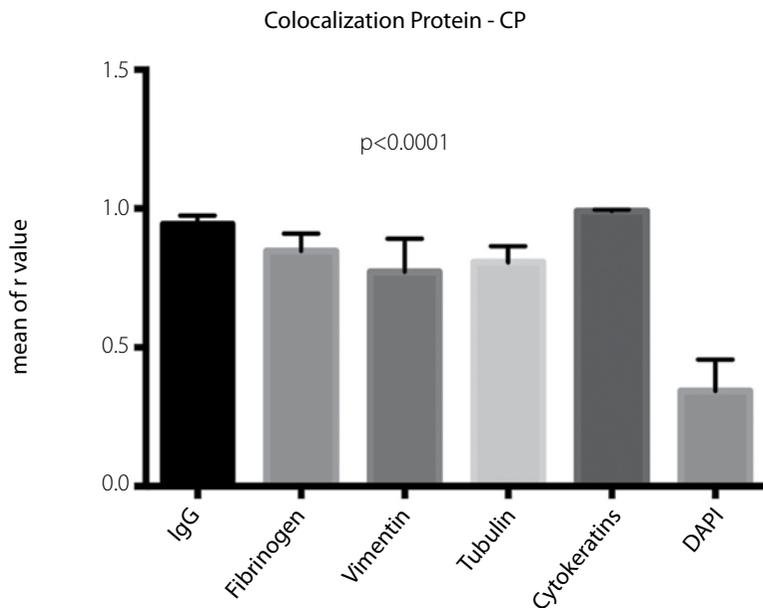
### Results

#### PAD2 and PAD4 expression exclusive to rheumatoid synovial tissues

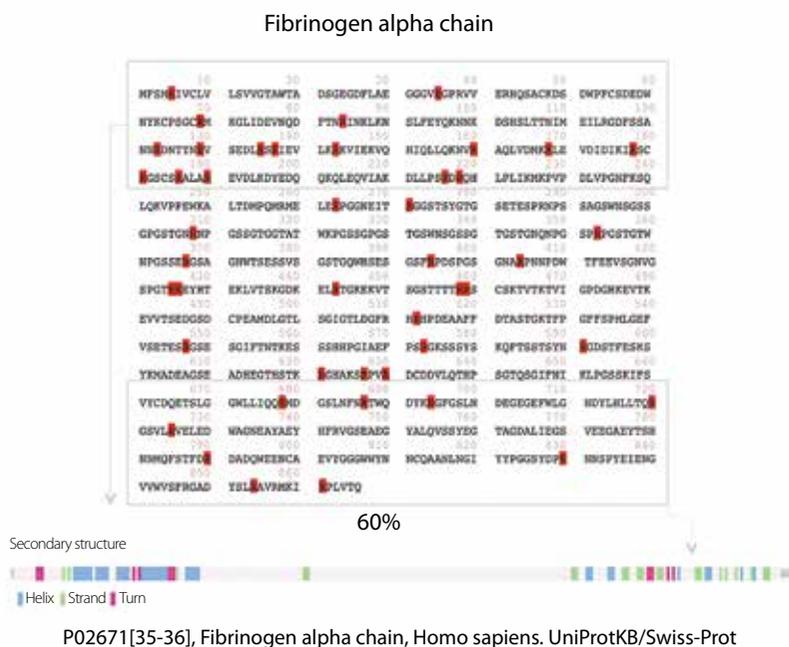
PAD2 and/or PAD4 enzymes were expressed in the synovial tissues of all patients with RA (100%). In sharp contrast, all of the OA controls tested negative for these enzymes, and the differences between both the groups were significant ( $p<0.0001$ ). In rheumatoid synovial tissue, PAD2 was expressed in the 10 biopsies of RA with a strong signal by immunohistochemistry assays; however, PAD4 expression was faint, and it was present in seven synovial biopsies. Notably, PAD expression was detectable at a range of levels, particularly at the synovial border and in some biopsies along the villi. Moreover, PAD expression was particularly prominent along the inflammatory infiltrates as well as in specific cells, such as fibroblast-like synoviocytes (Figure 1 and Table 1).

#### PAD enzymes citrullinate synovial proteins

This study also aimed to assess whether PAD2 enzyme could convert the residues of target proteins into citrulline in situ. Current results suggest that PAD2 and PAD4 are functional enzymes because they induced post-translational modifications of target proteins as suggested by the immunohistochemical tag induced by the anti-CCP antibody. A subset of the biopsies exhibited a widespread distribution of post-translationally modified proteins that correlated well with citrullinated areas that were identified by anti-citrulline antibodies. Therefore, the presence of these three immunoreagents (PAD2/PAD4, CP, and citrulline) suggests that the citrullination process depends on PAD activity, which was developed in the following locations: within villi, fibroblast-like cells, and inflammatory infiltrates (Table 2).



**Figure 4.** Cell-by-cell analysis of the co-localization graph of various candidate proteins for citrullination. Above, a graph of the mean and SD of correlation obtained by the co-localization of green and red fluorescence expressed as r-value means. The sum of these values obtained from cytoplasmic co-localization in individual cells was compared with the values of nuclear (DAPI) co-localization



**Figure 5.** An example of sites suitable for citrullination in the fibrinogen alpha chain, which are predicted by sequence analysis of data P02671 obtained in UniProtKB/Swiss-Prot data bank. Note that 60% of the arginine residues are located in the secondary structure

**Protein candidates for citrullination**

To address which proteins are the targets of PAD activity, we screened a group of five proteins using a double-fluorescence assay and were able to demonstrate a good degree of co-localization between candidate proteins tagged in red and citrullinated protein domains tagged in green or viceversa. Such a PAD activity induces an *in situ* protein citrullination that produces a yellow color, and a possible catalytic reaction was expressed as

an “r” value. The image analysis yields the following values: IgG, 0.993±0.0; fibrinogen, 0.846±0.06; vimentin, 0.771±0.01; β-tubulin, 0.806±0.05; and cytokeratin, 0.990±0.04. The tentative citrullination areas were mainly observed in the cytoplasm; however, the nuclear cellular domains (blue staining by DAPI) had lower values (0.353±0.12). Consequently, the differences between nuclear and cytoplasmic citrullination were significant with a p value of <math>< 0.0001</math> (Figure 2-4).

**Discussion**

This study aimed to identify the possible protein targets of PAD activity in rheumatoid synovial tissue. To accomplish this goal, a double-fluorescence approach was used to detect the possible protein targets of PAD2 and PAD4 enzymes in the synovial tissues of patients with RA. The primary results of this study can be summarized as follows: 1) PAD2 and PAD4 were exclusively detected in rheumatoid synovial tissue and 2) to demonstrate whether synovial PADs were functional, an anti-CCP antibody was purified from rheumatoid serum and then used in immunohistochemistry and in a double-fluorescence assay to co-localize candidate substrates for PAD, including fibrinogen, IgG, β-tubulin, vimentin, and cytokeratin. The results of this assay suggested that a variety of proteins suitable for citrullination are potential targets of PAD2 and PAD4 enzymes.

Various attempts to disclose the possible targets of PAD activity have been proposed (28), and on the basis of recent data, we know that PAD activity may exhibit a range of substrate specificities. For example, PAD2 citrullinates β/γ-actin, whereas PAD4 citrullinates the H3 histone. Additionally, functional PAD2 and PAD4 isoforms are predominantly active in rheumatoid synovial tissue (29-31). Furthermore, the *in vitro* assays demonstrated that fibrinogen, α-enolase, and other PAD-modified proteins become autoantigens, and patients with RA produce autoantibodies against citrullinated peptides. Notably, some of these citrullinated peptides can be detected *in vivo* in synovial fluid (30). Considering the aforementioned results, we assumed that the citrullination process occurs in rheumatoid synovial tissue. It is important to note that all these works suggest that the citrullination process is developed in the synovial tissue or synovial fluid, and proteins, such as fibrinogen or vimentin, are stereochemically modified and become potential sources of autoantigens (32). The main limitation of this study and other studies is the limited number of samples. Another limitation is the lack of association between synovial citrullinated proteins and HLA-DRB1 (shared MHC epitope) as reported by Verpoort et al. (33); finally, another limitation is that most of the studies indirectly demonstrated citrullination by an anti-CCP antibody rather than directly elucidating the changes in protein structure induced by citrulline that are demonstrated by magnetic resonance spectroscopy (32-34).

These results confirmed synovial PAD activity, suggesting that this catalytic mechanism is important in the pathophysiology of RA. Addi-

tionally, we demonstrated that a few candidate targets of PAD activity were post-translationally modified in rheumatoid synovial tissue. Our results suggested that certain modified proteins are potential triggers of autoimmune responses in patients with RA. Finally, taking into account the present results, some of the candidate proteins were further analyzed using citrullination-prediction analysis. For this purpose, we used the UniProtKB/Swiss-Prot data bank to explore the distribution of accessible arginine residues in the secondary structures of the studied proteins. This analysis yielded the following results: the fibrinogen alpha chain displays 60% of the residues suitable for citrullination in its secondary structure and the fibrinogen beta chain displays 79% of the residues suitable for citrullination in its secondary structure distributed near the extreme N- and C-terminal ends, whereas vimentin displays only 42% of the residues suitable for citrullination in its secondary structure (Figure 5).

Theoretically, the steric shift induced by citrullination affects the electrostatic properties of the candidate proteins. This modification consequently affects the structure and relaxation dynamics of citrullinated proteins, causing the conformational modification of native proteins to produce neo-epitopes. Notably, this phenomenon could be induced by calcium-dependent reactions, which is the case for PAD enzymes (35, 36).

Our results support the conclusion that the synovial citrullination of proteins is PAD dependent. Moreover, there is a collection of candidate proteins that can be citrullinated, and it appears possible that in some patients, the citrullination process can be initially triggered in the synovial tissue. Taking into account the results obtained in other studies, various tissues, such as the gingiva and lung, may serve as post-translational locations of PAD enzymes. Consequently, this process can occur at multiple sites in the bodies of patients with RA (37-42). Regardless of where citrullination occurs, the result may be similar, representing a formidable source of autoantigens; nevertheless, despite the broad range of candidate proteins for citrullination as suggested by current results, the most important variable to trigger an autoimmune response is the high-affinity binding of citrullinated peptides to the class II molecules, followed by strong co-stimulatory signals and the appropriate cytokine feeding. Therefore, we can assume that citrullination is an important mechanism from the pathogenic perspective of rheumatoid autoimmunity.

**Ethics Committee Approval:** Ethics Committee approval was received for this study from participant institutions.

**Informed Consent:** Written informed consent was obtained from patients who participated in this study.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept - A.B.S., M.R.R., M.E.P.P., M.A.B.S., R.H.E.; Collection and/or Processing - L.D.B., M.A.C.J., M.R.R., M.E.P.P.; Materials and Data - J.J.B.G., Supervision - E.A.D.; Writer and Critical Review - R.H.E., E.A.D.

**Conflict of Interest:** No conflict of interest was declared by the authors.

**Financial Disclosure:** This project was financially supported by PROMEP UAZ-CA5 Autoinmunidad.

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