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Autoantibodies against citrullinated histone H3 in rheumatoid arthritis and periodontitis patients

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Abstract

Aim: To determine the presence of citrullinated histones in inflamed periodontal tissue and to determine the presence of anti-citrullinated histone autoantibodies in sera from patients with rheumatoid arthritis (RA) and periodontitis (PD) patients.

Methods: The presence of citrullinated histone H3, PAD4 and CD68 was determined in 15 periodontal tissue biopsies from PD patients by immunohistochemistry. Sera from 36 healthy controls (HC), 113 PD patients and 84 patients with RA were assessed on presence of autoantibodies against citrullinated histones by Western blot and against citrullinated histone H3 by ELISA.

Results: Citrullinated histone H3, PAD4 and CD68 were present in periodontal tissue from nine (60%), 14 (93%) and 13 (87%) PD patients, respectively. Anti-citrullinated histone H3 autoantibodies were found in 33 (39%) patients with RA compared to three (8%) HC and 11 (10%) PD patients. Anti-citrullinated histone H3 levels were higher in anti-cyclic citrullinated peptide (anti-CCP)-positive compared to anti-CCP-negative patients with RA (p = .0008) and correlated moderately with anti-CCP levels (ρ = .22). No associations were found between anti-citrullinated histone H3 levels and periodontal status or smoking behaviour of patients with RA.

Conclusion: PD patients are exposed to citrullinated histone H3 in inflamed periodontal tissue. Citrullinated histone H3 is targeted by autoantibodies present in RA sera. This supports a role for periodontitis in generation of antigens targeted by autoantibodies directed against citrullinated proteins.

KEYWORDS

ACPA, autoantibodies, citrullinated histone H3, periodontitis, rheumatoid arthritis

1 | INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease affecting 0.5%–1% of the adult population and is characterized by synovial inflammation and joint destruction (Willemze, Trouw, Toes, & Huizinga, 2012). The majority of patients with RA (50%–80%) is seropositive for autoantibodies. The two most common autoantibodies are rheumatoid factor (RF) and autoantibodies against citrullinated proteins (ACPA), with the latter being most specific for RA. Citrullination is a post-translational modification of peptide-bound arginine which is modified to citrulline by the enzyme peptidyl arginine deiminase (PAD). The number of citrullinated proteins that is known to be targeted by ACPA is limited; citrullinated fibrinogen, vimentin, α-enolase, filaggrin are the major known citrullinated antigens (Wegner et al., 2010a). In humans, five different isotypes (PAD1–PAD4, PAD6) are present of which PAD2 and PAD4 are considered to be involved in the generation of citrullinated autoantigens in RA as these two PAD isotypes have been found in RA synovium (Foulquier et al., 2007).

PAD2 is mainly present in macrophages, whereas PAD4 is expressed by neutrophils. Smoking, the main environmental risk factor for RA, is able to enhance PAD expression in bronchoalveolar lavage cells and lung mucosa which in turn may lead to the generation of citrullinated proteins (Makrygiannakis et al., 2008).

In recent years, neutrophils have been studied for their role in the pathology of RA, specifically for their possible role in ACPA initiation (Wright, Moots, & Edwards, 2014). Neutrophils are important during the innate immune response against microbes via phagocytosis of bacteria, degranulation and formation of neutrophil extracellular traps (NETs). These NETs consist of a chromatin meshwork of which histones are the major component. NETs are able to trap and kill microbes with antimicrobial peptides that are typically present in neutrophil granules (Brinkmann et al., 2004). Neutrophils from patients with RA show enhanced NETosis compared to neutrophils from healthy controls (HC) both in the absence or presence of added stimuli (Khandpur et al., 2013). Importantly, citrullination of histones by PAD4 is considered to be an essential step in the formation and stabilization of NETs (Li et al., 2010). Recently, citrullinated histones H2A, H2B (Corsiero et al., 2015) and H4 (Pratesi et al., 2014) have been described as targets of autoantibodies in patients with RA. Therefore, NETs can be considered as a potential source of citrullinated proteins which could be targeted by ACPAs (Dwivedi & Radic,2014).

The development of RA has been hypothesized to be associated with periodontitis (PD) by reason of one periodontal pathogen, *Porphyromonas gingivalis* (Rosenstein, Greenwald, Kushner, & Weissmann, 2004), that has its own PAD enzyme (PPAD) (McGraw, Potempa, Farley, & Travis, 1999). In consequence, *P. gingivalis* has the unique capability to citrullinate (human) proteins in periodontal lesions, thereby potentially creating targets for ACPA (Wegner et al., 2010b). Earlier work from our group has demonstrated that the inflamed periodontium is indeed a source of citrullinated proteins (Nesse et al., 2012). Neutrophils are abundantly present in inflamed periodontal tissue and are the first cells to migrate to periodontal lesions upon infection or inflammation (Cooper, Palmer, & Chapple, 2013; White, Chicca, Cooper, Milward, & Chapple, 2016); this is in sharp contrast to synovial tissue in which neutrophils are scarce. Also, NETs have been found in the gingival pockets and in the purulent crevicular exudates of patients with chronic PD (Vitkov, Klappacher, Hannig, & Krautgartner, 2009). A study by Neeli, Khan, & Radic (2008) showed that histone H3 is citrullinated by PAD4 in neutrophils after an inflammatory response.

This study investigated whether citrullinated histone H3 is present in inflamed periodontal tissue. In addition, this study assessed whether citrullinated histone H3 is a target of autoantibodies in the serum of patients with RA. Furthermore, associations between the presence of autoantibodies against citrullinated histone H3 and periodontal status or smoking status in patients with RA were studied.

Clinical Relevance

Scientific rationale for the study: Periodontitis has been hypothesized to play a role in the initiation of rheumatoid arthritis via excessive citrullination in the periodontium which could induce autoantibody formation directed to citrullinated proteins (ACPAs). Neutrophils are abundantly present in inflamed periodontal tissue and express peptidylarginine deiminase 4 (PAD4), the enzyme involved in citrullination and neutrophil extracellular trap (NET) formation.

Principal findings: In periodontal tissue, citrullinated histone H3 is present, which is a target for autoantibodies in patients with RA.

Practical implications: The presence of citrullinated histone H3 in inflamed periodontal tissue supports a role for periodontitis in generation of antigens that are targeted by ACPA.

2 | MATERIALS AND METHODS

2.1 | Study groups

Serum samples were collected from adult patients with established RA (n = 84), severe untreated PD (n = 113) and HC (n = 36) without systemic disease and without PD. Serum samples of patients with RA and HC were previously used as control groups in a study in which sera from patient groups of various sizes with or without mucosal inflammation were assessed and compared on the presence of arthritis-associated autoantibodies (Janssen et al., 2015).

Patients with established RA were consecutively recruited between March and September 2011 at the outpatient clinic of the Department of Rheumatology and Clinical Immunology of the University Medical Center Groningen in Groningen. The periodontal status of patients with established RA, all fulfilling the ACR 1987 criteria for RA (Arnett et al., 1988), was assessed according to the Dutch Periodontal Screening Index (DPSI) (Van der Velden, 2009). Patients with RA were categorized according to the DPSI scores in categories A (no PD, DPSI scores 0, 1 and 2), B (moderate PD, DPSI score 3−) and C (severe PD, DPSI scores 3+ and 4).

Patients with untreated generalized severe PD were excluded if they had any other systemic disease or they had antibiotic use <3 months before inclusion (Janssen et al., 2015).

HC were recruited among subjects planned for first consultation at the Department of Dentistry of the University Medical Center, Groningen. Periodontal health was assessed using the DPSI, with the inclusion criterion defined as DPSI score ≤2 (absence of PD). Exclusion criteria were antibiotic use <3 months before inclusion and the presence of any systemic disease (Janssen et al., 2015). Patient characteristics are summarized in Table 1.

Histological staining was performed on inflamed periodontal tissue sections of 15 randomly selected PD patients (DPSI 4), without any other systemic disease, obtained from a former study (Nesse et al., 2012). Non-inflamed periodontal tissue samples from six patients undergoing prophylactic removal of impacted third molars (wisdom teeth) were used as control samples (Nesse et al., 2012).

All participants provided written informed consent before study enrolment in compliance with the Declaration of Helsinki. The study

TABLE 1 Characteristics of study participants

	Healthy controls (HC)	Periodontitis patients (PD)	Patients with rheumatoid arthritis
N	36	113	84
Age, yrs, mean (SD)	34 (15)	51 (9)*	56 (11)*
Female (%)	56	59	69
Smoking (former or current), *n* (%)	8 (22)	88 (78)*	34 (40)
No PD (%)	100	0	31
Moderate PD (%)	0	0	40
Severe PD (%)	0	100	29
DAS28, median (IQR)	NA	NA	2.2 (1.7–2.7)
CRP (mg/L), median (IQR)	0.4 (0.3–1.5)	1.0 (0.6–2.4)	1.9 (1.0–6.0)*
IgM RF positive, *n* (%)	1 (2.8)	8 (7.1)	62 (74)*
Anti-CCP positive, *n* (%)	0 (0)	1 (0.9)	63 (75)*

*$p < .0001$ compared to HC.

DAS28, Disease Activity Score of 28 joints; CRP, C-reactive protein; IgM RF, IgM rheumatoid factor (positive score defined as ≥10 IU/ml); anti-CCP, anti-cyclic citrullinated peptide antibody, measured by Euro Diagnostica anti-CCP2 kit (positive score defined as ≥25 U/ml).

was conducted with approval of the Medical Ethical Committee of the University Medical Center of Groningen (UMCG 2011/010).

2.2 | Immunohistochemical detection of citrullinated histone H3, PAD4 and CD68

Human paraffin-embedded gingival tissue samples were collected from PD patients. Five-micrometre sections were prepared on glass slides. Before staining, endogenous peroxidase activity was inhibited by incubating 0.3% H_2O_2 in methanol and followed by blocking off non-specific antibody binding with 1% BSA and 1% normal goat serum in PBS. Subsequently, tissue samples were stained with rabbit anti-citrullinated histone H3 (ab5103; Abcam, Cambridge, UK) 1:250 in PBS + 1% BSA, rabbit anti-PAD4 (ab3877; Abcam) 1:200 in PBS + 1% BSA or mouse anti-CD68 (clone KP1; Dako, Glostrup, Denmark) 1:100 in PBS + 1% BSA. Next, sections were incubated with goat anti-rabbit IgG-HRP (P0448; Dako) in PBS + 1% BSA or rabbit anti-mouse IgG-HRP (P0260; Dako), followed by using a DAB kit (K3467; Dako). Tissues were additionally stained with a rabbit IgG control (Southern Biotech, Birmingham, AL, USA) to control for non-specific binding, as previously described (Nesse et al., 2012). Sections were counter-stained with haematoxylin and mounted with glycerin. In each periodontal tissue sample, positive cells were counted in 10 adjacent fields with a 40× objective (magnification 400×). Mean cell numbers per tissue sample were calculated for each staining. Immunohistochemical scoring was performed by one trained person.

2.3 | Neutrophil isolation and stimulation

Blood was obtained from healthy volunteers. Neutrophils were isolated from buffy coat using Lymphoprep (Axis Shield, Oslo, Norway) centrifugation, followed by lysis of erythrocytes using ice-cold 0.15 M ammonium chloride solution. Neutrophils were rinsed with Hank's balanced salt solution supplemented with 140 mg/ml calcium and 100 mg/ml magnesium (HBSS++). Neutrophils were re-suspended at 1×10^6 cells/ml HBSS++. After seeding, neutrophils were stimulated 3 h with 1.9 μM calcium ionophore (A23187; Sigma, Zwijndrecht, NC) or left untreated. Then, neutrophils were re-suspended and centrifuged; the resulting pellet containing neutrophils was subsequently used for histone extraction.

2.4 | Histone extraction

Histones were obtained using an acid extraction method (Shechter, Dormann, Allis, & Hake, 2007). Briefly, pellets of 1×10^6 cells/ml stimulated and unstimulated neutrophil cells were incubated overnight in 0.2 M H_2SO_4 at 4°C with agitation. Subsequently, acid-extracted proteins were precipitated with 33% trichloroacetic acid for 2 h at 4°C, followed by two wash steps with 100% acetone and finally re-suspension in dH_2O. Protein concentrations of re-suspended histones were determined using bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

2.5 | SDS-PAGE and immunoblotting

Extracted histones, from unstimulated and stimulated neutrophils, were resolved by 15% polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride membranes (Millipore, Amsterdam, NC, the Netherlands). Membranes were blocked with Odyssey blocking buffer (Li-Cor, Lincoln, CA, USA) for 1 hr followed by incubation overnight at Li-Cor (Lincoln) 4°C with human serum diluted 1:100 in PBS, anti-citrullinated histone H3 (ab5103; Abcam) dilution 1:500, or anti-histone H3 (ab1791; Abcam) dilution 1:1000. Detection was performed with secondary antibody goat anti-human

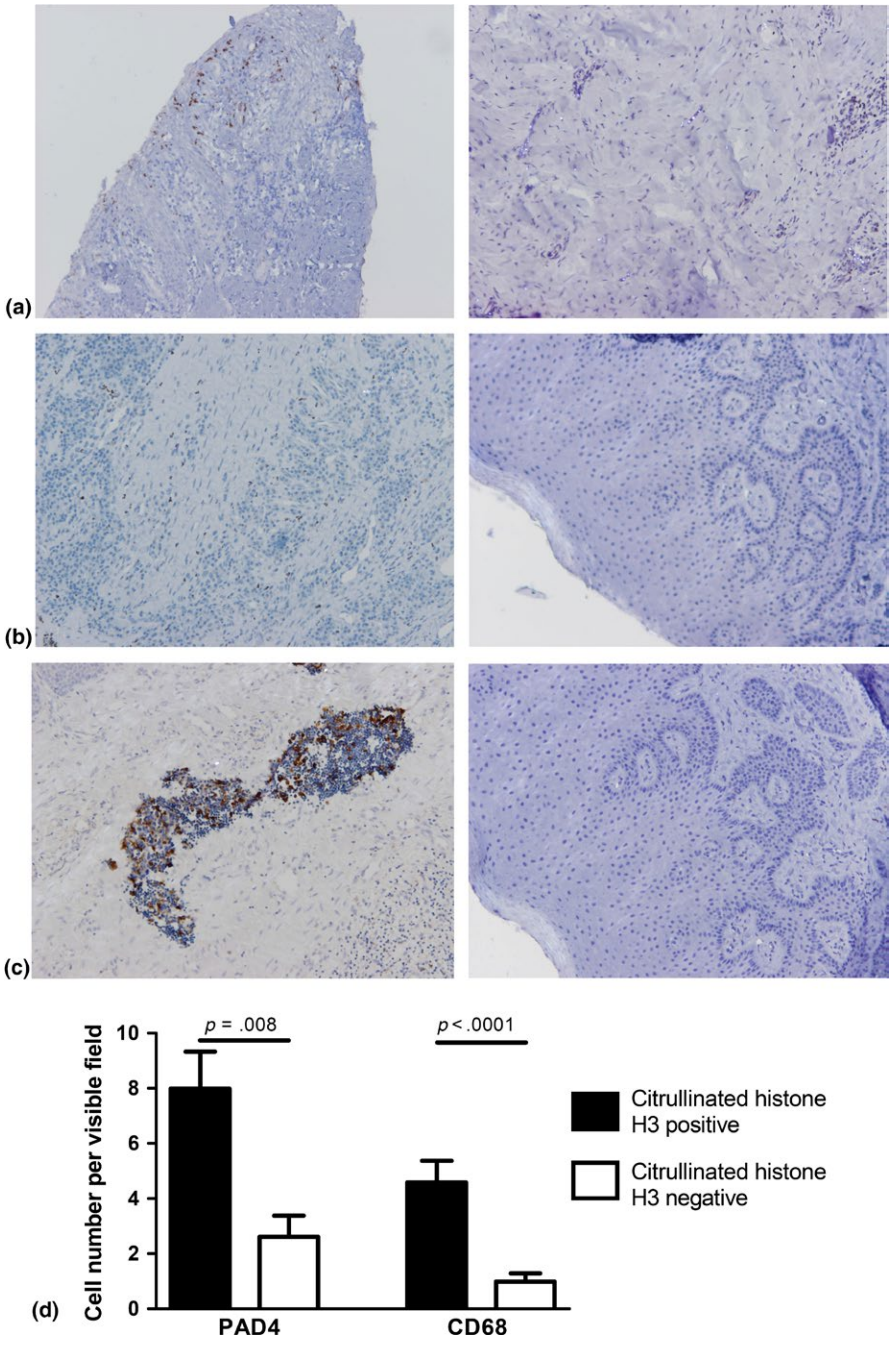

FIGURE 1 Immunohistological staining in inflamed periodontal tissue from periodontitis (PD) patients and healthy controls (HC). Representative stainings of (a) citrullinated histone H3 (b) PAD4 and (c) CD68 in periodontal tissue from PD patients (left) and HC (right). The brown depicts the presence of stained proteins. Magnification 100×. (d) Mean cell numbers of PAD4-positive cells and CD68-positive cells in citrullinated histone H3-positive and citrullinated histone H3-negative tissues. Results represented in mean ± SEM

IgG labelled with IRD680RD (Li-Cor) or goat anti-rabbit IgG labelled with IRD680RD (Li-Cor). Membranes were scanned and analysed using the Odyssey infrared imaging scanner (Li-Cor).

2.6 | ELISA detecting anti-citrullinated histone H3 in human sera

Costar ELISA plates (Corning, New York, NY, USA) were coated overnight at room temperature (rt) with citrullinated histone H3 (citrulline 2 + 8 + 17) peptides (ab32876; Abcam) (1 μg/ml) or non-citrullinated histone H3 peptides (ab12149, Abcam) (1 μg/ml) in PBS. Subsequent blocking was performed by 2% BSA-PBS for 1 h following incubation with human sera diluted 1:100 in 1% BSA in PBS + 0.05% Tween 20 for 1 hr at rt. After washing, mouse anti-human IgG-HRP 1:2000 (9040-05; Southern Biotech) was added to the wells and plates were incubated for 1 hr at rt. Bound antibodies were visualized using tetramethylbenzidine and hydrogen peroxide. Reactivity was determined separately by measuring the difference in reactivity against the peptide and background (BSA), with the cut-off defined as the difference in optical density (ΔOD) >2SD above the mean of HC.

2.7 | Statistical analysis

Data were analysed using GraphPad Prism 5 (Graphpad Software, San Diego, CA, USA). For comparisons between groups, unpaired two-tailed *t* tests were used for variables with Gaussian distribution

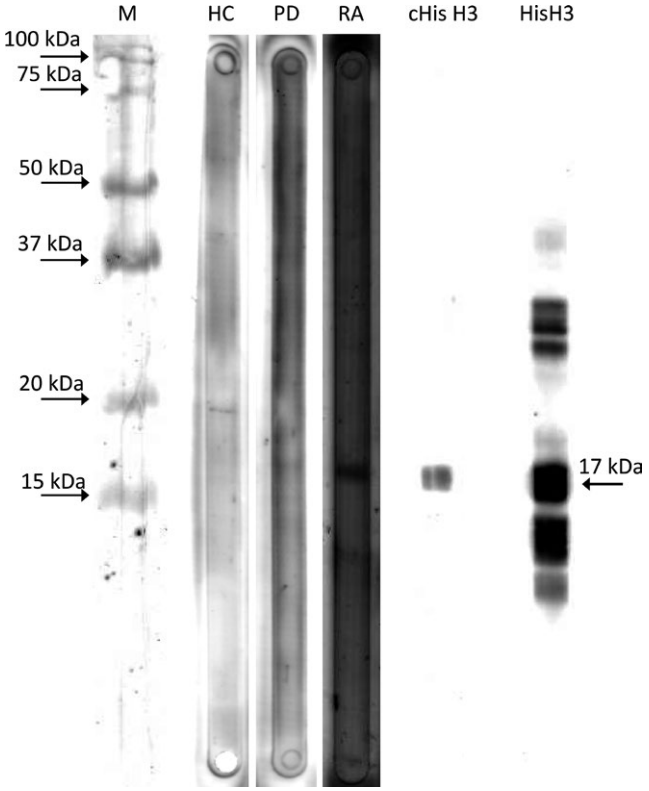

FIGURE 2 RA sera bind to citrullinated histone H3. Extracted histones (2 μg per lane) from stimulated neutrophils were subjected to Western blotting and incubated with HC, PD and RA sera and with anti-citrullinated histone H3 (cHis H3, Abcam 5103) and anti-histone H3 (HisH3, Abcam 1791) polyclonal antibodies. RA sera and anti-citrullinated histone H3 antibodies recognize a band of the same size. Representative sera are shown. HC and PD sera are anti-citrullinated histone H3 negative, whereas RA serum is anti-citrullinated histone H3 positive (as measured by ELISA)

and two-tailed Mann–Whitney tests for skewed variables. For group comparisons between three groups, Kruskal–Wallis tests were used with one-way analysis of variance with Dunn's multiple comparison post-test if overall significance level α was <.05. The Fisher exact test was used to analyse contingency tables, and Spearman ρ was used for correlation between different parameters.

3 | RESULTS

3.1 | Citrullinated histone H3 is present in inflamed periodontal tissue

Citrullinated histone H3 was detected in nine of 15 (60%) PD tissue samples (Figure 1a). PAD4 and CD68 were detected in most of the PD tissue samples (Figure 1b,c). Periodontal tissues from HC were negative for citrullinated histone H3, PAD4 and CD68 (Figure 1a–c). Citrullinated histone H3-positive tissue samples had significantly higher positive cell counts for neutrophils (PAD4, $p = .008$) and macrophages (CD68, $p < .0001$) per visible field compared to citrullinated H3-negative tissue samples (Figure 1d).

3.2 | RA sera contain autoantibodies that target citrullinated histones

Immunoblotting showed that antibodies in four of 13 tested sera from patients with RA bound to histones from stimulated neutrophils, while only one of 15 PD sera tested positive and whereas none of nine HC sera had antibodies that bound to histones. This is depicted in Figure 2, where representative immunoblots for HC, PD and RA sera are shown. Of note, the PD patient (not depicted in Figure 2) whose serum reacted to citrullinated histone H3 was anti-cyclic citrullinated peptide (anti-CCP) positive. Some RA sera also bound to histones from unstimulated neutrophils (data not shown), which suggests that unstimulated neutrophils may have been activated by the isolation procedure that could have induced histone citrullination. Therefore, an ELISA was set up to measure reactivity against a citrullinated peptide from histone H3 to ascertain that the measured reactivity was directed against citrullinated histone H3 and not to any of the other histone proteins. The ELISA system also ensured that a large number of sera could be tested. A cut-off was set, which was based on >2SD above the mean of HC, which resulted in seropositivity for IgG anti-citrullinated histone H3 in three (8%) HC, 11 (10%) PD patients and 33 (39%) patients with RA, with the latter having significantly increased levels compared to HC and PD patients ($p < .0001$, Figure 3a). In patients with RA, anti-citrullinated histone H3 levels were higher in anti-CCP-positive compared to anti-CCP-negative individuals ($p = .0008$, Figure 3b), and anti-citrullinated histone H3 levels showed a small but significant correlation ($\rho = .22$, $p = .0462$) with anti-CCP levels (Figure 3c). Anti-citrullinated histone H3 levels were not different between patients with RA stratified according to their periodontal status (Figure 3d). Additionally, no differences in anti-citrullinated histone H3 levels were found between non-smokers and smokers in PD patients (Figure 3e) and patients with RA (Figure 3f).

4 | DISCUSSION

PD has been hypothesized to be a risk factor for RA development (Rosenstein et al., 2004), which is supported by the fact that a higher incidence of PD is present among patients with RA (de Smit et al., 2012). Our study showed the presence of citrullinated histone H3 in inflamed periodontal tissue from PD patients, while the presence of PAD4 in neutrophils indicates that citrullination is a process that is existent in inflamed periodontal tissue. Besides possessing PAD2 expression which plays a role in citrullination, local activity of macrophages might play a role in ACPA generation by engulfing NET fragments and presenting citrullinated histone H3 to T cells, leading to an ACPA response in susceptible individuals. Whether ACPAs are produced locally in inflamed periodontal tissue is currently unclear. However, a previous study showed the presence of T and B cells in inflamed periodontal tissue (Nesse et al., 2012).

In this study, we identified citrullinated histone H3 as a target for ACPAs in patients with RA. Anti-citrullinated histone H3 levels in PD patients were comparable to HC which is in agreement with a previous

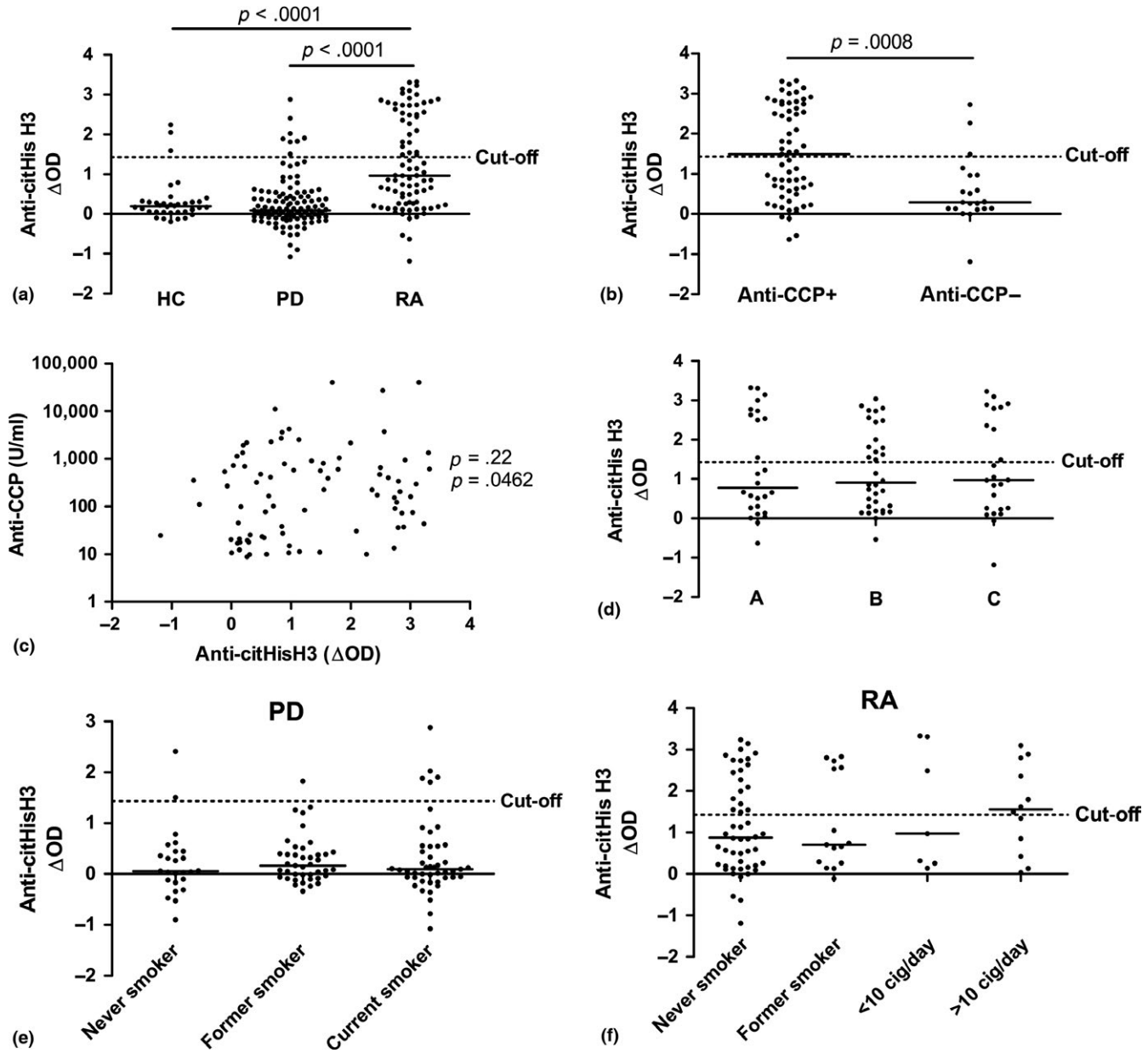

FIGURE 3 Levels of anti-citrullinated histone H3 antibodies: (a) in sera from healthy controls (HC), periodontitis (PD) patients and patients with rheumatoid arthritis (RA). (b) In patients with RA, anti-citrullinated histone H3 levels were significantly higher in anti-CCP-positive individuals compared to anti-CCP-negative individuals. (c) Anti-citrullinated histone H3 levels showed a small but significant correlation with anti-CCP levels in patients with RA. (d) Anti-citrullinated histone H3 levels in patients with RA, stratified according to periodontal status: no PD (A), moderate PD (B) and severe PD (C). No significant differences were observed. (e) Anti-citrullinated histone H3 levels in PD patients, stratified according to smoking status. No significant differences were observed. (f) Anti-citrullinated histone H3 levels in patients with RA, stratified according to smoking status. No significant differences were observed. Horizontal bars indicate the median values. Anti-citHis H3, anti-citrullinated histone H3; ΔOD, delta optical density; anti-CCP, anti-cyclic citrullinated peptide antibodies; U/ml, Units/ml

study of our group in which reactivity against specific citrullinated peptides was assessed in PD patients (Janssen et al., 2015). In recent years, several studies have reported citrullinated histones H2A, H2B (Corsiero et al., 2015) and H4 (Pratesi et al., 2014) as potential targets for ACPAs. Autoantibodies against citrullinated histone H4 were found in 63%–67% of patients with RA (Pratesi et al., 2014). Besides differences in experimental methods, the difference could also be explained by the setting of the cut-off value in our ELISA, given the limited group size and data distribution in our HC group.

In addition, we recognized specific binding to citrullinated histone H3. Previously, Dwivedi et al. (2012) identified that anti-citrullinated histone H3 in patients with Felty's syndrome, which can be characterized as a form of "active extra articular rheumatoid" disease with splenomegaly and neutropenia being present besides joint involvement. The latter study did find a low frequency (two of 37 patients) of anti-citrullinated histone H3 in patients with RA, although differences in experimental methods and limited patient with RA group size in that study could explain for the different results compared to our study.

ACPA and RF have been found to be present years before the onset of RA pre-symptomatic individuals (Nielen et al., 2004), but their role in disease development and pathogenesis is still unclear. Especially individuals seropositive for ACPA are at increased risk for developing RA (van de Stadt et al., 2011). Whether anti-citrullinated histone H3 autoantibodies also play a role in the initiation of RA is yet unclear and should be assessed in seropositive arthralgia patients.

We found no association between the presence of anti-citrullinated histone H3 and periodontal status and smoking behaviour of patients with RA. This may indicate that the development of anti-citrullinated histone H3 is more dependent on genetic factors (HLA-DRB1-SE) than exposure to citrullinated antigens via PD or smoking. The presence and degree of gingivitis, however, may be more important for citrullination. The periodontal status of the patients, as scored with DPSI, does not necessarily reflect ongoing gingival inflammation.

The pathogenic role of autoantibodies that recognize citrullinated histones is currently unclear. Autoantibodies directed against histone H2B were found to be arthritogenic in a collagen-induced arthritis mouse model (CIA) (Sohn et al., 2015). This is in contrast to a study that recognized anti-citrullinated histone H2A autoantibodies, directed against the N-terminal region of histone H2A, as a possible therapeutic treatment for RA (Chirivi, Jenniskens, & Raats, 2013). Administration of these therapeutic ACPAs (tACPAs) resulted in reduced inflammation and joint damage in collagen-antibody-induced arthritis and CIA mouse models. The therapeutic effect of tACPA is proposed to lie in inhibition of NET formation and clearance of already formed NETs, thereby reducing formation of citrullinated autoantigens. Whether anti-citrullinated histone H3 autoantibodies are pathogenic or therapeutic remains to be determined. In our study, anti-citrullinated histone H3 levels were not (negatively) correlated with DAS28 and C-reactive protein levels in patients with RA (data not shown). However, the patients with RA were all on treatment and displayed relatively low disease activities during the study period. Regarding whether these tACPAs are pathogenic or not, studies should be performed looking at epitope recognition and glycosylation status.

In conclusion, we showed that RA sera contain increased levels of autoantibodies against citrullinated histone H3 compared to sera of PD patients and HC. Also citrullinated histone H3 is present in inflamed periodontal tissue, which supports a role for PD in the generation of antigens that are targeted by ACPA.

CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflict of interests in connection with this article.

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