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SYSTEMIC DISEASES (M BARTOLD, SECTION EDITOR)

# Is Citrullination the Missing Link between Periodontal Disease and Rheumatoid Arthritis?

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**Abstract** Connections between periodontitis (PD) and rheumatoid arthritis (RA) are suggested by an increased prevalence of PD in RA, shared environmental and genetic risk factors, and correlating levels of severity when the two diseases occur together. Here, we compare and contrast the results from numerous studies documenting this association, and highlight the involvement of citrullination in the development of autoimmunity leading to the destructive pathology that characterizes RA. The contribution of citrullination to RA may occur in at least three distinct phases: (i) the initial breakdown of tolerance leading to autoimmunity; (ii) the maturation of the citrulline specificity of the autoantibody response; and (iii) the pro-inflammatory effect of citrullinated proteins themselves in established disease. We conclude that citrullination is more than a 'missing link'; rather, it is an active process in the evolution of low levels of autoimmunity found in PD into the pathogenic anti-citrullinated protein response specific to RA.

**Keywords** Rheumatoid arthritis · Periodontitis · Citrullination · Anti-citrullinated protein antibodies (ACPA) · Peptidylarginine deiminase (PAD) · Etiology · *Porphyromonas gingivalis* · PPAD

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## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by inflammation and destruction of the joints. The etiology of RA is unknown, but increasing evidence suggests it fulfills the paradigm of the interaction between genetic and environmental factors leading to a disease-specific autoimmune response [1]. Periodontitis (PD) is one candidate environmental risk factor. In this review we consider the main elements of the interaction between RA and PD, and examine citrullination as the process linking them.

## Citrullination in Health and Disease

The post-translation modification of citrullination is the conversion of an arginine residue to a citrulline residue by peptidylarginine deiminase (PAD) enzymes, and occurs constitutively in humans by five known PAD isoforms (PAD1–4 and PAD6). With broad tissue distribution, PADs carry out citrullination in a wide range of processes, from keratinization of skin to innate immunity [2]. Of particular interest in RA and PD are isoforms PAD2 and PAD4 as both have been found in the RA synovium [3] and in periodontal tissues in PD [4]. PAD2 is found in multiple tissues and skeletal muscle, while PAD4 is found predominantly in macrophages and neutrophils [2]. Deregulated levels of citrullination have been implicated in a number of pathological conditions including psoriasis and prion disease [5, 6]. A well-characterized example is multiple sclerosis (MS), a neurodegenerative disease characterized by the destruction of myelin around nerve axons. In MS, citrullination of myelin basic protein in brain tissues is increased, altering the myelin structure, increasing susceptibility to degradation by proteases, and potentially exposing neoepitopes for responding T cells [7]. Citrullination is also a feature of a number of inflammatory diseases, including both

RA and PD, but antibodies to citrullinated protein/peptide antigens (anti-citrullinated protein antibodies; ACPA) remain relatively specific for RA.

## The Chain of Events Between Periodontitis and Rheumatoid Arthritis

An association between PD and RA has been theorized since Hippocrates described pulling teeth as a cure for arthritis circa 400 BC. In more recent years the link between the two diseases has been substantiated by several studies based on different populations from several continents. Despite the definition of periodontal disease status and RA manifestations often differing between studies, the vast majority reported a positive association, with increased prevalence of PD in patients with RA compared to osteoarthritis (OA) or controls without joint disease (Table 1). The association between the two diseases appeared to be independent of smoking status in several studies [8–12], as did other parameters and confounders such as age, gender, and medication [10, 11, 13–15]. However, it is important to note that these parameters do vary between studies and, in particular, the use of anti-inflammatory drugs such as corticosteroids, non-steroidal anti-inflammatory drugs, and disease-modifying anti-rheumatic drugs can mask true periodontal conditions, especially when evaluating less severe periodontal conditions [16, 17] (reviewed in [18]). The influence of oral microbes normally associated with PD and their effect on RA has also been looked at in some studies [8, 9, 15, 19•, 20]. The presence of, and antibodies to, *Porphyromonas gingivalis*, one of the main bacterial species implicated in the etiology and pathogenesis of PD, often correlates with RA parameters. One study found an association between new-onset RA and bacterial species other than *P. gingivalis* (*Prevotella* and *Leptotrichia*) irrespective of severity of PD. These bacteria were not present in the control group. Interestingly, the genus Porphyromonas was universally present in all patient groups but *P. gingivalis* specifically was the most prevalent in PD patients. The study also showed that in the new-onset RA group, *P. gingivalis* was more prevalent and abundant in patients with advanced PD than in those without PD [8].

A number of studies have observed a correlation in the severity of the conditions: de Smit et al. [9] found RA patients with severe PD had a higher 28-joint Disease Activity Score (DAS28) than RA patients with no or moderate PD. Similarly Mercado et al. found periodontal bone loss correlated to swollen joint count and health assessment status in RA [21]. In a large and comprehensive study, Gonzalez et al. [20] also measured loss of periodontal bone in relation to a variety of ACPA specificities in 287 RA patients and 330 OA controls. They observed significantly increased bone loss in ACPA-positive patients, which correlated with total ACPA levels and health assessment questionnaire, tender joint count and DAS28-CRP (C-reactive protein) scores. Interestingly, of 19 reactivities tested, levels of anti-citrullinated vimentin and anti-citrullinated histone antibodies also correlated with increased bone loss [20]. In another study on this cohort, Mikuls et al. [19•] investigated ACPA level and specificity in relation to PD status and antibodies to *P. gingivalis* surface proteins. They found a correlation between anti-*P. gingivalis* antibodies and total ACPA level, and a correlation between anti-*P. gingivalis* antibodies and ACPA specificity, which appeared to be dependent on smoking status. Anti-citrullinated-clusterin, enolase, filaggrin, and fibrinogen antibodies were associated with anti-*P. gingivalis* levels in the 'ever-smokers' group, while 'never smokers' had correlating anti-*P. gingivalis* and citrullinated-filaggrin, histone, and vimentin antibodies [19•]. These studies demonstrate a strong correlation between PD and RA severity, which is associated with ACPA production. There also appears to involvement of *P. gingivalis*, which is modulated by smoking status, demonstrating the importance of environmental factors in disease pathogenesis.

## Interaction with Genetics

The most well-established genetic risk factor for RA is the 'shared epitope' (SE), a number of *HLA-DRB1* alleles encoding five amino acids around the binding cleft of the major histocompatibility complex (MHC) class II molecule. These include members of the *HLA-DRB1**04 group, *HLA-DRB1**0101 and *0102, as well as lesser-known alleles (reviewed by Holoshitz [22]). These particular amino acid arrangements influence binding of peptides for antigen presentation, and generally exhibit higher affinity for negative or uncharged amino acids over positive, thus making binding of neutral citrulline preferential to positively charged arginine [23]. SE alleles are also associated with chronic aggressive PD [24], though it remains unclear whether the SE is associated with PD as a whole. The PTPN22 *R620W* gene variant is another known susceptibility factor in RA [25] and with a stronger association with ACPA-positive RA [26]. However, the role of this variant of PTPN22 in relation to PD remains to be established.

## Interactions with Smoking

Cigarette smoking is an established environmental risk factor for RA, and has a stronger association with the ACPA-positive phenotype [27]. It has been suggested that this may be due to increased expression of PAD enzymes in the lungs of smokers [28], leading to citrullination of native proteins in the lung,

**Table 1** Prevalence and severity of periodontitis in rheumatoid arthritis in various studies

| Periodontol disease status | Periodontol evaluation | Prevalence in RA | Prevalence in controls | *p* value | Ref. |
|---|---|---|---|---|---|
| | | ***n*=287** | ***n*=330 (OA)** | | [19•] |
| Periodontitis (%) | CAL ≥6 mm<br>PrDp of ≥5 mm | 35 | 26 | 0.022 | |
| | | | ***n*=75** | ***n*=75** | [16] |
| None/mild (%) | No PrDp/CAL ≥3 mm | 29 | 31 | 0.547 | |
| Severe (%) | CAL ≥6 mm with PrDp≥5 mm | 21 | 16 | 0.547 | |
| | | ***n*=100** | ***n*=112** | | [17] |
| None (%) | CAL=0 mm | 0 | 18 | <0.05 | |
| Mild (%) | CAL=1–2 mm | 42 | 75 | <0.05 | |
| Moderate (%) | CAL=3–4 mm | 48 | 5 | <0.05 | |
| Severe (%) | CAL ≥5 mm | 10 | 2 | <0.05 | |
| | | ***n*=13,779** | ***n*=137,790** | | [61] |
| Periodontitis (%) | ICD9-CM: 523.3–5) | 39 | 35 | <0.001 | |
| Severe (%) | ICD9-CM: 523.3–4 | 23 | 20 | <0.001 | |
| | | ***n*=34** | ***n*=18** | | [8] |
| None (%) | No bleeding upon probing. | 6 | 45 | <0.01 | |
| Severe (%) | CAL ≥5 mm and PrDp ≥5 mm | 53 | 22 | <0.01 | |
| | | ***n*=95** | ***n*=420** | | [9] |
| None (%) | Bleeding pockets ≤3 mm | 30 | 70 | <0.001 | |
| Moderate (%) | PrDp 4–5 mm without gingival recession | 43 | 18 | <0.001 | |
| Severe (%) | PrDp ≥4 mm with gingival recession | 27 | 12 | <0.001 | |
| | | ***n*=80** | ***n*=80** | | [13] |
| Mild (%) | CAL ≤1.17 mm | 65 | 88 | 0.006 | |
| Moderate/severe (%) | CAL >1.17 to ≤3.5 mm/>3.5 mm | 35 | 12 | | |
| | | ***n*=69** | ***n*=35 (OA)** | | [10] |
| None (%) | Healthy peridontium | 19 | 37 | <0.05 | |
| Moderate/severe (%) | <50 % bone loss, BOP, and tooth mobility <2 to ≥2 | 51 | 26 | 0.03 | |
| | | ***n*=103** | ***n*=4,358** | | [11] |
| None (%) | Dentate and no PD | 28 | 56 | <0.001 | |
| Periodontitis (%) | Dentate, CAL, and PrDp ≥4 mm | 16 | 10 | <0.0001 | |
| Edentulism (%) | No natural teeth | 58 | 34 | <0.0001 | |
| | | ***n*=57** | ***n*=52** | | [14] |
| None (%) | CAL <4.0 mm | 65 | 90 | 0.001 | |
| Moderate (%) | CAL >4 to <6 mm | 23 | 6 | 0.001 | |
| Severe (%) | CAL >6 mm | 12 | 4 | 0.001 | |
| | | ***n*=65** | ***n*=65** | | [21] |
| No/mild (%) | PrDp 0–6 mm | 55 | 75 | <0.05 | |
| Moderate/severe (%) | PrDp 6.2 to >8.2 mm | 45 | 25 | <0.05 | |
| Periodontitis | | ***n*=50** | ***n*=101** | | [12] |
| | PrDp (mm) | 2.9±0.8 | 2.3±0.4 | <0.0001 | |
| | CAL (mm) | 2.6±1.7 | 0.95±0.7 | <0.0001 | |
| Periodontitis | | ***n*=37** | ***n*=37** | | [15] |
| | Mean alveolar bone loss (%) | 23.8 | 18.9 | <0.05 | |
| | Mean tooth loss (n) | 17.9 | 22.6 | <0.05 | |

*CAL* clinical attachment loss, *ICD9-CM* International Classification of Disease, 9th revision, clinical modification, *OA* osteoarthritis, *PrDp* probing depths

which in turn results in the formation of ACPA. Smoking is also a risk factor for PD and tooth loss in a dose-dependent manner [29, 30], but it is now accepted that the links between PD and RA cannot be explained by smoking as a common risk factor [8–10].

## Anti-Citrullinated Protein Antibodies: the Link in the Chain

The discovery of ACPA has provided the biggest step in recent years towards refining the diagnosis of RA [31] and increasing

our understanding of its etiopathogenesis (reviewed in Wegner et al. [1]). Diagnostic tests for ACPA commonly use cyclic synthetic peptides such as the anti-cyclic citrullinated peptide (anti-CCP) assays, or mutated forms of recombinant proteins citrullinated in vitro such as the anti-mutated citrullinated vimentin assay. These generic tests for ACPA are now included in the revised diagnostic criteria for RA [32] and are associated with more aggressive disease measured by DAS28, Visual Analogue Scale, and radiographic scores of joint damage [33–35] as well as an increased risk of secondary disorders of the cardiovascular system [36]. ACPA are present in serum years before disease onset [37, 38] and increase in titre and range of specificities prior to development of pathology [34, 39]. A recent study reported reduced bone volume and bone mineral density loss in a small group of ACPA-positive individuals with no arthralgia or joint swelling [40], suggesting that ACPA may induce bone loss even before clinical presentation of symptoms.

In addition to clinical correlations of ACPA with disease severity, there is also evidence of direct involvement of ACPA in pathogenesis. The ACPA tests, which are so valuable in diagnosis, are of limited use when it comes to examining pathogenic mechanisms. This is because the peptides used in the CCP assays, for example, were selected on the basis of their ability to maximize the diagnostic sensitivity of the antibody tests, and do not represent the real antigens that occur in vivo. Therefore, the so-called 'specific antigens' are those that provide the best tools for examining the pathogenicity of ACPA. The best-established specific antigens are the citrullinated forms of fibrinogen, vimentin, enolase, and type-2 collagen (reviewed in Wegner et al. [1]). Harre et al. demonstrated that anti-citrullinated vimentin antibodies stimulate osteoclastogenesis by binding citrullinated vimentin on the cell surface [41]. In addition, binding of immune complexes containing antibody and citrullinated fibrinogen induced increased production of tumor necrosis factor-α by simultaneously binding both toll-like receptor 4 (TLR4) and the Fc-gamma receptor [42•]. In this study, Sokolove and colleagues also demonstrated that citrullinated fibrinogen alone induced a more potent cytokine response than native fibrinogen through TLR4 and MyD88 pathway activation, indicating citrullinated proteins themselves may be pathogenic.

## Citrullination: the Missing Link?

In light of increasing evidence that ACPA are directly involved in RA disease pathogenesis, it is reasonable to propose that citrullination of autoantigens is an essential process that forms a link (or links) in the chain of events between etiological agents and the downstream events in disease pathology. PD is particularly relevant in this respect, because one of its major pathogens, *P. gingivalis*, expresses the only known bacterial PAD enzyme known as *P. gingivalis* PAD (PPAD). PPAD has attracted considerable attention as a possible molecule underlying the mechanisms for tolerance breakdown in RA. It differs from mammalian PADs in its ability to citrullinate free arginine and also shows a marked preference for C-terminal peptidyl arginines generated by another *P. gingivalis* enzyme, arginine gingipain, whereas eukaryotic PADs only citrullinate internal arginines. PPAD also differs in its lack of $Ca^{2+}$ dependency and therefore does not depend on cell lysis, apoptosis, or secretion for its activity. The citrullinating capabilities of PPAD are thought to promote bacterial survival of *P. gingivalis* by inactivating the killing defenses of the host, such as antimicrobial peptides and complement [43, 44]. In vitro PPAD citrullinates peptides with C-terminal arginines from RA-associated antigens, enolase and fibrinogen [45]. Given C-terminally citrullinated peptides would not be generated by host PAD enzymes such as PAD2 and PAD4, these would be true neopeitopes and thus could potentially react with T cells to drive an antibody response against both bacterially derived and host proteins. One problem with this hypothesis is the lack of evidence that C-terminally citrullinated peptides bind to MCH molecules or to responding T cell receptors. However, one group has shown that a highly immunogenic peptide from hen egg lysozyme (HEL) was citrullinated by endogenous PADs in a C-subterminal position (flanked only by a downstream C-terminal glycine) and bound to MHC. This interaction resulted in autoantibodies to uncitrullinated HEL and autophagy in HEL transgenic mice [46, 47]. Thus, it remains possible that C-terminally citrullinated peptides from enolase or fibrinogen may be bound to MHC and break tolerance by similar mechanisms. In support of this idea, is the observation that patients with PD have antibodies to peptides from enolase and other RA autoantigens and that these antibodies, as in the HEL transgenic mice, reacted mainly with uncitrullinated variants of the peptides [48, 49••].

A second mechanism for PPAD breaking tolerance to citrullinated proteins is via autocitrullination, which occurs during the production and purification of recombinant PPAD [50]. Citrullinated peptides from PPAD are targeted by antibodies in RA [50, 51] but autocitrullination of PPAD has yet to be demonstrated in vivo, so it is possible that the antibodies to citrullinated PPAD peptides are simply part of the cross-reactive ACPA response. However, if autocitrullination of PPAD does prove to be a mechanism for inducing autoimmunity in RA, it has profound therapeutic implications, in that PPAD inhibitors could be a novel and targeted approach to treating patients whose RA has been induced by PD. Since PPAD is structurally and genetically unrelated to mammalian PADs, such treatment should be antibacterial and therefore devoid of side effects.

A third mechanism for citrullination breaking tolerance may not involve PPAD at all but the host PADs, such as PAD2 and PAD4. A polymorphism in the *PADI4* gene, which results in increased stability of PAD4 mRNA, has been shown to be a susceptibility factor in RA in Asian populations [52]. This suggests that increased citrullination by PAD4 could be directly linked to ACPA formation, possibly by generating neoepitopes that bind favorably to the MHC. One of the first studies to examine this mechanism showed that a peptide derived from the autoantigen vimentin (amino acids 66–77), with arginine-71 replaced by a citrulline, binds with high affinity to HLA-DRB1*0401 MHC class II molecules, eliciting T cell activation [23].

In PD, citrullinated proteins and enzymes PAD2 and PAD4 have been detected in gingival tissues [4], and have been shown in higher levels in inflamed tissues, correlating with clinical parameters of pathology such as depth of periodontal pockets [53]. Both PAD2 and PAD4 are important in the formation of neutrophil extracellular traps (NETs), which have been detected in abundance in purulent crevicular fluid from patients with PD [54]. It has been proposed by Dwivedi and Radic that NET formation may induce autoimmunity to citrullinated proteins in RA through internalization of bacteria complexed with citrullinated proteins, inducing an antibody response to both bacterial and native proteins [55]. Expression of key citrullinated RA antigens vimentin and enolase on the surface of NETs demonstrated by Sur Chowdhury et al., [56] supports this hypothesis. In addition, an antibody response to the citrullinated histones mediating NET formation has been observed in RA patients [57].

A fourth possible mechanism for inducing ACPA may not involve citrullination at all, at least not in the initial stages. Of relevance in this respect is the immunodominant epitope of citrullinated alpha enolase known as citrullinated enolase peptide 1 (CEP-1). The sequence KIHA-cit-EIFDS-cit-GNPTVE is 97 % homologous to the corresponding sequence in citrullinated *P. gingivalis* enolase [58]. Thus, *P. gingivalis* infection could initiate tolerance breakdown through cross-reactivity of antibacterial enolase immunity with mammalian enolase. This was demonstrated in an animal model by Kinloch et al., where immunization of DR4 transgenic mice with both citrullinated and uncitrullinated *P. gingivalis* enolase induced autoantibodies to mammalian enolase. Importantly, the antibody response was not citrulline specific as judged by reactivity of serum from the immunized mice with the arginine control peptide (REP-1) [59]. This indicates that citrullination is not required for inducing an immune response, and that when the response occurs, the antibodies are not citrulline specific. This is in keeping with the findings of largely not citrulline-specific autoantibody response to CEP-1 and REP-1 in PD [48, 49••] and a very similar pattern of non-citrulline-specific ACPA responses in another chronic bacterial infection, bronchiectasis [60]. At first sight, the lack of citrulline specificity in these immune responses would seem to conflict with the numerous studies demonstrating ACPA in the serum of RA patients years before the onset of disease. However, one very recent re-analysis of a large pre-RA study showed that the earliest serum samples had a significantly higher response to the arginine control peptides from several citrullinated peptide antigens [59].

## Conclusion

The links between PD and RA are now well-established in a number of epidemiological studies, both in terms of frequency and the severity of the two diseases. The evolution of pathogenic antibodies to citrullinated proteins is integral to this chain of events, suggesting that citrullination of autoantigens must occur as part of the process. Citrullination could promote tolerance breakdown, but the evidence to date suggests that it is likely to be at the T cell level, since the B cell response in diseases that predispose to RA (including PD) is not citrulline specific. By contrast, by the time the first signs of arthritis appear, the ACPA response is already strikingly citrulline specific, suggesting that constant exposure to citrullinated autoantigens at the site of inflammation, be it in the periodontium, lungs or possibly even the gastrointestinal tract, causes a second link, the gradual evolution of citrulline specificity of the ACPA response, which further matures as a result of exposure to citrullinated proteins in the joint. Once clinical disease becomes established, citrullination of autoantigens may acquire a third pathogenic role by interacting with TLR receptors, already well-characterized in the cases of fibrinogen and vimentin, with other proinflammatory citrullinated proteins awaiting discovery.

Thus, we can conclude the citrullination is more than a ‘missing link’, it is an essential process in the evolution of autoimmunity in those patients with PD who go on to develop RA. This is important because inhibition of the bacterial enzyme PPAD, or perhaps the host PADs, PAD2 and PAD4, could be a completely novel approach to treating RA by specifically targeting this link.

### Compliance with Ethics Guidelines

**Conflict of Interest** Anna B. Montgomery, Elena B. Lugli, and Patrick J. Venables declare that they have no conflict of interest.

**Human and Animal Rights and Informed Consent** Both human and animal studies, all with appropriate ethical approval, informed consent and observation of national guidelines for animal welfare.

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