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Citrullination as a plausible link to periodontitis, rheumatoid arthritis, atherosclerosis and Alzheimer's disease

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ABSTRACT

Periodontitis, rheumatoid arthritis (RA), atherosclerosis (AS), and Alzheimer's disease (AD) are examples of complex human diseases with chronic inflammatory components in their etiologies. The initial trigger of inflammation that progresses to these diseases remains unresolved. *Porphyromonas gingivalis* is unique in its ability to secrete the *P. gingivalis*-derived peptidyl arginine deiminase (PPAD) and consequently offers a plausible and exclusive link to these diseases through enzymatic conversion of arginine to citrulline. Citrullination is a post-translational enzymatic modification of arginine residues in proteins formed as part of normal physiological processes. However, PPAD has the potential to modify self (bacterial) and host proteins by deimination of arginine amino acid residues, preferentially at the C-terminus. Migration of *P. gingivalis* and/or its secreted PPAD into the bloodstream opens up the possibility that this enzyme will citrullinate proteins at disparate body sites. Citrullination is associated with the pathogenesis of multifactorial diseases such as RA and AD, which have an elusive external perpetrator as they show epidemiological associations with periodontitis. Therefore, PPAD deserves some prominence as an external antigen, in at least, a subset of RA and AD cases, with as yet unidentified, immune/genetic vulnerabilities.

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Introduction

Investigating the effect of complex human diseases on specific organs has previously been the norm. However, new collaborative research assesses the knock on effect of diverse pathologies on conditions that develop because of the human ageing processes. Periodontitis (PD), rheumatoid arthritis (RA), atherosclerosis (AS), and Alzheimer's disease (AD) are examples of complex diseases. There is a strong correlation of contributions from oral pathogens in their development without specific understanding of the mechanisms leading to the disease pathogenesis. The focus of this review is on the periodontal keystone pathogen *Porphyromonas gingivalis* [1,2], and its secreted peptidyl arginine deiminase (PPAD) enzyme in the development of the extraoral autoimmune and inflammatory diseases mentioned above [3–7] (Figure 1).

A chronic form of PD becomes prevalent in tricenarian/quadragenarian vulnerable hosts, whilst sporadic AD is common in octo-nonagenarians. *P. gingivalis* manipulates the host's cellular immune responses and undermines host-microbe homeostasis [5], thereby leading to dysbiosis in a previously symbiotic microbiome [2]. Breakdown of cellular barriers allows dissemination of this pathogen to the rest of the body. Undoubtedly, human polymorphic genes do influence the susceptibility of the host to disease but they also affect the direction taken by the pathogens that use them for their survival and proliferation. Thus, *P. gingivalis* is a typical example of a pathogen that shows this trait by adapting to challenging inflammophilic environments of the host directed to kill it [8]. The virulence and potential pathogenic effects of *P. gingivalis* are diverse and, through them, this bacterium can affect many different organs and diseases [3,6,7,9,10]. The virulence factor under focus here is the enzyme *P. gingivalis* peptidyl-arginine deiminase (PPAD). This enzyme modifies both bacterial and host proteins by deimination of arginine residues in proteins and peptides, converting them to citrulline [11–13] (Figure 2). Protein citrullination causes deregulation of the host's inflammatory signalling network by altering the spatial arrangement of the original 3D-structure and function of the protein [3,6]. This may lead to exposure of damage- and/or pathogen associated molecular patterns (PAMP/DAMP) which can then be used by pattern recognition receptors (PRR), to provide entry and immune evasion [14]. Subsequent immune responses directed against the bacterial antigens by the infected organ can lead to tissue damage. In some individuals, this can initiate autoimmune

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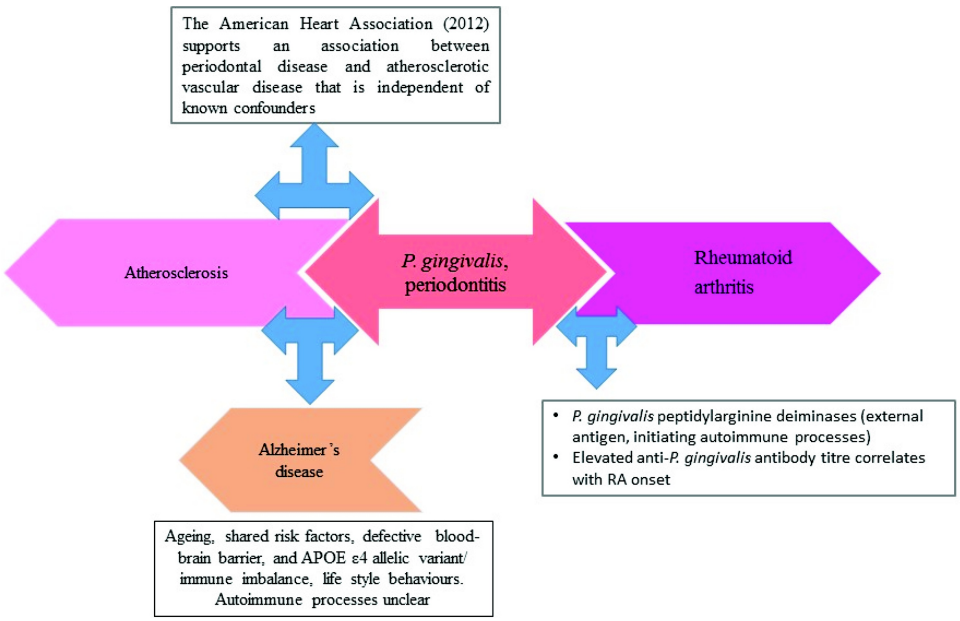

Figure 1. Schematic to show additive effect from an oral condition such as periodontitis to the development of mixed pathologies through smoking, atherosclerosis, and rheumatoid arthritis with direct inflammatory mediator input from *P. gingivalis* infection to Alzheimer's disease. The major arrows point to major risk factors with plausible effect on each condition. The three-way arrows provide explanation of the link with periodontitis.

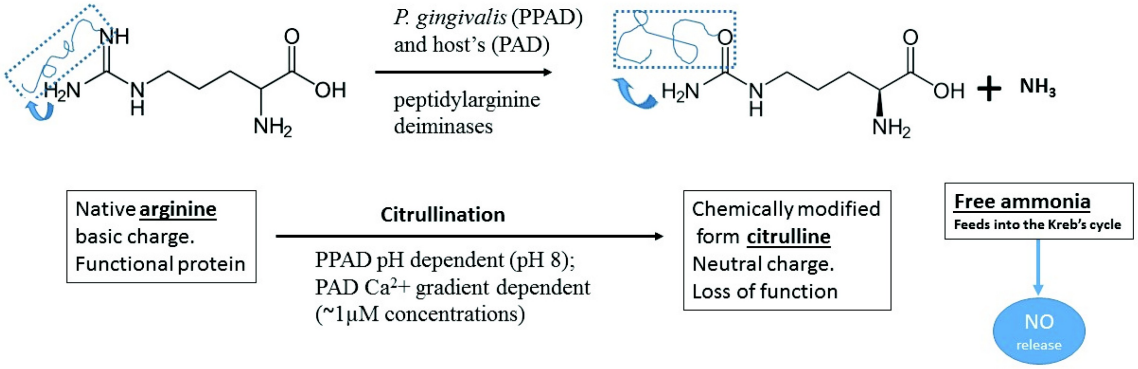

Figure 2. Chemical modification of arginine amino acid residue by *P. gingivalis* and host-mediated peptidylarginine deiminases resulting in the conversion of arginine on functional peptide to citrulline (defective protein). This posttranslational modification alters the spatial arrangement of the original 3D-structure and function of the protein peptide as indicated by arrows (arginine-peptide and citrulline-peptide). Ammonia released during the chemical reaction is beneficial for PPAD activation.

responses [3,6] (Figure 3). At present, *P. gingivalis* is the only known pathogen that produces PPAD [3]. This gives the bacterium prominence in causing both periodontitis and extraoral diseases, especially RA.

The host also has intrinsic sources of citrullination due to genes coding for a family of enzymes called peptidyl arginine deiminases (PADs). Although the human peptidylarginine deiminase family contains five isotypes (PAD 1, 2, 3, 4/5 and 6) with tissue specific expression [15], there is a paucity of information on the *P. gingivalis* PPAD(s) infecting different tissues and cells [16]. Protein citrullination is important for many normal physiological processes such as epithelial terminal differentiation, regulation of gene expression, apoptosis, and inflammation [14,15,17]. However, the posttranslational modification involving the citrullination process can affect the function of several signalling molecules as well as protein structures and functions. One such example is C5a anaphylatoxin. This is a glycoprotein with a number of arginine residues that are released following complement activation. Functional C5a induces vasodilation and chemotaxis of inflammatory cells in the site of injury. On citrullination by PPAD, C5a loses this function [18]. It is not surprising therefore that an increased citrullination of cytoskeletal filaments and PAD enzymes have been found in numerous chronic inflammatory and autoimmune diseases like AD, RA, and multiple sclerosis (MS), respectively [15,19]. Owing to their similar etiologies, PPAD deserves some consideration as an extrinsic antigen in the pathogenesis of RA and MS, thus contributing to autoimmune processes. The physiological conditions and the specific arginine residues targeted for

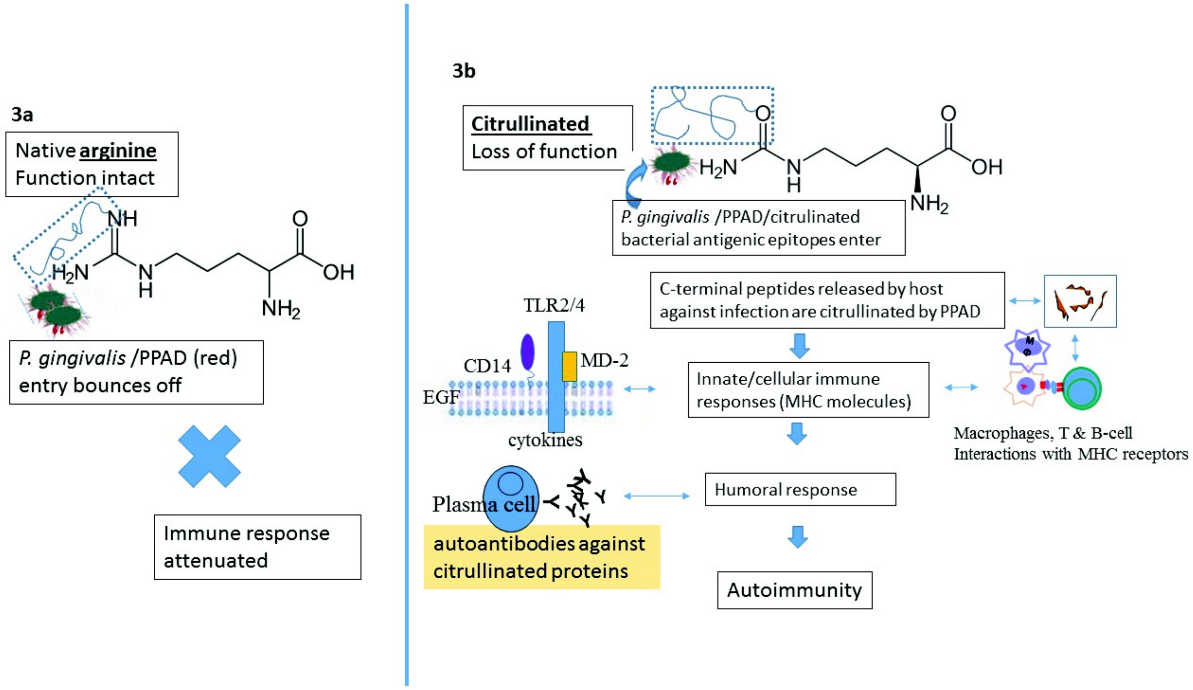

Figure 3. Schematic to illustrate immune events leading to autoimmunity resulting from PPAD-mediated citrullination of C-terminal arginine residues. These citrullinated peptides produced by the combined action of gingipains cleave polypeptides into fragments. This results in PPAD structurally becoming closer to gingipains on the bacterial surface membrane.

P. gingivalis also citrullinates its own proteins generated by PPAD. These represent a pool of potent antigenic epitopes that can break the tolerance to specific citrullinated host peptides. The loss of tolerance can generate autoantibodies against citrullinated proteins. The arrows point to direction(s) of immune processes with the endpoint being autoimmunity. Text in boxes clarifies the symbols alongside the cascade of events as visualized by the authors. TLR 2/4 = toll like receptors 2 and 4, MD-2 = adapter protein in the NFkB inflammatory cascade, EGF = epidermal growth factor.

deimination by PPAD and/or host PADs remain differential. For example, PPAD requires a higher pH for its activity and preferentially targets carboxy-terminal and free arginine residues [20] cleaved by arginine-gingipains (Rgps). The human PADs, on the other hand, exclusively citrullinate internal peptidyl arginine residues [11,21], which are activated following an influx of calcium ions from the extracellular environment or from the cytosol [22]. Although both (host and bacterial) enzymes catalyse the same chemical reaction [12] (Figure 2), identification of either PPAD or PAD citrullinated arginine residues in cells presents technical challenges. This may limit progress in our ability to differentiate PPAD citrullinated arginine residues from those of the host. The aim of the present review is to discuss the possible importance of citrullination in the pathogenesis of PD, RA, AS, and AD, which are common, complex, chronic inflammatory diseases with unclear etiologies.

Periodontitis and citrullination

Periodontitis is an inflammatory oral disease affecting the tissues supporting teeth in their bony sockets and occurs in two forms, aggressive and chronic. If left untreated, periodontitis will lead to loss of teeth. Although, PD is not an autoimmune disease *per se*, *P. gingivalis* infection has the potential to induce autoimmune responses in oral tissues [23]. Periodontitis affects approximately 65 million (47%) US adults, 30 years and older [24]. By adopting a more resilient phenotype through selecting different signalling pathway molecules, *in vitro* studies demonstrate the survival 'instincts' of this pathogen under both poor and sufficient bioavailability of haemin [25,26]. This keystone pathogen with its companion species is associated with the initiation and progression of chronic periodontitis by secretion of several virulence factors including Rgps and PPAD in the periodontal pocket [27,28].

Citrullinated peptides initiated by *P. gingivalis* are produced by the combined action of Rgps that cleave polypeptides into fragments with C-terminal arginine, followed by citrullination with PPAD [29]. Thus, citrullination of surface proteins depends on the action of Rgp proteases. The modification of C-terminal arginine residues is the result of PPAD becoming structurally closer to Rgps on the bacterial surface. This dual action of modification initially reported when production of citrullinated peptides derived from fibrinogen and α-enolase by PPAD has been reported [30]. These two proteins are major auto-antigens in RA [3]. The secreted PPAD may spread deeply within the connective tissue by shedding *P. gingivalis* outer membrane vesicles, or through tissue diffusion of the soluble enzyme [13].

The soluble enzyme modifies the epidermal growth factor (EGF) located in the inflamed periodontium which subsequently interferes with EGF function by blocking the recognition between the epithelium and the EGF signalling pathway molecules [13]. This is a mechanism for breaking local protective epithelial cell–periodontal tissue barriers and delaying the healing process [13]. In addition, PPAD inhibits the ability of EGF to stimulate epidermal cell proliferation and migration and prevents epidermal growth factor receptor (EGFR)–EGF interaction-dependent stimulation by suppressing cytokine signalling 3 and interferon regulatory factor 1 signalling [13]. Ammonia produced as a byproduct during deimination can promote the survival of *P. gingivalis* in the periodontal pocket [12] and potentially optimize the pH-dependent function of Rgp and PPAD. In this way, Rgps and PPAD may inactivate hemagglutinins, promote ATP production, and exert negative effects on neutrophil functions [11,13,27,31].

P. gingivalis also citrullinates the proteins associated with its cell envelope [3], thus generating in a PPAD-dependent manner, a pool of potent antigenic epitopes that can break the tolerance to specific citrullinated host peptides [14]. The loss of tolerance can generate autoantibodies against citrullinated proteins (ACPAs). Increased levels of ACPAs are detected in patients with aggressive periodontitis [32] compared to controls [33]. ACPA-positive patients are also more inclined to have moderate to severe PD than ACPA-negative patients [34]. *P. gingivalis* infection in PD implies the induction of autoimmune responses that are characteristic of RA. Shimada et al. [35] found an association between anti-PPAD IgG and anti-cyclic citrullinated peptide (CCP) IgG responses, and proposed a role for PPAD in protein citrullination of patients with both PD and RA.

Rheumatoid arthritis and citrullination

RA is an autoimmune disorder that occurs when the host is unable to differentiate self from non-self-antigens. This can give rise to an immune system mistakenly attacking the host (self) tissues. RA manifests as painful and chronic inflammation of the joints. It also affects other areas of the body including skin, eyes, lungs, heart and blood vessels. Patients with RA have a higher frequency of morbidity and mortality from cardiovascular dysfunctions [36]. Most data from the developed world estimates an RA prevalence between 0.5 and 1% [37], with a mortality risk of 1.5–1.6 greater than that of the general population [38]. Citrullinated protein and anti-citrullinated protein antibodies play important roles in RA development [39]. The *PAD4* gene encoding the PAD4 protein is one of the RA risk genes associated with protein citrullination [40], and anti-PAD4 antibodies are specific markers of RA [18]. Badillo-Soto et al. [41] suggested that citrullination of synovial proteins is PAD2- and PAD4-dependent and both these enzymes have been detected in the RA synovium [42]. These authors also found that RA patients have high titres of antibodies preferentially binding to fibrinogen citrullinated by PAD4. Seri et al. [43] reported data from mice suggesting that PAD4 deficiency reduced the severity of arthritis in a glucose-6-phosphate isomerase-induced arthritis model. An explanation could be that chronic exposure to citrullinated proteins in the periodontal pocket may predispose susceptible individuals to generate ACPA in the synovia with subsequent development of RA. This is because ACPA titres in RA patients correlate with the presence of PD [34].

Atherosclerosis and citrullination

Atherosclerosis occurs when the arteries become narrower and harden due to an excessive build-up of plaque within the lumen. The plaque reduces the blood flow around the body, causing ischaemia, which in turn may lead to cardiovascular complications. Atherosclerosis is common in the elderly and remains the major cause of death and disability in this group. The American Heart Association (AHA) recognizes that PD is independently associated with arteriosclerotic vascular disease (ASVD) [44]. The connection between citrullinated peptides and the development of atherosclerosis remains unclear in comparison to RA. Citrullinated proteins are detected in atherosclerotic plaques [45,46], but their true relevance here has not been clarified. This observation has provided a rationale for using citrullinated histone seroreactivity as a biomarker for atherosclerosis [45]. It may reveal citrullinated fibrinogen (cFb) within atherosclerotic plaque and therefore could underpin the reason for accelerated atherosclerosis in RA patients. Geraldino-Pardilla et al. [46] reported that higher levels of ACPAs targeting citrullinated histone 2B were associated with higher coronary artery calcium (CAC) scores when compared with lower antibody levels, suggesting a potential role of seroreactivity to citrullinated histone in the pathogenesis of atherosclerosis [46].

PPAD acting together with arginine-specific proteinases from *P. gingivalis* may promote the growth of this pathogen in the periodontal pocket [11]delete space. PPAD deiminates the guanidine group of C-terminal arginine residues on a variety of peptides, including the vasoregulatory peptide-hormone bradykinin yielding a citrulline residue and ammonia [11]. Citrullinated bradykinin must be resistant to inactivation by ubiquitous Arg-specific tissue and cell-surface associated carboxypeptidases thus prolonging its vasodilatory activity. Such pathophysiological events may allow bacteria to penetrate the vasculature, and

advance the development of cardiovascular disease [47]. As *P. gingivalis* has a high dependency on environmental haem [48], sourced from lysing erythrocytes, this activity implies host deprivation of its ample supply of oxygen. This would also promote hypoxic conditions as well as development of atherosclerotic plaque.

A recent report described citrullination that was unique to the cardiac proteome [49]. Protein citrullination appeared to have caused important structural alterations in the cardiac sarcomere with subsequent detrimental consequences to the myocardium of patients who died of heart failure. This implied that pathogenic citrullination occurs in systemic diseases but further research is needed to understand its adverse role in heart-related tissues.

Citrullinated proteins such as fibrinogen and vimentin are associated with the CAC, score. Sokolove et al. [45] demonstrated fibrinogen and vimentin CAC scores in 134 patients with RA diagnosis. Previously mentioned PAD4 enzymes and citrullinated proteins have been detected within atherosclerotic plaques and ACPAs from atherosclerotic plaques in RA patients [45]. In a cohort of 3,052 healthy males, Cambridge et al. [50] monitored ACPAs for the development of coronary artery disease. Their results showed that 10.4% of the cases were ACPA positive compared to 3.8% of controls. Statistical significance remained after controlling for classical risk factors such as smoking and C-reactive protein ($p = 0.02$). If PD had been monitored in the same cohort this would have demonstrated a prominent role for *P. gingivalis* in the production of ACPAs. Strong staining for citrulline was detected in the myocardial interstitium of RA patients compared to rheumatoid disease and controls [51] and staining for citrullination was higher in the myocardial interstitium of RA patients compared to other diseases. Since there is extensive citrullination in the normal myocardium of RA patients as well as in the atherosclerotic plaque, there is potential for protein citrullination to promote cardiovascular pathology within the population at large [52]. In this context, serum antibodies to citrullinated proteins may be a risk factor for coronary heart disease.

Alzheimer's disease and citrullination

AD is the most common form of dementia, constituting 60–80% of all dementias, and has two forms. The sporadic form is common with potential for the role for susceptibility genes and pathogens as well as co-morbidities, similar to those implicated in the aforementioned human complex diseases [7]. Due to the rising number of dementia cases, and the paucity of adequate treatment for AD, there is a consensus for preventing the disease in at least a third of all sporadic AD cases by modifying behavioural life-styles [10]. A person showing symptoms of AD can have difficulty in remembering words, suffer from depression and show behavioural changes and confusion. The benchmark of AD confirmation is the presence of two key neurohistological hallmarks namely beta amyloid (Aβ) plaques, and neurofibrillary tangles (NFTs) comprising hyperphosphorylated tau protein in specific anatomical regions of the brain. All risk factors that apply to heart disease also apply to AD. An underlying feature of stroke pathology includes vascular infections where *P. gingivalis* is often identified [53]. Increased evidence links peripheral infections with AD, and lipopolysaccharide (LPS) entry into the ageing brain generates cytokines via innate immune responses (Figure 3) [9]. LPS ischaemia from atherosclerosis and hypoxia link *P. gingivalis* with early death of erythrocytes for the supply of haem. All are potential causes for the development of sporadic AD. *P. gingivalis* was shown to migrate from its oral location to the brain [54,55] where it invoked inflammatory responses typical of neurodegenerative diseases in mice with blood–brain barrier (BBB) damage [55,56]. Ishigami et al. [57] identified glial fibrillary acidic protein (GFAP), a marker of astrocytes in the AD brain, to be a substrate for host PAD2, and suggested a role for the citrullinated GFAP in the progression of this neurodegenerative disease. GFAP deimination was characteristic for AD in humans and in experimental autoimmune encephalomyelitis (EAE) in mice where the BBB was breached [58–60]. Although not conclusively shown, citrullinated GFAP (dysfunctional protein) would link with defects in BBB integrity because the foot processes of astrocytes tightly cover capillary openings in the endothelial cell junctions to protect neurons from extrinsic insults. Another report implicated brain-reactive autoantibodies to AD in relation to BBB breakdown and to certain cytoskeletal proteins such as tubulin, GFAP, and S-100 [61] (Figure 3). The relevance of humoral responses in the pathophysiology of AD, is little understood; however, by analogy, RA patients having a high titre of antibodies in their serum suggests a strong possibility of PPAD/host PAD mediated loss of tolerance against citrullinated nerve tract covering myelin sheath proteins in AD.

Acharya et al. [62] confirmed citrullination of pyramidal neuronal intracellular proteins in the AD hippocampus. Antibodies against myelin basic protein (MBP) are detectable in serum of patients with active demyelinating lesions in MS [63]. Analogous to MS as an autoimmune condition, damaged myelin interacts with Aβ deposits in AD and antibodies to glial derived antigens are reported [64]. Acharya et al. [62] suggested that autoantibodies in AD associate with host PAD4 and protein citrullination, although it is not

clear if this refers to the antibodies reported by Papuc et al. [64]. Matsuomi et al. [65] found an abnormal accumulation of citrullinated proteins and an increase of the PAD2 content in the hippocampi of AD patients. The most over-citrullinated proteins in AD were structural proteins such as vimentin, MBP and GFAP [15]. Neurodegenerative processes following *P. gingivalis* infection in animal models are currently under-investigated and further research is essential to determine PD-related causal factors in AD.

Over expression of PADs and protein citrullination are abnormal features of neurodegeneration and inflammatory diseases [66] and have actually been proposed as a possible cause of AD [67]. During neurodegenerative processes, it has been hypothesized that a higher concentration of Ca^{++} activates citrullination [15] but, *P. gingivalis* LPS and intact citrulline constitute potential contributory factors [68].

PAD2 and PAD4 enzymes are detected in astrocytes and neurons, and there is a concomitant accumulation of citrullinated proteins within PAD4-expressing cells including neurons of the hippocampus and the cerebral cortex [62,65]. This implies that citrullination of neuronal cytoskeletal proteins may be toxic, because disease-associated neuronal loss appears to result in the release of their cellular contents into the cerebral parenchyma from which they enter the blood and lymph circulation. Some of them are able to elicit an immune response that results in the production of autoantibodies [62]. Inhibition of PAD may therefore be worth serious therapeutic consideration in the treatment/prophylaxis of diseases where citrullination takes place.

Concluding remarks

Conversion of arginine to citrulline is a post-translational modification that occurs during normal physiological processes. Conversely, abnormal citrullination can lead to severe human diseases. Epidemiologically, there seems to be a correlation between citrullination caused by *P. gingivalis* and PD [69,70] and between *P. gingivalis* and RA [14,71–73]. There is also an association between AS and RA [74]. In reality, cardiovascular disease, including coronary heart disease is a significant cause of death in RA [75] and AD patients [76]. *P. gingivalis* secreting PPAD is related to both RA [14] and AS [77]. It seems plausible that PPAD through its ability to citrullinate proteins could contribute to PD, RA, AS, and AD through increased inflammation, although currently the anti-PPAD antibody response remains unique to RA. *P. gingivalis* infection may precede RA but whether it is a direct cause is controversial despite supporting data from studies on animals in the development and aggravation of experimental arthritis [72]. Overall, citrullination may contribute to a better understanding of host proteins [14]. There is great heterogeneity in the extracellular proteome and citrullinome of *P. gingivalis* [78]. This adds to the well-known fact that different strains of *P. gingivalis* have different degrees of virulence. The suggestions outlined above would benefit from more scientific support and drawing firm conclusions on them at this time would be inappropriate. However, if future research shows them to be correct; it would emphasize the need for prevention and aggressive treatment of advancing periodontitis where *P. gingivalis* is a keystone pathogen, but not necessarily the only one. It is also clear that *P. gingivalis* as a keystone pathogen is only present at high prevalence rates in the host subset susceptible to PD. Therefore, although the *P. gingivalis*-PPAD-systemic disease axis is a compelling line of thought, this may not be true for all patients with RA or AD where *P. gingivalis* has been implicated.

Disclosure statement

No potential conflict of interest was reported by the authors.

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