Periodontitis and rheumatoid arthritis

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rijksuniversiteit groningen

PERIODONTITIS AND RHEUMATOID ARTHRITIS

A search for causality and role of *Porphyromonas gingivalis*

Proefschrift

ter verkrijging van de graad van doctor aan de

Rijksuniversiteit Groningen

op gezag van de

rector magnificus prof. dr. E. Sterken

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 4 november 2015 om 12.45 uur

door

Menke Janke de Smit

geboren op 22 november 1976

te Deventer

Opmaak en druk: RCG Grafimedia, Groningen

ISBN: 978-90-367-8362-0, digitale versie: 978-90-367-8363-7

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INTRODUCTION TO THE THESIS

Periodontitis

Periodontitis is a chronic inflammatory disease that leads to destruction of the soft and hard tissues supporting the teeth (the periodontium). If left untreated, advanced periodontitis may ultimately result in loss of teeth. The essential role of dental plaque in the etiology of periodontitis has been well established by studies that have shown that removal of supra- and subgingival dental biofilm normally results in disease resolution. Extensive microbial composition analyses have identified oral bacteria, such as *Porphyromonas gingivalis,* as strong markers of disease status.
Periodontal health requires a controlled immuno-inflammatory state that can maintain host–microorganism homeostasis in the periodontium [1]. However, in periodontitis, the host immune response is deregulated - either because it is subverted by the microbial community or because of host immunoregulatory defects- and is therefore ineffective at restraining bacterial outgrowth and overt pathogenicity [2].

Periodontitis is not a local phenomenon

Periodontitis is not only a cause of tooth loss but has also been shown to affect systemic health [3]. It is linked to the initiation, progression and/or disease activity of systemic autoimmune or inflammatory diseases including diabetes, cardiovascular disease, inflammatory bowel disease and rheumatoid arthritis [4, 5]. However, it is yet difficult to determine whether periodontitis is a cause or a consequence of these complex and multifactorial diseases. Before periodontitis can be admitted into the causal chain of a disease, the evidence of the association of periodontitis with a particular disease has to be extremely high. In addition, the relationship can be bidirectional because of common environmental and genetic risk factors and parallel pathogenic pathways [6].

Association with rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease that is characterized by chronic destructive polyarthritis of synovial joints. Immune dysfunction causes accumulation of destructive pro-inflammatory mediators in the synovial membrane leading to synovitis and destruction of cartilage and bone tissue of the joint.
In the past decade, the interest in the epidemiological and pathological relationships between periodontitis RA has been rising, driven in part by interest in the role of citrullination and attendant autoantibody responses as a disease-defining feature of RA, and the recognition that oral bacteria and inflammation may play important roles [7]. Citrullination is a post-translational modification catalyzed by a family of enzymes called peptidylarginine deiminases (PAD) [8]. In this reaction, an arginine residue within a protein is converted into the non-coded amino acid citrulline. This modification leads to a loss of positive charge, reduction in hydrogen-bonding ability and subsequently in conformational and functional changes of the protein. Citrulline is not a natural amino acid in proteins and may therefore induce an immune response. Citrullination is involved in several physiological processes including terminal differentiation of the epidermis, apoptosis, and gene expression regulation, but it has also been implicated in pathological processes and autoimmunity like in RA [9].

Autoimmunity in periodontitis

Autoimmune reactions to native and post-translationally modified self-antigens may play a role in the pathogenesis of aggressive periodontitis [10]. Overproduction of reactive oxygen species (ROS) within the inflamed lesion, as a result of influx of oxygen consuming inflammatory cells, leads to post-translational modification by ROS [11]. Furthermore, break of tolerance may be initiated by enzymatic post-translational modification, for example, cleavage of extracellular proteins by matrix metalloproteases [12], bacterial proteases [13] or citrullination by PAD enzymes [14]. The exact etiology of autoimmune reactivity in periodontitis is not known, but may be linked to the inflammatory process resulting from infection with *P. gingivalis*, which expresses both arginine-specific proteases (gingipains) and PAD [10, 15].

Inflammation and auto-immunity

Chronic inflammation as a result of infection may play a role in the initiation, progression, and perpetuation of chronic autoimmune diseases. Although many other factors are necessary to develop autoimmunity, a number of mechanisms have been postulated by which infection can trigger autoimmune disease. Infectious agents might induce T- or B-cell responses that can cross-react with self antigens, due to similarities between microbial and self-proteins or peptides that are sufficient to result in the activation of auto-reactive T- and B- cells (molecular mimicry). In addition, microbial processing of self peptides, such as posttranslational modification, can result in 'foreign' antigens or antigens that activate cross-reactive T- or B- cells (cryptic antigens). Activation of the cross-reactive T- and B- cells results in the release of cytokines and chemokines that recruit and activate monocytes and macrophages, which mediate self-tissue damage, cell apoptosis and/or necrosis (bystander activation) and enhanced processing and presentation of self-antigens (epitope spreading) (see Fig. 1, page 12, reused from [16]).

Self-reactive T-cells in RA

The human leukocyte antigen (HLA) system is the locus of genes that encodes for proteins on the surface of cells (the major histocompatibility complex, MHC), and is responsible for regulation of the immune system in humans. The major function of MHC is to bind to peptide fragments derived from pathogens and display them on the cell surface of an antigen-presenting cell (APC) for recognition by the appropriate T-cells via the T-cell receptor (TCR). In RA, HLA is considered to be the major genetic factor determining disease susceptibility because MHC has to interact with citrullinated self-peptides with a certain affinity in order to elicit an antigen-specific immune response. In 1987 a 'shared epitope hypothesis' was postulated for the association of particular MHC-II molecules and RA [17]. The shared epitope is located in one of the substrate binding sites (the P4 pocket) of the MHC class II molecule in the HLA-DR β-chain. Individuals carrying positively charged P4 pockets (with shared epitope of HLADRB*0101, *0401 or *0404) can mount an immune response to citrullinated peptides and are susceptible to RA [18]. In contrast, those who express negatively charged P4 pockets (HLA-DRB*0402) might be protected from the disease [19].

AIM AND OUTLINE OF THE THESIS

A search for causality and role of Porphyromonas gingivalis

The aim of the thesis was to assess possible causality in the association between periodontitis and RA, with the focus on the role of *P. gingivalis*. Causality was analyzed with assistance of the Bradford Hill criteria [20]. These criteria have been designed to be considered before deciding that the most likely interpretation of an association between two diseases is causation. The criteria to be considered involve: strength and consistency of the association, biological plausibility, temporal relationship, specificity regarding to *P. gingivalis*, dose-response relationship, experimental evidence and coherence of clinical findings, and analogy.

In **chapter 1**, we started our research by summarizing at the time present available knowledge and hypotheses regarding the disease association between periodontitis and RA.
In **chapter 2**, epidemiological data are presented from a population of RA patients of the northern part of the Netherlands. In this study several Bradford Hill criteria were considered, including strength and consistency of the association between RA, periodontitis, and *P. gingivalis*, and dose-response relationship, i.e., whether severity of periodontitis is linked to severity of RA.
In **chapter 3**, temporal relationship, i.e., cause (infection with *P. gingivalis*) precedes consequence (RA), was investigated by assessment of the antibody response against *P. gingivalis* in a cohort of patients at risk for RA and who were prospectively followed for RA development.

Because RA auto-antibodies often precede clinical signs of RA and because of the assumption that mucosal inflammation, such as periodontitis, can precede RA, in **chapter 4**, presence of RA auto-antibodies was assessed in patients without RA but with oral (periodontitis) or lung mucosal inflammation.
Objective of the study in In **chapter 5** was assessment of *P. gingivalis* PAD gene expression and citrullination patterns in representative samples of *P. gingivalis* isolates from patients with and without RA and in related microbes of the *Porphyromonas* genus. In **chapter 6**, available evidence for possible causality between periodontitis, *P. gingivalis* and RA, according to the Bradford Hill criteria, is summarized.

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Figures

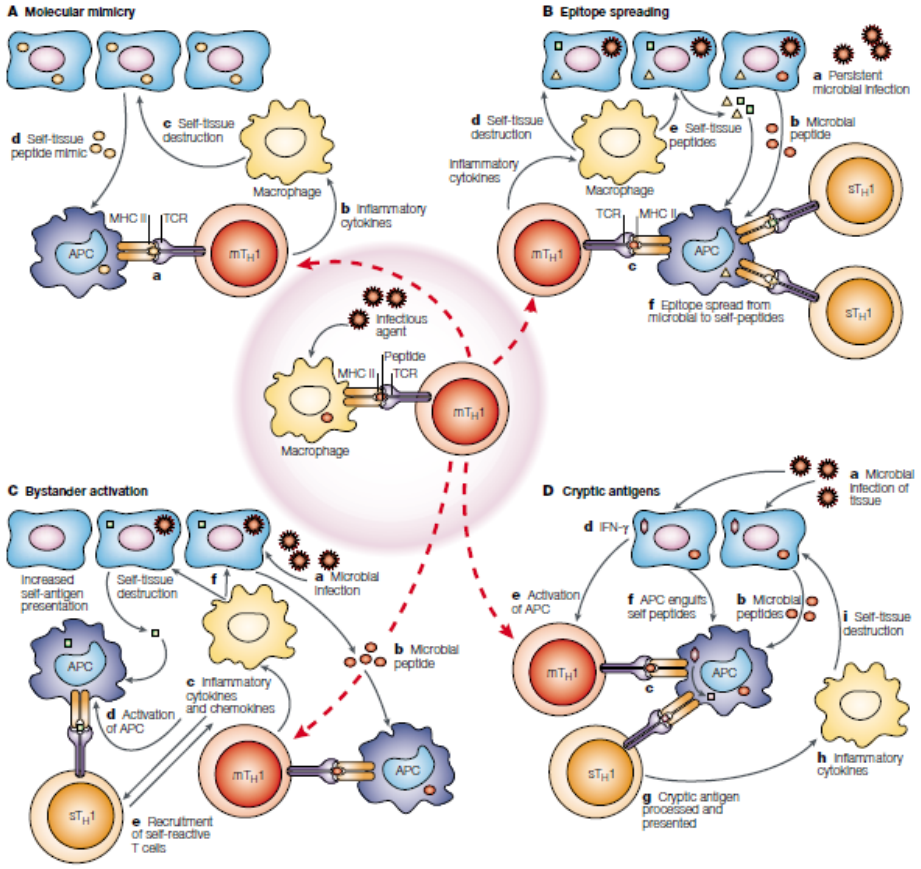

Fig. 1 Mechanisms of infection-induced autoimmunity via self-reactive T-cells. Reused from [16].

A. Molecular mimicry displayed as the activation of cross-reactive TH1 cells that recognize both the microbial epitope (mTH1) and the self epitope (sTH1) (a). Activation of the cross-reactive T-cells results in the release of cytokines and chemokines (b) that recruit and activate monocytes and macrophages, which mediate self-tissue damage (c). The subsequent release of self-tissue antigens and their uptake by APCs perpetuates the autoimmune disease (eptitope spreading, see also figure B)

B. Epitope spreading involves a persistent microbial infection (a) that causes the activation of microorganism-specific TH1 cells (b, c) which mediate self-tissue damage (d). This results in the release of self peptides (e), which are engulfed by APCs and presented to self-reactive TH1 cells (f). Continuous damage and release of self peptides results in the spread of the self-reactive immune response to multiple self-epitopes (f).

C. Bystander activation is the nonspecific activation of self-reactive TH1 cells. Activation of microorganism-specific TH1 cells (a, b) leads to inflammation (c, d) and results in the increased infiltration of T-cells at the site of infection and the activation of self-reactive TH1 cells by TCR-dependent and -independent mechanisms (e) Self-reactive T-cells activated in this manner mediate self-tissue damage and perpetuate the autoimmune response (f).

D. Cryptic antigen model describing the initiation of autoimmunity by processing of self peptides, such as post-translation modification. Following microbial infection (a) pro-inflammatory cytokines are secreted by both activated microbe-specific TH1 cells (b, c) and microbe-infected tissue cells (d). This activates APCs (e) and can lead to APC engulfing self-antigens (f). Cytokine activation of APCs can induce increased protease production and different processing of captured self-antigens, resulting in presentation of cryptic epitopes. The presentation of these cryptic epitopes can activate self-reactive TH1 cells (g), leading to self-tissue destruction (h, i).

TH1: T helper 1 cell that recognize the microbial epitope (mTH1), the self epitope (sTH1) or both (cross-reactive TH1), APC: antigen-presenting cell, MHC II: major histocompatibility complex class II, TCR: T-cell receptor.

CHAPTER
01

General introduction

Rheumatoid arthritis and periodontitis; a possible link via citrullination

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Anaerobe 2011 Aug;17(4):196-200. doi: 10.1016/ j.anaerobe.2011.03.019. Epub 2011 Apr 16.

Abstract

Rheumatoid arthritis (RA) and chronic and aggressive periodontitis are chronic inflammatory disorders characterized by deregulation of the host inflammatory response. Increased secretion of pro-inflammatory mediators results in soft and hard tissue destruction of the synovium and periodontium respectively. Both diseases share risk factors and have pathological pathways in common, resulting in loss of function and disability as a final clinical outcome. This article discusses possible interactions, particularly related to the periodontal pathogen *Porphyromonas gingivalis*, which could explain the observed association between these two prevalent diseases.

1. Periodontal disease

Supragingival plaque accumulation results in an inflammatory response of the gums and is called gingivitis. This infection can be eliminated by reduction of the total bacterial load through simple oral hygiene measures. When the infection proceeds, destructive periodontal disease or periodontitis can develop in susceptible individuals. Periodontitis can be characterized as chronic (slowly progressive) or aggressive (highly destructive), further classification can be made on the extent (localized/generalized) and severity (mild/moderate/severe) [1]. Clinical signs of the disease are bleeding gums, deepened periodontal pockets, suppuration and in an advanced stage, mobility of the teeth with tooth loss as the final disease outcome due to extensive loss of alveolar bone. Periodontitis is a multifactorial, bacterial driven, chronic inflammatory disorder; severe periodontitis occurs in 10–15% of an adult population, independent of ethnicity and geographic location [2]. Bacteria play a major role in etiology; it is thought that the biofilm in the subgingival area causes a chronic inflammatory response that is responsible for destruction of the alveolar bone and soft tissue surrounding the teeth (the periodontium). The subgingival biofilm in periodontal lesions consists of hundreds of bacterial species, most of which are strict anaerobic and of which a significant part is non-cultivable. Cultivable microbial indicators for periodontitis are, among others, *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Parvimonas micra, Treponema species* and *Tannerella forsythia* [3]. Although bacteria are essential for periodontal disease to occur, a susceptible host is also required. It is thought that susceptibility is determined by genetic traits and lifestyle factors such as smoking and stress. Identified risk factors for the initiation of periodontitis are subgingival calculus and subgingival detection of *A. actinomycetemcomitans* [4]. Risk indicators for progression of the disease include smoking [5], age [4], stress and psychological factors [6] and existing attachment loss [7]. Other putative risk factors involve gender, education, socio-economic status [8], nutritional factors [9] and body mass index [6, 10].

2. Periodontitis is not a local phenomenon

In generalized severe chronic and aggressive periodontitis the infected and necrotic epithelium surface area amounts up to 20 cm^2. Periodontal lesions may lead to bacteremia that can be the cause of focal infection of dental origin [11-13]. Severe periodontitis also results in a continuous systemic inflammatory response [14-16]. Periodontitis has been associated with a number of other chronic and inflammatory diseases such as diabetes mellitus [17], atherosclerosis, cardiovascular disease and stroke [18], rheumatoid arthritis [19, 20], Crohn's disease and ulcerative colitis [21] and preterm birth and low birth weight [22]. In this paper we review current knowledge on the association of periodontitis and rheumatoid arthritis and discuss possible mechanisms of interactions between the two disorders.

3. Rheumatoid arthritis and anti-citrullinated protein antibodies

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis with a prevalence of 0.5%–1.0% of adults in industrialized countries. The disease is far more common among women than men (3:1) and prevalence rises with age, with a peak in the fifth decade [23]. The etiology is multifactorial and the pathogenesis is poorly understood. Autoimmunity to citrullinated proteins is highly specific for RA and may be of pathogenic significance [24]. Risk of developing RA is of 50% attributable to genetic factors [25]. Smoking is the major known environmental risk factor for RA. Smoking and genetic risk factors interact in providing an increased risk of RA [26]. Immune responses with several inflamma-

tory cascades lead toward a final common pathway with persistent synovial inflammation and associated damage to articular cartilage and underlying bone as a consequence. There is evidence of a preclinical or asymptomatic phase of the disease, in which auto-antibodies can be be present [27]. The auto-antibodies most frequently found in patients with RA are antibodies which bind to the constant domain of IgG molecules (IgM rheumatoid factor, IgM RF) and antibodies against citrullinated proteins (anti-citrullinated protein antibodies, ACPA). The majority of individuals with RA (50–80%) have serum positive titers for IgM RF and/or ACPA. ACPA have a higher specificity (98%) and sensitivity (up to 80%) for diagnosis of RA than IgM RF [28]. ACPA seem to be better predictors of poor prognosis of RA; ACPA positive RA is associated with increased joint damage and low remission rates [29]. ACPA are rare (<1%) in other inflammatory conditions [30]. ACPA were originally measured as antibodies against keratin (the anti-perinuclear factor) [31], and more recently as anti-cyclic citrullinated peptides (anti-CCP) [32]. These auto-antibodies recognize epitopes containing citrulline. Citrulline is a nonstandard amino acid, and is therefore not incorporated in proteins during translation. However, it can be generated by post-translational modification (citrullination) of protein-bound arginine by peptidylarginine deiminase (PAD) enzymes. This post-translational modification may have a big impact on the structure and function of the target protein, partly due to a change of charge. Citrullination is an inflammation-associated phenomenon, occurring in a wide range of tissues. It is predominantly observed in proteins of the cytoskeleton. It seems to represent a general regulatory mechanism, particularly occurring during apoptosis. So far, five isotypes of PAD have been described in humans. All these enzymes rely strongly on the presence of calcium for activity and are unable to convert free L-arginine into L-citrulline, which can be done by nitric oxide synthase in eukaryotes or by arginine deiminase in bacteria. Because of their calcium dependency, PAD enzymes are more likely to be active in the extracellular compartment. PAD2 and PAD4 are found in synovial fluid and in synovial tissue of RA patients and are therefore the most likely candidate PAD isotypes for the citrullination of synovial proteins in RA [33]. Smoking enhances PAD2 expression in human lungs with consequent generation of citrullinated proteins in the bronchoalveolar compartment [12]. Recently, PAD2 expression and citrullinated proteins have also been detected in the periodontium [34]. Whereas citrullination is associated with inflammation in general, the development of antibodies against them (ACPA) is specific to RA. The high specificity of ACPA is therefore most likely the result of an abnormal humoral response to these proteins. Citrullinated proteins and ACPA are present in the inflamed synovium [35], suggesting that the resulting immune complexes are directly involved in the disease pathogenesis of the chronic inflammation in the rheumatoid joint. If there is local ACPA production in the periodontium or in the bronchoalveolar compartment remains to be established, albeit higher ACPA reactivity in serum samples of aggressive periodontitis patients has been reported [36].

3.1. Similarities between RA and periodontitis

There are remarkable similarities between RA and chronic and/or aggressive periodontitis. Both diseases are chronic destructive inflammatory disorders characterized by deregulation of the host inflammatory response. The etiology of both diseases is multifactorial and susceptibility to the diseases is influenced by shared genetic and lifestyle factors. Both diseases are cumulative, i.e. severity, loss of function and quality of life decrease with longer disease duration. There are common pathological mechanisms; both conditions are potentiated by an exaggerated inflammatory response featuring an increase

in localized and perhaps circulating pro-inflammatory mediators, resulting in soft and hard tissue destruction of the periodontium and synovium respectively.
A number of clinical studies point toward an association between periodontal disease and RA [37], despite the fact that patients suffering from RA are often treated with immune suppressant corticosteroids, thereby possibly reducing clinical evidence of periodontal disease. An important observation is that treatment of periodontal disease has a positive effect on disease activity of RA [38], although this observation needs further confirmation. Surprisingly, none of the studies on RA and periodontal disease considered microbiology, although bacteria play a primary role in the etiology of periodontal disease. Similarities in risk factors, common pathological pathways, association in prevalence and the effect of periodontal treatment on RA make us look further to explore the relation between periodontitis and RA, with a special focus on microbiology.

4. The Bradford Hill approach

To describe the strength and nature of an association between two disorders, the widely used Bradford Hill criteria to determine a causal association are applied [39]. These involve strength, consistency, specificity, temporality, biological gradient, plausibility, coherence, experimental evidence and analogy of the association.
Starting from epidemiologic evidence, four issues need to be addressed: strength, consistency, temporal relation, and analogy. The third National Health and Nutrition Examination Survey (NHANES III) is a nationally representative cross-sectional survey of non-institutionalized US population. Using this data, de Pablo et al. [40] included participants aged >60 years who had undergone both musculoskeletal and dental examinations (n = 4461). They found that subjects classified with RA, according to the American College of Rheumatology (ACR) criteria of 1987 (n = 103), were more likely to suffer from periodontitis, after adjusting for age, sex, ethnicity and smoking. Considering three out of six ACR criteria, they found an odds ratio (OR) of 1.8, considering four out of six ACR criteria an OR of 4.1. Participants with RA had significant more missing teeth than participants without RA. Comparing periodontal status in 65 RA patients (according to the ACR criteria 1987) with an age- and gender-matched control group (age range 20-70 years) without RA, Mercado et al. [41] found that individuals with RA are more likely to experience more periodontal disease (OR 2.2) compared to individuals without RA. Individuals in the RA group showed significant more missing teeth compared to the non-RA group, an observation that confirms previous findings [42, 43]. Indicators of disease activity for RA most positively correlated with periodontal bone loss were the number of swollen joints, health assessment questionnaire scores, levels of C-reactive protein and erythrocyte sedimentation rates. The other way around, in 1412 individuals attending the University of Queensland's School of Dentistry, Mercado et al. [44] found that self-reported RA was significantly higher in patients referred for periodontal treatment (n = 809) compared to patients not referred for periodontal treatment (3.95% vs. 0.66%). Nesse et al. [20] found in a cross-sectional study an increased prevalence of RA in patients with periodontitis, which could not be explained by the confounding factors sex, age and smoking. Coherence of the association is influenced by variation in design, setting, methods, selection bias and the fact that the majority of the studies on this association are low prevalence case-control studies with no consistent criteria to define periodontitis. With respect to temporal relation, specific auto-antibodies (IgM RF and ACPA) precede the symptoms of RA [27]. Half of patients with RA have specific serologic abnormalities several years (median 4.5 years) before the development of clinical symptoms. Besides the analogy of the characteristics of the population to which

the diseases are exposed, the two diseases have pathological mechanisms in common and they share environmental and genetic risk factors. If there is a dose-response relation between the two diseases is currently unknown. The available studies on association of periodontitis and RA did not quantify the extent of periodontal disease, but is now possible with the periodontal inflamed surface area (PISA) index for inflammatory burden [45]. Nevertheless, antibody titers to the periodontal pathogen *P. gingivalis* are increased in patients with RA and there are significant positive correlations between *P. gingivalis* antibody titers, CRP concentrations and antibody titers to citrullinated proteins, i.e. to disease specific immunity [46]. Biological plausibility is partly explained by this association, and the fact that periodontitis causes an inflammatory burden by eliciting a systemic inflammatory response. Antibody response to *P. gingivalis* and DNA of *P. gingivalis* self have been found in synovial fluid of RA patients [47-49]. Experimental evidence is drawn from two controlled studies that have been conducted on the effect of periodontal treatment on RA [38 ,50]. Both studies showed that periodontal therapy had a beneficial effect on laboratory RA parameters and clinical symptoms of RA. Because these studies had a small sample size and did not consider microbiology, there is a crying need for better designed experimental studies on the effect of periodontal treatment on RA disease activity.

5. Genetic factors in RA and periodontal disease

In both diseases, candidate gene approach revealed mainly genetic variations in genes encoding for elements of the innate immune system as a risk indicator. More than 30 genetic regions are associated with RA. Genetic variations in the major histocompatibility complex, class II, DR beta 1 (HLA-DRB1) and protein tyrosine phosphatase (PTPN22) genes are the major genetic risk indicators that have been reproducibly identified so far. The association of a number of specific HLA-DRB1 alleles is seen exclusively for the ACPA positive subset of RA [51]. These HLA alleles share a common peptide-binding motif known as the shared epitope (SE). Antigen modification by protein citrullination is thought to allow antigens to fit in the HLA alleles that hold this SE. The result is breaking of tolerance and antibody formation against these antigens [52]. The PTPN22 gene codes for a tyrosine phosphatase, with a potential function in the regulation of T-cell and B-cell activation. The best-studied environmental factor in RA is smoking and this seems to be a risk factor for ACPA positive disease, especially in the context of positivity for HLA-DRB1 SE alleles [53]. Studies have also shown an additive interaction between PTPN22 and smoking, however no gene-gene interaction was observed between PTPN22 and HLADRB1 SE [54]. Genetic and lifestyle factors (smoking) have become the leading susceptibility factors in periodontal disease. The family background and the familial aggregation of early onset aggressive periodontitis have long been recognized. This supports the connection between certain genes' mutation and periodontal disease manifestation. Like RA, among candidate genes possibly associated with increased host immune susceptibility to periodontitis are HLA-DR polymorphisms. A significant association was found between HLA-DRB1 SE and aggressive periodontitis, stratified according to ethnogeographic origin [55]. Several single nucleotide polymorphisms, notably in the IL-1, IL-6, IL-10, vitamin D receptor, and CD14 genes have been linked to severity and presence of destructive periodontal disease [56] Genes that encode for IL-1 production have received attention as potential predictors of periodontal disease progression, because of its involvement in the regulation of the host's inflammatory response and bone resorption. IL-1 is not only involved in signaling processes resulting in autoimmune induced bone destruction but also in several hereditary

autoinflammatory syndromes. Meta-analysis of four common promoter SNPs in the IL-1 region in British Caucasian patients revealed an association with increased susceptibility to RA [57]. Irrespective of smoking and presence of *P. gingivalis* and *A. actinomycetemcomitans*, patients with severe periodontitis (chronic and/or aggressive) showed a significantly higher frequency of the positive IL-1 genotype than periodontally healthy individuals (42% vs. 11%, all Caucasian subjects) [58]. In a study of 42 patients (1044 teeth) in maintenance care for 14 years, the combined effect of a positive IL-1 genotype and smoking did increase the risk of tooth loss by 7.7 times, compared to 2.7 and 2.9 times for positive IL-1 genotype and smoking separately [59]. Also, gene polymorphisms in pro-inflammatory cytokines IL-6 and the IL-1 cluster are associated with systemic inflammation in patients with severe periodontitis (chronic and/or aggressive, 65% European Caucasians) [15].

5.1. A link via citrullination

Given the fact that antibody formation against citrullinated proteins plays a major role in autoimmunity in RA, and given the fact that citrullination seems to be a unique feature for the periodontal pathogen *P. gingivalis*, Rosenstein et al. [60] were the first to hypothesize that the onset and progression of RA is influenced by the presence of periodontal infection with *P. gingivalis*.
The bacteria involved in periodontitis accumulate in a subgingival biofilm that comprises predominantly Gram-negative strict anaerobic rods. The group of dark-pigmented anaerobic rods is strongly associated with destructive periodontal disease and the major pathogen in this group is *P. gingivalis* [61]. The prevalence of *P. gingivalis* in severe periodontitis is 70% and it has been infrequently isolated from subjects without periodontitis [3], suggesting that this bacterium is not a normal inhabitant of a healthy periodontium [62]. To date, the single prokaryotic enzyme that can citrullinate proteins, has been identified in *P. gingivalis* [63]. Based on the biochemical characteristics and properties of this PAD enzyme, it could be a virulence agent. *P. gingivalis* PAD deiminates the guanidine group of carboxyl-terminal arginine residues on a variety of peptides, to yield ammonia and a citrulline residue. In contrast to human PAD, it can convert both peptidylarginine and free L-arginine and is not dependent on calcium [64].
Known antibodies to citrullinated proteins, the specific serological markers for RA, include anti-citrullinated keratin (the anti-perinuclear factor), anti-citrullinated vimentin (formerly known as the Sa-antigen), anti-citrullinated filaggrin, anticitrullinated fibrin(ogen) and anti-citrullinated α-enolase antibodies. Alpha-enolase is a multifunctional protein, best known for its role in glucose metabolism and more recently as a plasminogenbinding protein on the surface of various mammalian and prokaryotic cells [65, 66]. In RA, the immunodominant epitope of human α-enolase is citrullinated-enolase-peptide-1 (CEP-1). This epitope (amino acids 5-21) shows 82% sequence similarity with CEP-1 of *P. gingivalis*. The amino acids 13-21 are 100% identical. Antibodies purified for affinity to human CEP-1 cross-reacted with CEP-1 of *P. gingivalis* [67]. Recently, Wegner et al. [68] showed that PAD from *P. gingivalis* is able to citrullinate its endogenous proteins and more strikingly, also human fibrinogen and human α-enolase. This seems to be a unique characteristic of *P. gingivalis*. Thus, the immune system in patients with periodontal infection with *P. gingivalis* is exposed to citrullinated antigens that might become systemic immunogens; directly, or via molecular mimicry and cross reactivity [60, 68]. Periodontal infection with *P. gingivalis* could contribute to the total antigenic load of citrullinated proteins, generated by host PAD during the inflammatory response and by bacterial PAD produced as a virulence factor of *P. gingivalis*. In a genetic susceptible host, for example in context of HLA-DRB1 SE, this

could result in a pathologic immune response, with the formation of ACPA and joint inflammation as a consequence. How periodontitis and RA could be related through common genetic and lifestyle risk factors, inflammatory burden, and in particular in presence of *P. gingivalis* is graphically represented in the illustration at the end of this chapter (Fig. 1, page 27).

To come back to the Bradford Hill criteria, biological plausibility is partly explained by the fact that periodontitis causes a systemic inflammatory response. The association of *P. gingivalis* with the RA-related anti-citrullinated protein antibody response could be another explanation. Sequence similarity and cross-reactivity with immunodominant epitopes of citrullinated proteins and their bacterial variants may indicate a role for *P. gingivalis* in autoimmunity in patients with RA. To fulfill the Bradford Hill criteria in detail, studies linking periodontal disease and RA need further investigation. If there is a distinct relation, treatment of periodontitis is thought to be of influence on disease activity of RA. By studying (pre)clinical and (micro)biological markers of both diseases, we intend to further unravel the pathogenic relation between periodontitis and RA. Recognition of the association between RA and periodontitis on both a clinical and biologic level may result in new opportunities for intervention that will modify the course of these prevalent debilitating chronic inflammatory disorders.

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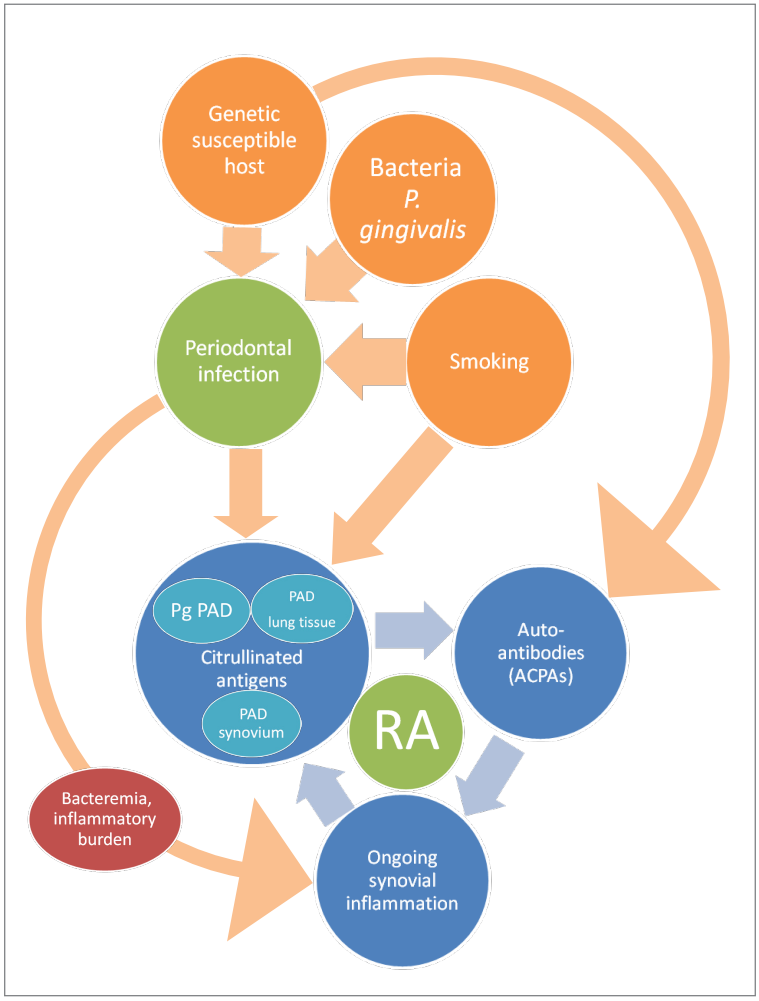
Figures

Fig. 1. Possible interactions of periodontal infection with *P. gingivalis* in etiology and pathogenesis of ACPA positive RA.

RA and periodontal infection share genetic traits, lifestyle risk factors as smoking and gene-environmental interactions (for details see text). Infection with P. gingivalis can cause bacteremia and generates a systemic inflammatory response, thereby contributing to the total inflammatory burden. In addition, P. gingivalis is able to citrullinate proteins. Given the fact that citrullination is an inflammation-associated process, P. gingivalis contributes in two ways to the total antigenic load of citrullinated proteins. Smoking contributes to citrullination as well. A susceptible host forms antibodies against the citrullinated proteins (ACPA), which are highly specific for RA. Immune-complex formation sustains synovial inflammation, representative for the laboratory parameters and clinical symptoms of RA.



CHAPTER

02

Periodontitis in established rheumatoid arthritis patients: a cross-sectional clinical, microbiological and serological study

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Arthritis Research & Therapy 2012 Oct 17;14(5):R222. doi: 10.1186/ar4061.

Abstract

Introduction:
The association between rheumatoid arthritis (RA) and periodontitis is suggested to be linked to the periodontal pathogen *Porphyromonas gingivalis*. Colonization of *P. gingivalis* in the oral cavity of RA patients has been scarcely considered. To further explore whether the association between periodontitis and RA is dependent on *P. gingivalis*, we compared host immune responses in RA patients with and without periodontitis in relation to presence of cultivable *P. gingivalis* in subgingival plaque.

Methods:
In 95 RA patients, periodontal condition was examined using the Dutch Periodontal Screening Index for treatment needs. Subgingival plaque samples were tested for presence of *P. gingivalis* by anaerobic culture technique. IgA, IgG and IgM antibody titers to *P. gingivalis* were measured by ELISA. Serum and subgingival plaque measures were compared to a matched control group of non-RA subjects.

Results:
A higher prevalence of severe periodontitis was observed in RA patients in comparison to matched non-RA controls (27% versus 12%, $p<0.001$). RA patients with severe periodontitis had higher DAS28 scores than RA patients with no or moderate periodontitis ($p<0.001$), while no differences were seen in IgM RF or ACPA reactivity. Furthermore, RA patients with severe periodontitis had higher IgG and IgM anti-*P. gingivalis* titers than non-RA controls with severe periodontitis ($p<0.01$ resp. $p<0.05$), although subgingival occurrence of *P. gingivalis* was not different.

Conclusion:
Severity of periodontitis is related to severity of RA. RA patients with severe periodontitis have a more robust antibody response against *P. gingivalis* than non-RA controls, but not all RA patients have cultivable *P. gingivalis*.

Introduction

Several studies have demonstrated an increased prevalence of periodontitis and a higher rate of tooth loss in patients with rheumatoid arthritis (RA) in comparison with the general population in the US [1, 2], Northern Europe [3-6], and Australia [7]. RA may also be more prevalent among patients with periodontitis [4, 8]. Differences in disease criteria and methods for evaluation of periodontal status, however, form a problem in interpretation of the literature [9].

Periodontitis and RA are both chronic destructive inflammatory disorders and result from deregulation of the host inflammatory response. Both conditions are potentiated by an exaggerated inflammatory response featuring an increase in local and perhaps circulating pro-inflammatory mediators, resulting in destruction of the soft and hard tissues surrounding the teeth (the periodontium) and the synovial joint [10-13]. Susceptibility is influenced by shared genetic and lifestyle factors. Both diseases are cumulative; that is, severity and loss of function increase with longer disease duration. There are a number of postulated mechanisms by which infections can trigger autoimmune disease, but most evidence in animal models supports the idea that cross-reactive immune responses cause autoimmunity because of molecular mimicry between microbiological and self-antigens [14]. Rosenstein and colleagues [15] have hypothesized that *Porphyromonas gingivalis*, a major periodontal pathogen, plays a role in the pathogenesis of RA. *P. gingivalis* is a prokaryote that uniquely contains a peptidyl arginine deiminase enzyme [16] necessary for citrullination and can induce an immune response to citrullinated self-proteins [15, 17]. Citrullination changes the structure and function of proteins and has been demonstrated in several physiological and pathological processes [18]. Antibodies against citrullinated proteins (ACPA) are 95% specific and 68% sensitive for RA [19, 20]. These antibodies can be present several years before the clinical onset of RA [21] and are associated with a more destructive disease course than RA without detectable ACPA [22]. Moreover, periodontitis can contribute to the total inflammatory burden by eliciting bacteremia and systemic inflammatory responses [23, 24].

Given the observed epidemiological association and the hypotheses mentioned above, periodontitis may be regarded as a risk factor for initiation and progression of RA. At present, disease management of RA is based on early diagnosis, aggressive treatment, and regular monitoring, and disease remission is the ultimate treatment goal. To achieve this goal, reduction of total inflammatory burden is necessary. This may involve periodontal infection control in patients with periodontitis. Studies have reported higher antibody titers against *P. gingivalis* in RA patients and a positive correlation with ACPA [25-27], suggesting that infection with this periodontal pathogen may play a role in the risk and progression of RA. However, oral colonization by *P. gingivalis* in patients with RA is barely considered. To study whether the association between periodontitis and RA is dependent on *P. gingivalis*, we compared host immune responses in RA patients with or without periodontitis in relation to the presence of cultivable *P. gingivalis* from subgingival plaque. Because of the recent observation that the inflamed periodontium contains citrullinated proteins [28], we also investigated the presence of ACPA in the inflammatory exudates from the gingival crevice (gingival crevicular fluid, or GCF).

Methods

Patients

Patients with rheumatoid arthritis

Established RA patients matching the inclusion criterion were consecutively recruited between March and September 2011 at

the outpatient clinic of the Rheumatology and Clinical Immunology Department of the University Medical Center Groningen in Groningen. The inclusion criterion was fulfilling the American College of Rheumatology classification criteria for RA [29], and exclusion criteria were age under 18 years, edentulism, diabetes, active thyroid disease, presence of non-oral infections, present malignancy, myocardial infarction or stroke fewer than 6 months prior to the study, pregnancy including a 6-month post-partum period as well as breastfeeding and antibiotic use fewer than 3 months prior to the study. Assuming that the prevalence of severe periodontitis is 10% to 15% [30] and the odds ratio of periodontitis in RA is 1.8 to 2.0 [7, 31], we calculated that we needed a minimum sample size of 75 patients with RA to find a difference of 12.5% in the prevalence of periodontitis by using a two-sided binomial test.

Non-rheumatoid arthritis controls

As a reference group for microbiological and serological measurements, subjects without RA were recruited from among subjects planned for first consultation at the Department of Dentistry and Oral Hygiene of the University Medical Center Groningen. The procedures of recruitment and informed consent were the same as for patients with RA. The inclusion criterion was not having RA, and exclusion criteria were the same as mentioned for patients with RA. Non-RA controls were matched for age, gender, number of teeth, body mass index (BMI), and smoking and periodontal status. We aimed to include matched non-RA controls in a ratio of 2:1 (Table 1, page 41). Healthy controls were defined as subjects without periodontitis and without cultivable subgingival *P. gingivalis*.

Control cohort for estimating prevalence of periodontitis in the general population

The prevalence of periodontitis was assessed in subjects attending a general dental practice within the referral area (Clinic for General Dental Practice Solwerd in Appingedam, The Netherlands). This control population consisted of 420 age- and gender-matched consecutive patients in whom the Dutch periodontal screening index (DPSI) score was assessed during one year (2010). The DPSI is a validated index based on bleeding on probing, pocket probing depth, and clinical attachment loss [32]. On the basis of DPSI scores, patients were categorized as having no periodontitis (A), moderate periodontitis (B), or severe periodontitis (C). Assessment of the socioeconomic status of this group was made according to data of highest self-reported level of received education of the municipal public health service of the northeast region of Groningen [33].

Ethics approval

All participants provided written informed consent prior to study enrollment according to the Declaration of Helsinki (General Assembly October 2008), and this study was conducted with the approval of the Medical Ethical Committee of the University Medical Center Groningen (METc UMCG 2011/010).

Clinical examination of rheumatoid arthritis

RA disease activity was measured with the Disease Activity Score 28 tender and swollen joint count (DAS28) [34]. Other parameters were disease duration of RA, smoking status (current, former, or never), BMI, and RA medication, including steroids and anti-tumor necrosis factor biologic agents. Assessment of the socioeconomic status was made according to highest self-reported level of received education.

Clinical examination of periodontitis

Periodontal condition was examined by using DPSI. The DPSI score was taken by a periodontist blinded for the diagnosis of RA. In the general dental practice, the DPSI score was taken by the dentist at the first visit of the patient.

Sampling

Peripheral blood and subgingival samples were obtained from the RA and non-RA controls at the day of clinical examination. Subgingival samples were taken by using sterile paper points [35]. Microbiological sampling included selection of the deepest bleeding periodontal pocket in each quadrant of the dentition on the basis of pocket probing depth measurements. If there were no bleeding periodontal pockets, mesial sites of the first molars or, in absence of a first molar, the mesial site from the adjacent anterior tooth in the dental arch was selected. Sample sites were isolated with cotton rolls, and supragingival plaque was carefully removed with curettes and cotton pallets. Subsequently, two paper points were inserted to the depth of the pocket and left in place for 10 seconds. All paper points per subject were pooled in reduced transport fluid [36] and processed for microbiological examination immediately after sampling. GCF samples were collected in the same way. The deepest non-bleeding site per quadrant was used to collect GCF to avoid blood contamination of the sample. GCF samples were discarded for further analysis if they were visibly contaminated with blood. Paper points for the GCF sample were pooled per subject in phosphate-buffered saline (PBS) with 1% bovine serum albumin. The paper points were removed after centrifuging at 28,000 g for 10 minutes, and the supernatant was stored at -20°C until use.

Laboratory procedures

Serum was investigated for C-reactive protein (CRP) concentration by enzyme-linked immunosorbent assay (ELISA) (DuoSet; R&D Systems). IgM RF (in international units per milliliter) was measured by using an in-house validated ELISA (cut-off point for positivity: 25 U/mL) [37]. Total IgG anti-cyclic citrullinated protein (anti-CCP) antibodies (ACPA) (in units per milliliter) were determined by using the Phadia analyzer (Phadia Laboratory Systems, Phadia AB) with an upper detection limit of 340 U/mL (cut-off point for positivity: 10 U/mL). Antibody levels to five synthetic native peptides representing the best established auto-antigens in RA [38] (that is, enolase-1, fibrinogen-1, fibrinogen-2, fibrinogen-3, and vimentin-1) and their citrullinated forms were measured by using the same ELISA as described by van de Stadt and colleagues [39].
IgA, IgG, and IgM antibodies to *P. gingivalis* were determined by using an in-house developed ELISA, in which a pooled lysate of four randomly selected clinical isolates of *P. gingivalis* from patients with periodontitis was used as an antigen. These monocultures were suspended in ice-cold PBS with protease inhibitors (Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets; Roche Diagnostics). After centrifuging and discarding of the supernatant, pellets were resuspended in an ice-cold lysis buffer containing non-denaturing detergent (Noninet P-40; Sigma-Aldrich) and sonicated for 15 minutes (Bioruptor Standard sonication device; Diagenode s.a.). Protein concentration was determined by using the BCA Protein Assay Kit (Pierce Protein Biology Products, Thermo Fisher Scientific). Microtiter plates (Costar 96-Well; Corning) were coated overnight with 1 µg/mL of antigen. Standard curves were made from protein standard (N protein-standard SL; Siemens Healthcare Diagnostics) diluted twofold (highest standard curve values were for 300, 25, and 250 ng/mL for IgA, IgG, and IgM, respectively). For the standard curves,

the following capture antibodies were used: monoclonal anti-human IgA (1:4,000, clone GA- 112; Sigma-Aldrich), monoclonal anti-human IgM (1:10,000, clone MB-11, µ-chain-specific; Sigma-Aldrich), and goat anti-human IgG (1:5,000, F(ab')2 fragment-specific; Jackson ImmunoResearch Europe Ltd.), respectively. Serum samples were incubated in four-fold serial dilutions (1:100, 1:400, 1:1,600, and 1:6,400). Detection of anti-*P. gingivalis* antibodies was carried out with horseradish peroxidase-labeled goat anti-human IgA, mouse antihuman IgG (Fc fragment-specific, clone JDC-10), and mouse anti-human IgM (µ-chain-specific, clone SA-DA4; all from SouthernBiotech) followed by color reaction with tetramythylbenzidin and hydrogen peroxide. Absorbance was read at 450 nm in an EMax microplate reader and corrected for background binding, and concentration of antibodies was calculated by SOFTmax PRO software (Molecular Devices) according to the IgA, IgG, or IgM standard curves included on each ELISA plate and expressed in nanograms per milliliter. As an internal control, two serum samples with a repeatedly high and a low titer were tested at each plate. The variation coefficients were 22% for IgA, 20% for IgG, and 25% for IgM anti-*P. gingivalis*. Interference of IgM RF was investigated by spiking samples with sera with known high titers of RF. Adding RF had no measurable effect on anti-*P. gingivalis* titers.
In paired samples of serum and GCF of patients with RA, presence of IgG ACPA was assessed using the anti-CCP2 ELISA (Euro Diagnostica). Because the HLA-DRB1 shared epitope (SE) is a known genetic risk factor for RA and a possible genetic risk factor for periodontitis [40], HLA-DRB1-SE containing alleles were genotyped from genomic DNA in the patients with RA. The presence of an RA SE was analyzed by a polymerase chain reaction-based sequence-specific oligonucleotide probe hybridization (SSOP) approach by using a commercial kit (Hologic Gen-Probe Incorporated) and Luminex xMAP technology (Luminex Corporation) in accordance with the instructions of the manufacturer.

Microbiology

Microbiological samples were processed by using standard anaerobic culture techniques [41, 42]. Paper point samples were vortexed for 2 minutes, and appropriate 10-fold serial dilutions (100 µL) in PBS were plated on blood agar plates (Oxoid no. 2; Oxoid Ltd.), which were supplemented with horse blood (5% vol/vol), hemin (5 mg/L), and menadione (1 mg/L) and incubated in 80% N2, 10% H2, and 10% CO2 at 37°C for up to 14 days. Besides the presence and proportions of *P. gingivalis*, those of other established periodontal pathogens, including *Prevotella intermedia, Fusobacterium nucleatum, Parvimonas micra, Tannerella forsythia,* and *Campylobacter rectus*, were assessed [43]. Identification was based on microscopic morphology, Gram reaction, and the production of a set of metabolic enzymes (API/ID 32; BioMérieux). Additional tests for identification included detection of a trypsin-like activity based on the degradation of benzoyl- DL-arginine-2-naphthylamide (Sigma-Aldrich) (44). Finally, the total number of colony-forming units per sample was determined.

Statistical analysis

Data were analyzed by using GraphPad Prism 4 and Instat 3 (GraphPad Software Inc).
For group comparisons among two groups, the unpaired two-tailed t-test for variables with normal distribution and the two-tailed Mann-Whitney U test for skewed variables were used. For group comparisons among three groups, Kruskal-Wallis one-way analysis of variance was performed. The Fisher exact test or the chi-squared test for independence was used to analyze contingency tables. Significance level α was 0.05.

Results

Patients

Two hundred thirty-nine consecutive patients with RA were invited to participate in this study. Of the 167 patients who provided written informed consent, 66 patients met at least one of the exclusion criteria. Of the 101 included patients, six patients were excluded from analysis because of incomplete data. The final cohort consisted of 95 patients with RA. In addition, 203 consecutive patients were invited to join the control group. Of this group, 108 dentate persons without RA provided written informed consent. Twenty-eight of them had to be expelled on the basis of the exclusion criteria. The remaining 80 patients served as non-RA controls for blood and subgingival samples, matched for age, gender, BMI, and periodontal and smoking status (n = 44), or as healthy controls (no periodontitis and no cultivable subgingival *P. gingivalis*, n = 36).
Characteristics of the predominantly (99%) Caucasian patients with RA, non-RA controls, and the healthy controls are summarized in Table 1, page 41.
The general control population for estimating the prevalence of periodontitis consisted of 420 individuals matched for age and gender with the patients with RA (age of 56 ± 5.5 years, and 68% were female). The socioeconomic status of this group as assessed by the highest self-reported level of received education was comparable between the control population and the patients with RA (low: 27% versus 36%, middle: 37% versus 30%, and high: 36% versus 34%).

Presence of HLA-DRB1 shared epitope in patients with rheumatoid arthritis

No differences were found in the presence or absence of the HLA-DRB1-SE in RA patients with no, moderate, or severe periodontitis (n = 78). In RA patients with no, moderate, or severe periodontitis, 58%, 66%, and 62%, respectively, were positive for HLA-DRB1-SE, and 29%, 21%, and 15%, respectively, had two alleles.

Periodontitis

Forty-three percent of the patients with RA had moderate periodontitis, and 27% had severe periodontitis. These numbers are significantly higher than, respectively, the 18% and 12% found in the control population ($p < 0.001$). Compared with the control population, the relative risk for having RA and severe periodontitis was 3.7 (95% confidence interval of 2.4 to 5.9) (Table 2, page 41).
RA patients with severe periodontitis had significantly higher DAS28 scores ($p < 0.001$) than RA patients with no or moderate periodontitis (Fig. 1, page 42).
CRP levels in RA patients with severe periodontitis were higher than in RA patients without periodontitis (marginally significant, $p = 0.05$). In none of the DPSI categories was a difference in DAS28 scores between smokers and non-smokers or former smokers. Also, no difference was found in RA disease duration, although patients with severe periodontitis were significantly older ($p < 0.01$). In the control population, there were no age differences between the DPSI categories.

Serology

Between RA patients with no, moderate, or severe periodontitis, no differences were seen in IgM RF and ACPA levels or in reactivity against the citrullinated peptides enolase-1, fibrinogen-1, -2, and -3, and vimentin-1 (Fig. 2, page 43). Between RA patients culture-positive or -negative for *P. gingivalis*, only reactivity against citrullinated fibrinogen-2 was different; reactivity was higher in *P. gingivalis* positive patients ($p < 0.01$). A small number of RA patients with moderate (n = 2) or severe (n = 2) periodontitis and culture-positive for *P. gingivalis* had a higher reactivity against citrullinated α-enolase compared

with RA patients without periodontitis and without subgingival *P. gingivalis*. This *P. gingivalis* culture-positive subgroup also had high IgM RF (mean 539 ± 796 U/mL), ACPA (mean 340 ± 0 U/mL), and anti-*P. gingivalis* titers (IgM: mean 93 ± 135, IgG: 190 ± 357, IgA: 61 ± 81 mg/L).

Overall, patients with RA showed higher IgM anti-*P. gingivalis* titers compared with non-RA controls ($p < 0.05$). There were no differences in anti-*P. gingivalis* titers between RA patients and non-RA controls with no or moderate periodontitis; however, RA patients with severe periodontitis showed both higher IgG and IgM anti-*P. gingivalis* titers compared with non-RA controls with severe periodontitis ($p < 0.05$) (Fig. 3, page 44). RA patients with moderate periodontitis have a less pronounced anti-*P. gingivalis* response compared with RA patients with severe periodontitis but a higher one than non-RA controls with severe periodontitis for IgG (borderline significant, $p = 0.06$) and IgM ($p < 0.05$). Both RA patients and non-RA controls culture-positive for *P. gingivalis* showed higher anti-*P. gingivalis* titers compared with their culture-negative counterparts.

In patients with RA, serum levels of IgM RF and ACPA showed a strong correlation ($\rho = 0.51$, $p < 0.0001$), as did IgG anti-*P. gingivalis* with IgM anti-*P. gingivalis* ($\rho = 0.41$, $p < 0.0001$) and IgA anti-*P. gingivalis* ($\rho = 0.66$, $p < 0.0001$). Medication used for RA was not of influence on anti-*P. gingivalis* titers. In non-RA controls, IgG anti-*P. gingivalis* only correlated with IgA anti-*P. gingivalis* ($\rho = 0.65$, $p < 0.0001$). In patients with RA, there was a weak but significant correlation between IgM anti-*P. gingivalis* and IgM RF ($\rho = 0.33$, $p < 0.01$), ACPA ($\rho = 0.24$, $p < 0.05$), and reactivity against the citrullinated peptides fibrinogen-1 ($\rho = 0.27$, $p < 0.05$) and fibrinogen-3 ($\rho = 0.22$, $p < 0.05$). Likewise, IgG anti-*P. gingivalis* was correlated with IgM RF ($\rho = 0.26$, $p < 0.05$). In RA patients with severe periodontitis, there were no correlations between IgG, IgM, and IgA anti-*P. gingivalis* titers and IgM-RF and ACPA. In patients with RA, ACPA levels in serum were comparable to ACPA levels in paired GCF samples which were not visibly contaminated with blood ($n = 45$, $\rho = 0.89$, $p < 0.0001$) (Fig. 4, page 44).

Microbiology

The subgingival prevalence of *P. gingivalis* was not different between patients with RA (16%) and non-RA controls (20%). In both groups, *P. gingivalis* was infrequently cultured in the absence of periodontitis (6% to 12%). None of the other identified periodontal pathogens differed in prevalence between RA patients and non-RA controls (*P. intermedia*: 66% versus 73%, *T. forsythia*: 84% versus 70%, *P. micra*: 88% versus 89%, *F. nucleatum*: 100% versus 95%, and *C. rectus*: 33% versus 41%). However, the prevalence of anaerobic Gram-negative rods other than *P. gingivalis* and *P. intermedia* was higher in patients with RA (27% versus 8%, $p < 0.05$), as was the total bacterial load (in colony-forming units per milliliter) ($p < 0.05$), notwithstanding the comparable mean probing pocket depth between RA patients and matched non-RA controls (4.2 ± 1.0 mm).

Discussion

In this study, we found a significantly increased prevalence of periodontitis in RA patients and the highest RA disease activity in patients with severe periodontitis. Importantly, serological markers for systemic inflammation of periodontal origin further substantiated the connection of the two diseases in terms of higher antibody titers against *P. gingivalis* in RA patients with severe periodontitis compared with severe periodontitis patients without RA. These differences cannot be explained by differences in *P. gingivalis* colonization, since the distributions in RA and non-RA patients were similar.

Given that periodontitis is associated with

only moderate elevations of CRP and erythrocyte sedimentation rate levels [45, 46], the higher DAS28 scores observed in RA patients with periodontitis can only partly be explained by these higher levels due to periodontitis. This is the first study that focused on anti-*P. gingivalis* titers in RA and non-RA controls, equally distributed in prevalence of *P. gingivalis* and periodontitis. We confirm elevated antibody titers against *P. gingivalis* in RA patients as reported in former studies; however, these studies did not consider the periodontal status or the microbiology of the patients with RA [26, 27]. We found higher anti-*P. gingivalis* titers in RA patients compared with matched non-RA controls in cases of severe periodontitis. No differences in anti-*P. gingivalis* titers between these groups were found in cases of moderate periodontitis; however, RA patients with moderate periodontitis still had higher titers compared with severe periodontitis patients without RA. RA patients with cultivable *P. gingivalis* showed the highest anti-*P. gingivalis* titers, suggesting that RA amplifies the antibody response against *P. gingivalis* and that colonization of *P. gingivalis* could have contributed to the elevated titer, according to the fact that carriage of *P. gingivalis* is the strongest determinant of the systemic antibody response against this periodontal pathogen [47]. An explanation for the elevated anti-*P. gingivalis* titers could be a hyperinflammatory state of RA patients with periodontitis. ACPA directed to citrullinated peptides of *P. gingivalis* could be another explanation for the elevated titers. Lundberg and colleagues [48] showed cross-reactivity of human ACPA with bacterial enolase. We found a high reactivity in a small subgroup of RA patients with periodontitis and culture-positive for *P. gingivalis*, but overall we found a weak correlation between ACPA and anti-*P. gingivalis* titers. Whether anti-*P. gingivalis* antibodies are directed against the same epitopes as ACPA needs further investigation. To consider whether elevated titers are specific for *P. gingivalis*, antibody titers to other periodontal bacteria should be assessed.

Little is known about oral colonization by *P. gingivalis* in patients with RA. We found no differences in the presence and proportions of any of the assessed periodontal pathogens between RA patients and non-RA controls; however, the prevalence of anaerobic Gram-negative rods other than *P. gingivalis* and *P. intermedia* was higher in patients with RA. Recently, Scher and colleagues [49] assessed the complete subgingival microbiota by pyrosequencing and detected *P. gingivalis* more frequently in recently diagnosed, never-treated RA patients (n = 25) in comparison with established RA patients or healthy controls; this finding, however, could be explained by the higher prevalence of severe periodontitis in those patients.

An association between the presence of periodontitis and ACPA or IgM RF levels or both could not be established, in contrast to the findings of Dissick and colleagues [2], nor could we find differences in reactivity to the five synthetic citrullinated peptides between the DPSI categories, other than a higher reactivity against citrullinated peptide fibrinogen-2 in *P. gingivalis* culture-positive patients. As epitope spreading is related to RA disease duration [39], a widespread reactivity to different citrullinated peptides was found in our cohort with relatively long disease duration. In future studies, the (temporal) relation of RA, periodontitis, and antibodies specific for RA should be performed in periodontitis patients, in non-treated RA patients, and/or in patients at risk for RA, ideally in a prospective follow-up study.

We found no differences in the presence or absence of the HLA-DRB1-SE in RA patients with no, moderate, or severe periodontitis, although this should be interpreted with some caution because of the small numbers of patients. The strong correlation between ACPA levels in serum and GCF, with lower levels in GCF, is suggestive of diffusion of ACPA from plasma to GCF. Within the limitations of the method used in this study, we found no indication for local ACPA production in the periodontium.

Conclusions

We confirmed the previously reported disease association between RA and periodontitis and the increased prevalence of periodontitis in patients with RA. In addition, severity of periodontitis appeared to be related to RA disease activity. Furthermore, severity of RA was not associated with cultivable *P. gingivalis* in established RA patients, although anti-*P. gingivalis* titers were higher in RA patients with severe periodontitis compared with matched non-RA subjects.

Acknowledgements

We thank Christelle Bal, of the Clinic for General Dental Practice Solwerd in Appingedam, The Netherlands, for providing their data for the control population and Annechien Lambeck, of the Department of Transplantation Immunology, University Medical Center Groningen, for HLA genotyping the cohort RA patients. This research was partly funded by the Jan Kornelis de Cock foundation (project number 11-71) and the foundation of the Dutch Periodontal Society.

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Tables and Figures

Table 1 Characteristics of patients with rheumatoid arthritis (RA), non-RA controls, and healthy controls.

	Patients with RA	Non-RA contols	Healthy controls
Number	95	44	36
Female, percentage	68	57	56
Age in years (SD)	56 (11)	54 (9.7)	34 (15)
Current smoker, percentage	23	27	14
Former smoker, percentage	40	43	0
Body mass index	25.8 (4.9)	25.4 (4.4)	n.a.
Number of teeth	23.4 (6.3)	24.2 (4.9)	27.8 (2.9)
RA duration in years (SD)	7.4 (5.9)	0	0
DAS28 (SD)	2.4 (0.93)	0	0
Seropositive for IgM RF, percentage	53	0	n.a.
Seropositive for ACPA, percentage	71	n.a.	n.a.
Medication for RA, percentage			
None	6	100	100
DMARDs	79	0	0
Anti-TNFα	15	0	0

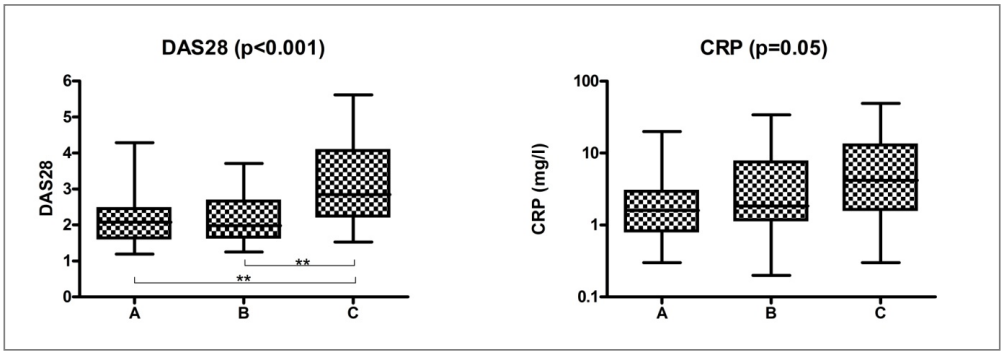
ACPA: anti-citrullinated protein antibodies, anti-TNFα: anti-tumor necrosis factor alpha, DMARD: disease modifying anti-inflammatory drug, IgM RF: immunoglobulin M rheumatoid factor, n.a.: not assessed, SD: standard deviation.

Table 2 Prevalence of periodontitis of rheumatoid arthritis (RA) patients compared with the age- and gender matched control population.

DPSI category	Patients with RA (n = 95)	Control population (n = 420)	*P* value	Relative risk	95% CI
A: no periodontitis, percentage	30	70	<0.001		
B: moderate periodontitis, percentage	43	18	<0.001	3.6	2.3-5.5
C: severe periodontitis, percentage	27	12	<0.001	3.7	2.4-5.9

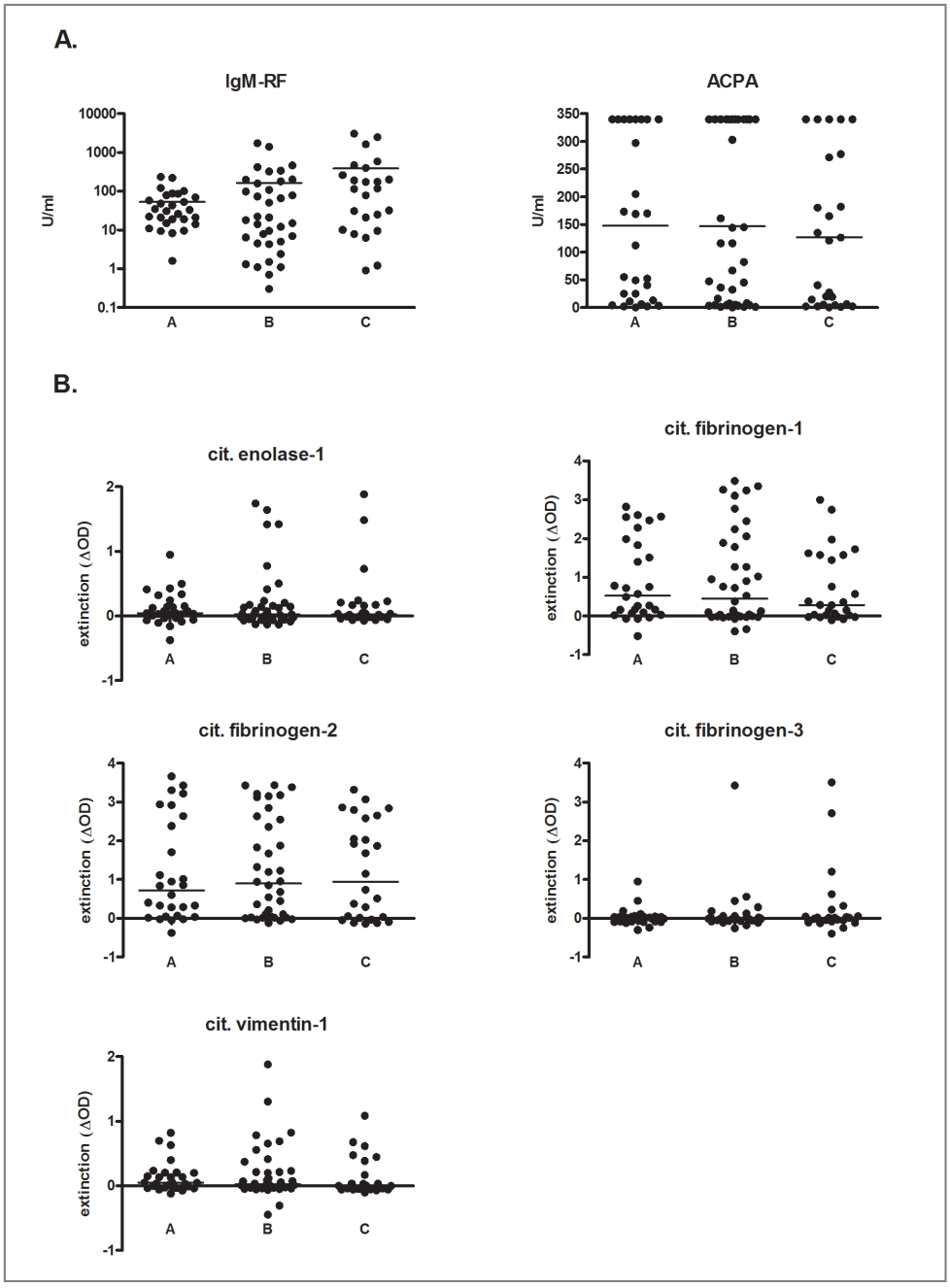
CI: confidence interval, DPSI: Dutch periodontal screening index, RA: rheumatoid arthritis.

Fig. 1 DAS28 scores and CRP- levels in patients with rheumatoid arthritis and no (a), moderate (b), or severe (c) periodontitis.



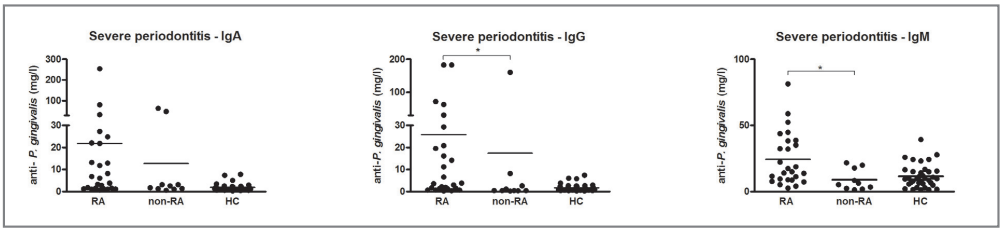
***p < 0.01, CRP: C-reactive protein, DAS28: disease activity score 28 tender and swollen joint count.*

Fig. 2 (A) IgM RF and ACPA reactivity and **(B)** reactivity against five citrullinated (cit.) peptides in patients with rheumatoid arthritis and no (a), moderate (b), or severe (c) periodontitis.



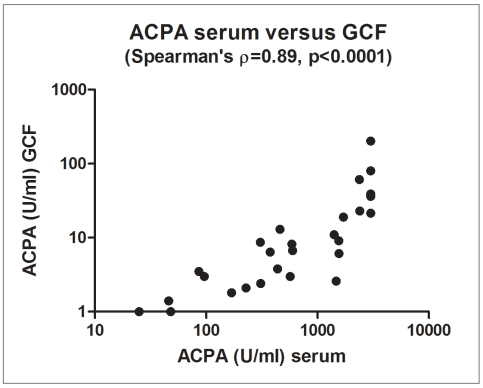
No significant differences were observed. ΔOD: optical density of the citrullinated form minus the native form of the peptide, ACPA: anti-citrullinated protein antibodies, IgM RF: immunoglobulin M-rheumatoid factor.

Fig. 3 IgA, IgG, and IgM antibody response against *Porphyromonas gingivalis* in rheumatoid arthritis (RA) patients and non-RA controls with severe periodontitis as well as in healthy controls (HC).



**p < 0.05, Ig: immunoglobulin.*

Fig. 4 Correlation of anti-citrullinated protein antibody (ACPA) in paired samples of serum and gingival crevicular fluid (GCF) of patients with rheumatoid arthritis (n = 45).



CHAPTER

03

Antibodies against *Porphyromonas gingivalis* in seropositive arthralgia patients do not predict development of rheumatoid arthritis

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Annals of the Rheumatic Diseases 2014 Jun;73(6):1277-9. doi: 10.1136/annrheumdis-2013-204594. Epub 2014 Jan 13.

Letter to the editor

Clinical studies point towards an association between periodontitis and rheumatoid arthritis (RA) [1, 2]. A pathogenic role is suggested for *Porphyromonas gingivalis* [3]. *P. gingivalis* may contribute to the pathogenesis of RA by breaking immune tolerance through formation of (bacterial and human) citrullinated proteins, leading to anticitrullinated protein antibody production (ACPA) [4, 5]. Since ACPA production precedes RA development [7] and because *P. gingivalis* IgG antibodies are long-term stable in untreated periodontitis patients [8], we investigated whether anti-*P. gingivalis* antibody levels are prognostic for development of RA, by assessing these antibodies in a cohort of 289 adults at risk for RA.

Patients with arthralgia and seropositivity for IgM rheumatoid factor (IgM RF) and/or ACPA were selected from a prospective follow-up study on arthritis development [9]. The occurrence of arthralgia in people with these autoantibodies probably represents a late stage in the preclinical development of (rheumatoid) arthritis, especially if the symptoms are symmetrically located in the small joints, a situation which could be named 'inflammatory arthralgia' [10]. They are further referred to as seropositive arthralgia patients (SAP); their median follow-up was 30 months (IQR 13–49).

Baseline sera were used for measurement of ACPA, IgM RF, C-reactive protein (CRP) and HLA-DRB1 SE carrier status [9]. IgA, IgG and IgM antibody levels against *P. gingivalis* were determined by in-house ELISA with a pooled lysate of clinical isolates of *P. gingivalis* as antigen [11]. Interference of IgM RF on anti-*P. gingivalis* antibody assays was excluded by spiking samples with sera with known high titres of RF.

Reference groups for antibody levels against *P. gingivalis* consisted of healthy subjects without periodontitis and without cultivable subgingival *P. gingivalis* (HC, n = 36, mean age 34 ± 15 years, 53% female, 14% current smoker) and severe periodontitis patients without systemic disease (PD, n = 117, mean age 51 ± 9.3 years, 58% female, 43% current smoker, 42% of n = 45 *P. gingivalis*-culture positive [12]. Both groups were recruited among subjects planned for first consultation at the dental department of the University Medical Center Groningen and a referral practice for periodontology (Clinic for Periodontology Groningen) [11].

IgA and IgG anti-*P. gingivalis* were higher in PD than in HC (both $p < 0.0001$). PD culture-positive for subgingival *P. gingivalis* had higher IgA and IgG anti-*P. gingivalis* than culture-negative PD ($p < 0.01$ and $p < 0.001$). No differences were found for IgM anti-*P. gingivalis*.

Cut-off values for anti-*P. gingivalis* positivity were set at >2 SD above the mean of HC.

Influence of anti-*P. gingivalis* positivity on RA development was analyzed using a multivariate Cox proportional hazards model with time until RA development as dependent variable and age, gender, HLA-DRB1 SE carriage, smoking, number of tender joints, and CRP- ACPA- and IgM RF-positivity at inclusion as other variables.

After 12 months (median, IQR 6–20), 33% (n = 94) of SAP had developed RA according to 2010 American College of Rheumatology/European League against Rheumatism criteria [13]. Baseline characteristics of SAP who developed RA (RA+) or did not develop RA (RA−) are listed in Table 1, page 50.

In SAP, IgG anti-*P. gingivalis* was higher than in HC, but lower than in PD, as was IgA anti-*P. gingivalis* (Fig. 1A, page 51). No differences in IgM anti-*P. gingivalis* were found, nor were differences found for anti-*P. gingivalis* antibody levels between ACPA-positive or ACPA-negative SAP.

SAP who developed RA did not have elevated anti-*P. gingivalis* antibody levels at baseline compared with SAP who did not develop RA

within the follow-up period (Fig. 1B, page 51). When using cut-off values for anti-*P. gingivalis* positivity, the proportion of IgA and IgG anti-*P. gingivalis*-positive patients was higher in SAP who did not develop RA (Table 1, page 50). Besides a weak correlation of IgM anti-*P. gingivalis* with ACPA in SAP who developed RA ($p < 0.05$, $\rho = 0.23$), no other correlation with anti-*P. gingivalis* was found.

The multivariate Cox proportional hazards model showed significant influence of ACPA (HR 11, 95% CI 5.1 to 24, $p < 0.0001$), IgM RF (HR 2.5, 95% CI 1.6 to 4.1, $p < 0.0001$), number of tender joints (HR 1.05, 95% CI 1.01 to 1.09, $p < 0.05$) and HLA-DRB1 SE carriage (HR 1.7, 95% CI 1.1 to 2.6, $p < 0.05$) on RA development. Influence of anti-*P. gingivalis*, CRP, age, gender and smoking could not be established. Within the limitations of this study, we conclude that anti-*P. gingivalis* antibody levels are not prognostic for development of RA.

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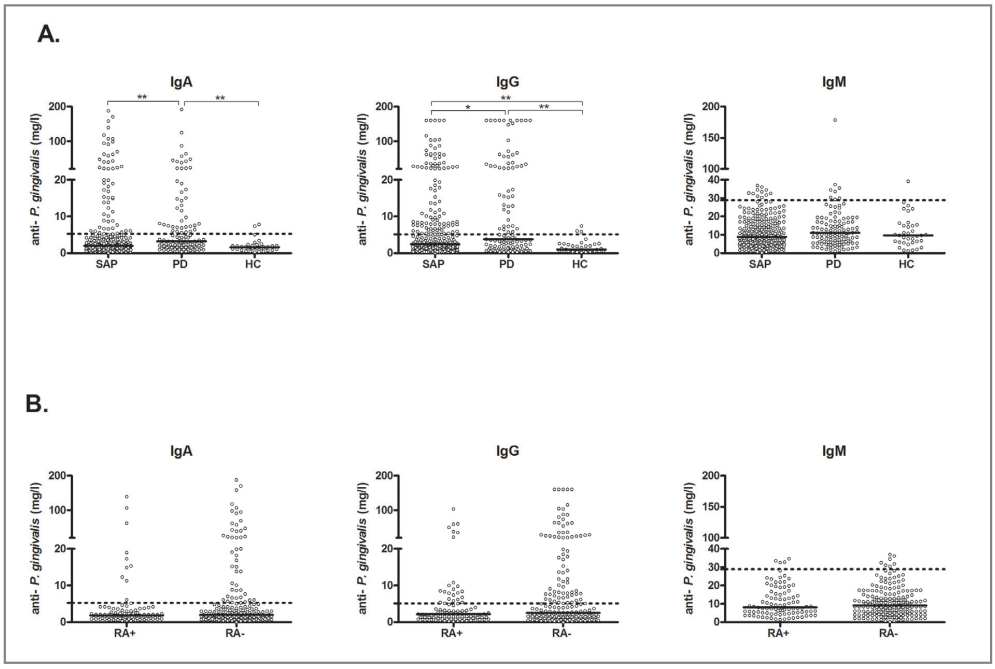
Tables and Figures

Table 1 Baseline characteristics of seropositive arthralgia patients (SAP) who did (RA+) or did not (RA−) develop rheumatoid arthritis within the follow-up period.

	All SAP	RA+	RA-	*P* value RA+ vs. RA-
Number	289	94	195	
Female, percentage	79	81	78	0.76
Mean age in years (SD)	50 (12)	48 (11)	50 (12)	0.19
Smoking at inclusion, percentage	29	35	26	0.13
HLA-DRB1 SE, percentage	40	45	37	0.19
Seropositive for IgM-RF, percentage	61	57	63	0.37
Seropositive for IgG ACPA, percentage	65	90	53	0
Median (IQR) hsCRP (mg/L)	2.2 (1.0-4.8)	2.6 (1.0-4.6)	2.0 (0.9-5.1)	0.47
Median (IQR) TJC53 at inclusion	0 (0-3)	1 (0-4)	0 (0-3)	0.1
Median (IQR) follow-up in months	30 (13-49)	25 (12-46)	34 (15-49)	0.05
Median (IQR) time until RA development	-	12 (6-20)	-	-
Positive for anti- *P. gingivalis* IgA, percentage†	20	11	25	0.01
Positive for anti- *P. gingivalis* IgG, percentage†	34	26	37	0.05
Positive for anti- *P. gingivalis* IgM, percentage†	6.9	5.3	7.7	0.62

**Variables reflected in percentages: Fisher's exact test with two sided p value, other variables: unpaired t-test with Welch's correction (Gaussian distribution) or Mann–Whitney U test (no Gaussian distribution).*
*†Positivity is defined as >2 SD above the mean anti-P. gingivalis levels of healthy controls. ACPA: anti-citrullinated protein antibodies, cut off level for positivity 5 U/mL, HLA-DRB1 SE: one or two copies of the HLA-DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410 or *1001 alleles, hsCRP: high-sensitivity C-reactive protein, RA: rheumatoid arthritis, RF: rheumatoid factor, cut off level for positivity 30 IU/mL, TJC53: tender joint count 53 joints.*

Fig. 1 (A) IgA, IgG and IgM anti-*Porphyromonas gingivalis* antibody levels in seropositive arthralgia patients (SAP) compared with severe periodontitis patients without other systemic disease and healthy controls with a healthy periodontium and no cultivable subgingival *P. gingivalis* (HC). **(B)** IgA, IgG and IgM anti-*P. gingivalis* antibody levels in SAP who developed rheumatoid arthritis (RA+) and SAP who did not develop rheumatoid arthritis (RA−) according to the 2010 American College of Rheumatology (ACR)/European League against Rheumatism (EULAR) criteria.



Solid lines represent median values. Dotted lines indicate arbitrary cut-off values for anti-P. gingivalis positivity defined as >2 SD above the mean of the healthy controls.
*Comparison of three groups: Kruskal-–Wallis one-way analysis of variance with Dunn's multiple comparison post-test if overall p < 0.05. Comparison of two groups: Mann–Whitney U test with two-sided p value. *p < 0.05, **p < 0.001.*

CHAPTER

04

Rheumatoid arthritis associated autoantibodies in non-rheumatoid arthritis patients with mucosal inflammation: a case control study

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Arthritis Research & Therapy 2015 Jul 9;17:174. doi: 10.1186/s13075-015-0690-6.

Abstract

Introduction:
Rheumatoid arthritis associated autoantibodies (RA-AAB) can be present in serum years before clinical onset of RA. It has been hypothesized that initiation of RA-AAB generation occurs at inflamed mucosal surfaces, such as in the oral cavity or lungs. The aim of this study was to assess systemic presence of RA-AAB in patients without RA who had oral or lung mucosal inflammation.

Methods:
Presence of RA-AAB (IgA and IgG anti-cyclic citrullinated peptide 2 antibodies (anti-CCP2), IgM and IgA rheumatoid factor (RF), IgG anti-carbamylated protein antibodies (anti-CarP) and IgG and IgA anti-citrullinated peptide antibodies against fibrinogen, vimentin and enolase) were determined in serum of non-RA patients with periodontitis (PD, n = 114), bronchiectasis (BR, n = 80) or cystic fibrosis (CF, n = 41). Serum RA-AAB levels were compared with those of periodontally healthy controls (HC, n = 36). Patients with established RA (n = 86) served as a reference group. Association of the diseases with RA-AAB seropositivity was assessed with a logistic regression model, adjusted for age, sex and smoking.

Results:
Logistic regression analysis revealed that IgG anti-CCP seropositivity was associated with BR and RA, whereas the association with PD was borderline significant. IgA anti-CCP seropositivity was associated with CF and RA. IgM RF seropositivity was associated with RA, while the association with BR was borderline significant. IgA RF seropositivity was associated with CF and RA. Apart from an influence of smoking on IgA RF in RA patients, there was no influence of age, sex or smoking on the association of RA-AAB seropositivity with the diseases. Anti-CarP levels were increased only in RA patients. The same held for IgG reactivity against all investigated citrullinated peptides.

Conclusion:
Although overall levels were low, RA-AAB seropositivity was associated with lung mucosal inflammation (BR and CF) and may be associated with oral mucosal inflammation (PD). To further determine whether mucosal inflammation functions as a site for induction of RA-AAB and precedes RA, longitudinal studies are necessary in which RA-AAB of specifically the IgA isotype should be assessed in inflamed mucosal tissues and/or in their inflammatory exudates.

Introduction

The first autoantibody discovered in rheumatoid arthritits (RA) was rheumatoid factor (RF), which is directed against the constant domain of the immunoglobulin G (IgG) molecule. RF is not very specific for RA, as it is commonly found in other (autoimmune) diseases too, [1-4]. In contrast to RF, anti-citrullinated protein antibodies (ACPA) are highly specific (98%) for RA [5]. ACPA can be directed against a number of citrullinated autoantigens. Production of ACPA in RA is associated with distinct genetic risk factors [6] and worse disease outcome [7].
Recently, anti-carbamylated protein (anti-CarP) antibodies were described as a third autoantibody system in RA [8]. Carbamylation is, like citrullination, a post-translational modification and results in a chemically similar structure [9]. Antibodies, however, are able to distinguish between carbamylated and citrullinated antigens. As for RF and ACPA [10, 11], the presence of anti-CarP in serum can precede clinical onset of RA and is associated independently of ACPA with a higher risk of developing RA [12].
Although the presence of ACPA and RF is of great importance in RA diagnosis, the role of these antibodies in the initiation and pathogenesis of RA has been less well elucidated. It has been hypothesized that initiation of RA-associated autoantibody (RA-AAB) generation occurs at inflamed mucosal surfaces, such as in the lung and oral mucosa [13]. IgA is the predominant antibody of the mucosal immune system and IgA ACPA is elevated and highly specific for RA in individuals with preclinical and early RA [14-16].
Because smoking is a risk factor for RA development [17], the lungs have been speculated to play a role in RA initiation [18]. Smoking induces chronic inflammation at mucosal surfaces [19] and act as an environmental trigger for the appearance of specifically IgA ACPA before onset of RA [15]. Lung mucosal inflammation (e.g., bronchiectasis [BR]) is more commonly found in RA patients than in the general population [20]. The capability of plasma cells in inducible bronchus associated lymphoid tissue to produce ACPA and RF [21], as well as the increased presence of airway abnormalities in arthritis-free individuals with serum RF and/or ACPA positivity as compared with ACPA- and RF-negative controls [22], may indicate a role for the respiratory system in the initiation of RA-AAB.
The association between RA and BR suggests that RA may occur at an increased rate in patients with cystic fibrosis (CF). The prevalence of rheumatic symptoms increases with age and CF severity and is associated with lung superinfection. However, an association with definite RA is not yet established [23]. After years of antigen stimulation, episodic arthritis could progress to RA [24]. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene may play a role in this progression [25]. Compared with healthy controls (HC), RF is increased in patients with CF, specifically in patients with CF who have episodic arthritis [24]. Up to now, there are no data on the presence of other RA-AAB in patients with CF.
As well as being a risk factor for RA, smoking is a risk factor for mucosal inflammation in the periodontal region (periodontitis [PD]) [26]. Microorganisms organized in the subgingival biofilm are the primary etiologic agents in PD. The periodontal pathogen *Porphyromonas gingivalis* has been hypothesized to be involved in ACPA initiation owing to its own peptidyl arginine deiminase (PAD) enzyme necessary for protein citrullination [27, 28]. *P. gingivalis* peptidyl arginine deiminase (PPAD) is able to citrullinate endogenous and human proteins, thereby creating antigens that have been presumed to initiate the ACPA response in RA [29]. Recently, this hypothesis has been expanded by myeloperoxidase-mediated protein carbamylation and associated anti-CarP production in the inflamed oral mucosa of patients with periodontitis [30]. Therefore, PD has been posed as a candidate risk factor for RA [31].

Reactivity against native forms of citrullinated RA autoantigens was found in sera from patients with RA before clinical symptoms occurred [32]. Recently, reactivity against native forms of citrullinated RA autoantigens in patients with PD was found to be increased compared with non-PD controls [33]. These findings have raised the hypothesis that, at least in some individuals, reactivity against citrullinated autoantigens is preceded by reactivity against their native forms [33].
The aim of this study was to assess inflammation of the oral and lung mucosa as a potential cause for RA-AAB production. RA-AAB were assessed in sera of patients without RA with PD, BR or CF. In addition, reactivity against the native forms of citrullinated autoantigens was assessed. RA-AAB serum levels were analyzed within the context of HC and patients with established RA.

Methods

Patient groups

Serum autoantibody levels were measured in adult patients without RA with PD (n = 114), non-CF patients with BR (n = 80) and patients with CF (n = 41). Subjects without systemic disease and without PD served as the HC group (n = 36). Patients with established RA without lung disease and with known periodontal status served as a reference group (RA group, n = 86).
Patients with untreated severe PD were recruited from a referral practice for periodontology (Clinic for Periodontology Groningen). Inclusion criterion was >30% of sites involved with clinical attachment loss ≥5 mm on the basis of full mouth oral measurements [34]. The exclusion criteria were antibiotic use <3 months before inclusion and having systemic disease other than PD. To assess the inflammatory burden exerted by the periodontium, the periodontal inflamed surface area (PISA) [35] was quantified.
Sera from patients with BR without CF and without RA from a previously conducted randomized controlled trial were included [36]. Baseline serum samples were used to avoid possible influence of treatment on antibody levels. Sera from a cohort of patients with CF without RA who visited the Department of Pulmonology of the University Medical Center Groningen for routine checkups were included. For BR and CF patients, the number of exacerbations was based on number of antibiotic courses received 12 months before inclusion. The percentage predicted forced expiratory volume in 1 second (%FEV1) at inclusion was used as a disease activity measure.
HC subjects were recruited from among subjects planned for first consultation at the dental department of the University Medical Center Groningen. Periodontal health was assessed using the Dutch periodontal screening index (DPSI) [37]. An inclusion criterion was DPSI score <2 (absence of PD), and exclusion criteria were antibiotic use <3 months before inclusion and presence of systemic disease.
Patients with established RA without lung disease at the time of inclusion and with known periodontal status as assessed by the DPSI served as a reference group for serological measurements. RA disease activity was assessed using the Disease Activity Score 28 tender and swollen joint count (DAS28).
In PD, RA and HC subgingival microbiological samples samples were tested for presence of *P. gingivalis* by using anaerobic culture techniques (for details see [38]).
Participants provided written informed consent before study enrollment according to the Declaration of Helsinki. This study was conducted with the approval of the Medical Ethical Committee of the University Medical Center Groningen (METc UMCG 2009/356, METc UMCG 2011/010) and Medical Ethical Committee Noord-Holland (METc Noord-Holland M07-002).

Laboratory measurements

IgG anti-cyclic citrullinated protein antibody levels (anti-CCP) were measured using a commercial anti-CCP2 kit (Euro Diagnostica) according to the manufacturer's protocol. Samples with a value <25 U/ml were measured again with an adjusted protocol in which samples were diluted 1:10 instead of 1:50. The diagnostic cut-off value was defined as >25 U/ml according to the manufacturer's instruction. However, IgG anti-CCP was not used as a diagnostic test for RA in this study; therefore, seropositivity was defined as >2 SD above the mean of HC (2.2 U/ml), analogous to the other autoantibodies measured. IgA anti-CCP measurements were performed using a modification of the anti-CCP2 kit (Euro Diagnostica). Sera were diluted 1:50 using the dilution buffer provided by the manufacturer. The secondary antibody was horseradish peroxidase (HRP)-conjugated polyclonal goat anti-human IgA (SouthernBiotech), diluted 1:20,000 in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.05% Tween-20 (Sigma-Aldrich). The color reaction was performed using tetramethylbenzidine (Sigma-Aldrich) and hydrogen peroxide. A pool of four sera from RA patients with high levels of IgA anti-CCP served as a calibrator for the standard curve expressed in arbitrary units per milliliter (AU/ml) and starting at 200 AU/ml. Seropositivity was defined as >2 SD above the mean of HC. To assess the citrulline-specific nature of the response, sera were tested on plates coated with the arginine containing counterpart of CCP2: cyclic arginine peptide (CAP, kindly provided by Euro Diagnostica), following almost the same protocol as described for the IgG and IgA CCP2 enzyme-linked immunosorbent assay (ELISA). A dilution of patient serum with high levels of IgG anti-CAP served as a calibrator for the standard curve, starting at 100 AU/ml. Similarly, IgA anti-CAP reactivity was measured with a dilution of a high-level responding serum serving as a standard curve, starting at 100 AU/ml. Seropositivity was defined as >2 SD of HC.

Levels of IgG anti-CarP antibodies against carbamylated fetal calf serum were assessed using a protocol described by Shi et al. [8]. Seropositivity was defined as >2 SD above the mean of a distinctive HC cohort [8].

The specificity of the response against citrullinated proteins was assessed by testing reactivity against four synthetic citrullinated peptides that are known autoantigens in RA: fibrinogen-1 (Fib1, β-chain amino acids 36–52, NEEGFFSACitGHRPLDKK), fibrinogen-2 (Fib2, β-chain amino acids 60–74, CitPAPPPISGGGYCitACit), α-enolase (CEP-1, KIHACitEIFDSCitGNPTVE) and vimentin (Vim1, VYATCitSSAVCitLCitSSV). Every peptide was linked with its N terminus to biotin with a spacer (SGSG) in between. Reactivity against the citrullinated and native form of the peptides was measured according to the method of van de Stadt et al. [39] with some modifications. In short, 96-well Costar plates (Corning) were coated overnight with 5 µg/ml streptavidin (Rockland Immunochemicals) in PBS. Subsequently, plates were blocked for at least 1 hour with 2% BSA and 0.05% Tween-20 in PBS followed by incubation with the biotin-labeled peptides (0.5 µg/ml in PBS). Next, serum samples were diluted 1:100 in high-performance ELISA buffer (Sanquin) and incubated on the plates. Reactivity was detected with HRP-conjugated monoclonal mouse anti-human IgG (clone JDC-10; SouthernBiotech) diluted 1:2000 in PBS with 1% BSA and 0.05% Tween-20 or with HRP-conjugated polyclonal goat anti-human IgA (SouthernBiotech) diluted 1:4000 in the same dilution buffer. Bound antibodies were visualised by tetramethylbenzidine and hydrogen peroxide. Reactivity against a citrullinated peptide and its native counterpart was measured on the same plate. Every serum was measured in duplicate, and a positive control serum was applied on each plate. The citrulline-specific response was expressed as the difference in optical density (ΔOD) between the citrullinated peptide and its native form, and it was considered posi-

tive when ΔOD was >2 SD above the mean of HC. Likewise, the arginine-specific response was expressed as ΔOD between the native peptide and its citrullinated form, and it was considered positive when ΔOD was >2 SD above the mean of HC.
IgM and IgA RF levels were assessed using a validated in-house ELISA [40]. Levels were expressed in international units (IU) per milliliter, and seropositivity was defined as >10 IU/ml for IgM RF and >25 IU/ml for IgA RF [40]. C-reactive protein levels were measured by performing ELISA (DuoSet; R&D Systems). Absorbance was read at 450 nm in an EMax microplate reader, and antibody levels were calculated by using SoftMax PRO software (Molecular Devices).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 5.00 for Windows; GraphPad Software) and IBM SPSS Statistics for Windows (version 20.0; IBM). Normality was tested using D'Agostino-Pearson omnibus K2 test. For group comparisons, a Mann-Whitney U test was used for continuous variables and Fisher's exact test for categorical variables. Comparison of RA-AAB levels between the groups was done with Kruskal–Wallis one-way analysis of variance with Dunn's multiple-comparison post-test compared with HC if overall $p < 0.05$. A logistic regression model was used to analyze the association of the diseases with seropositivity for anti-CCP and RF. The model was adjusted for age, sex and smoking (former and current versus never). A logistic regression model appeared to be better compared to a linear regression model owing to non-normally distributed residuals. Associations of anti-CCP, RF and anti-CarP with disease activity were assessed by using a Mann-Whitney U test or an unpaired t-test with or without Welch's correction, depending on normality and equality of variances. Correlations between different parameters were assessed by Spearman's ρ.

Results

The vast majority of all patient groups was Caucasian. The demographic and clinical characteristics of patient groups are listed in Table 1, page 65. Age varied significantly between the groups, being related to the specific ages at which a certain disease is mostly present; for example, patients with CF are of young age owing to the low survival rate of the disease.

Anti-CCP and anti-CAP levels

Compared with HC, IgG and IgA anti-CCP levels were increased in patients with CF (both $p < 0.01$) and patients with RA (both $p < 0.0001$). IgG anti-CCP seropositivity was 13%, 21%, 24% and 86% in PD, BR, CF and RA patients, respectively, and 5.6% in HC. According to the diagnostic cut-off, IgG anti-CCP seropositivity was 0.9%, 3.8%, 2.4% and 76% in PD, BR, CF and RA, respectively, and absent in HC. Seropositivity for IgA anti-CCP was 16%, 10%, 27% and 74% in PD, BR, CF and RA patients, respectively, and 8.3% in HC (Fig. 1, page 65)

Reactivity against the native counterpart of CCP (anti-CAP) was, compared with HC, increased in RA patients for IgG anti-CAP ($p < 0.01$) and in CF patients for IgA anti-CAP ($p < 0.05$), although this was not necessarily reflected in increased seropositivity (Fig. 2, page 67).
Correlations for IgG anti-CCP and IgG anti-CAP levels were found in HC ($\rho = 0.57$, $p < 0.001$), patients with PD ($\rho = 0.32$, $p < 0.001$) and patients with BR ($\rho = 0.47$, $p < 0.0001$), and a trend was observed in patients with CF ($\rho = 0.28$, $p = 0.08$). IgA anti-CCP and IgA anti-CAP levels were correlated in HC ($\rho = 0.41$, $p < 0.05$), patients with PD ($\rho = 0.39$, $p < 0.0001$), patients with CF ($\rho = 0.38$, $p < 0.05$) and patients with RA ($\rho = 0.21$, $p < 0.05$).

Rheumatoid factor

Compared to HC, IgM RF levels were increased in patients with RA only ($p < 0.0001$), while IgA RF levels were increased in BR ($p < 0.0001$), CF ($p < 0.01$) and RA patients ($p < 0.0001$). IgM RF seropositivity was 7%, 23%, 7% and 74% in PD, BR, CF and RA patients respectively, and 2.8% in HC. Seropositivity for IgA RF was 5.3%, 24%, 17%, 50% in PD, BR, CF and RA patients respectively, and 2.8% in HC (Fig. 3, page 67).

Anti-CarP antibodies and peptide specific reactivity

Compared with HC, IgG anti-CarP levels were increased in patients with RA ($p < 0.0001$). Seropositivity for IgG anti-CarP was observed in PD, BR, and CF patients (3.5%, 3.8% and 7.3%, respectively), but not in HC (Table 2, page 66). The percentage of seropositive patients with RA (48%) was congruent with anti-CarP seropositivity in another Dutch cohort of patients with RA (45%) [41]. Compared with that in HC, IgG reactivity against all investigated citrullinated peptides was increased in patients with RA ($p < 0.0001$). With the exception of patients with RA, IgG seropositivity against the various citrullinated peptides was not increased in other patient groups studied (Table 2, page 66). Compared with that of HC, IgA reactivity against citrullinated fibrinogen-1 was increased in patients with RA ($p < 0.01$), as was seropositivity (19%) (Table 2, page 66).
Compared with that of HC, IgA reactivity against native fibrinogen-2 ($p < 0.0001$) and vimentin ($p < 0.01$) was increased in patients with CF. No differences in seropositivity were observed for the various native peptides between all groups for both immunoglobulin isotypes (Table 2, page 66).

Regression analysis

Logistic regression analysis, adjusted for age, sex and smoking (former and current versus never) revealed that IgG anti-CCP seropositivity was more frequent in BR (odds ratio [OR], 8.6, 95% CI 1.5-50, $p < 0.05$) and RA (OR, 226, 95% CI 39-1309, $p < 0.0001$). The association with PD was borderline significant (OR, 5.2, 95% CI 0.99-27, $p = 0.05$). IgA anti-CCP seropositivity was associated with CF (OR, 4.4, 95% CI 1.1-18, $p < 0.05$) and RA (OR, 43, 95% CI 10-187, $p < 0.0001$). IgM RF seropositivity was associated with RA (OR, 10, 95% CI 10-757, $p < 0.0001$), whereas the association with BR was borderline significant (OR, 9.1, 95% CI 1-84, $p = 0.05$). IgA RF seropositivity was associated with CF (OR, 10, 95% CI 1.1-97, $p < 0.05$) and RA (OR, 20, 95% CI 2.4-163, $p < 0.01$). Smoking was only of influence on the association of IgA RF with RA ($p < 0.01$), as there were no smokers among CF patients. Apart from that, smoking, age and sex had no influence on the association of the diseases with anti-CCP or RF seropositivity.

Association of disease activity with autoantibody status

Pulmonary function, measured as %FEV1, was significantly worse in BR and CF patients seropositive for IgA RF ($p < 0.01$ and $p < 0.05$, respectively). Disease activity, as measured by the number of exacerbations 12 months before inclusion based on the number of antibiotic courses, was associated with seropositivity for IgA anti-CCP in patients with CF ($p < 0.01$). Disease extent of PD, measured as PISA, was negatively associated with anti-CarP seropositivity ($p < 0.05$). RA disease activity, as measured by DAS28, was associated with seropositivity for IgG anti-CCP ($p < 0.05$), IgA RF ($p < 0.05$), whereas the association with anti-CarP was borderline significant ($p = 0.05$).

Discussion

To our knowledge, this is the first study in which serum IgA anti-CCP and anti-CarP levels have been assessed in non-RA patients with inflammation of oral or lung mucosal tissues. Both IgG and IgA anti-CCP levels were increased in patients with CF compared with HC, and IgA anti-CCP seropositiviy was associated with presence of CF.

Besides one study reporting 1 of 45 adult patients with CF seropositive for IgG anti-CCP [23], no data on anti-CCP levels in patients with CF have been published. IgG anti-CCP seropositivity was associated with presence of BR, whereas the association with PD was borderline significant. According to the diagnostic cut-off for IgG anti-CCP, similar seropositivity (3.3%) has been reported in a comparable BR patient cohort [42], whereas seropositivity of up to 8% has been reported in patients with PD [43-45]. However, these studies comprised PD patient groups of limited sample size compared with our PD group. Our results for patients with PD are better comparable with those reported by de Pablo et al. [33], who found, according to the diagnostic cut-off 1% IgG anti-CCP seropositivity in patients with PD.

Both IgM and IgA RF were increased in patients with CF compared with HC, and IgA RF seropositivity was associated with presence of CF. The former findings are in accord with other reports in the literature [24], however, Koch et al. [23] found only slightly elevated RF in a cohort of 65 adult and pediatric CF patients and concluded that these laboratory findings were mostly nonspecific. IgM RF was increased in patients with BR compared with HC, and the association of IgM RF seropositivity with presence of BR was borderline significant. Seropositivity for IgM RF in BR (23%) is in accord with a recent comparable study [42]. The importance of RF and anti-CCP in BR and CF patients is emphasized by the fact that pulmonary function was significantly worse in BR and CF patients seropositive for IgA RF, which has been reported for patients with CF [46]. In addition, the number of exacerbations in CF patients was associated with IgA anti-CCP seropositivity.

Anti-CarP seropositivity was rather specific for RA. The importance of anti-CarP was underlined by a correlation with RA disease activity, in accordance with the findings of Shi et al. [8]. Recently, next to citrullination, carbamylation has been hypothesized to play a role in the association of PD and RA [30]. Serum anti-CarP levels were not increased in patients with PD, although seropositivity in HC was absent. The extent of periodontal disease was not associated with anti-CCP or RF seropositivity, nevertheless, the unknown periodontal status of BR and CF patients remains a limitation of our study. The extent of periodontal disease was negatively associated with anti-CarP seropositivity, the implication of which is unclear.

Our HC should ideally be better age-matched, because a trend towards increased amounts of serum IgM RF with advancing age has been described [47], especially in advanced elderly people without RA (aged >78 years) [48]. However, the latter study found that only 1 of 300 advanced elderly subjects was IgG anti-CCP seropositive when the cut-off level was set at the 98th percentile of blood donors aged 40–65 years (mean age, 50 years), which represents ages of patients for whom immunological tests for RA are typically performed [48]. This age range is comparable to that of our PD, BR and RA patients. Together with the absence of significant influence of age in the logistic regression model, we assume that RF and anti-CCP seropositivity is not much influenced by age differences among patients groups. Regarding anti-CarP, we cannot comment on contribution of the age factor in our patient groups because its relation with age has not been tested in healthy populations.

Among the PD, BR, CF patients and HC, no differences were found in IgA or IgG seropositivity for the citrullinated peptides of candidate autoantigens in RA (e.g., citrullinated α-enolase). Increased IgG reactivity

against citrullinated α-enolase in patients with PD was reported previously [33, 45]. Of note, these studies showed increased reactivity against the native peptide of citrullinated α-enolase as well. Therefore, the observed increased levels of anti-citrullinated α-enolase were probably, at least partly, not citrulline-specific. To rule out this possibility, we assessed the difference in reactivity against the citrullinated peptide and its native counterpart. Likewise, the specific reactivity against the native forms of the peptides was assessed. Break of tolerance toward native forms of citrullinated autoantigens may lead to reactivity against citrullinated autoantigens via epitope spreading [33]. Brink et al. [32] supported this hypothesis by reporting IgG seropositivity for various native peptides in a limited number of presymptomatic patients with RA. The correlations between anti-CCP and anti-CAP levels in our patient groups suggest that at least part of the observed anti-CCP reactivity is not citrulline specific. A non-citrulline specific anti-CCP response has been reported in tuberculosis patients [49, 50]. In addition, in all our patient groups there was limited IgA and IgG seropositivity toward one or more native peptides. Especially the patients with CF showed an increased IgA response against CAP, native fibrinogen-2 and vimentin peptides, but this was not reflected in increased seropositivity for these antigens. Seropositivity for native antigens was also observed in HC and might not be of clinical relevance. It remains unclear whether or not reactivity toward citrullinated peptides can be preceded by reactivity against their native forms, since no longitudinal data of our study subjects were available.

P. gingivalis has been speculated to contribute to the initiation of ACPA generation because of PPAD expression. No increased reactivity was found against citrullinated α-enolase in patients with PD, the candidate RA autoantigen that shows sequence similarity with *P. gingivalis* enolase [51]. In contrast to Lappin et al. [45], in our study we found no differences in ACPA levels between patients with PD with or without subgingival *P. gingivalis* (data not shown). Differences in study methodology, including *P. gingivalis* detection, could have contributed to this different study result.

Low serum anti-CCP levels have also been reported for gastrointestinal mucosal inflammation [52, 53]. Because RA-AAB are thought to be induced locally, serum levels might not necessarily reflect local autoantibody production. ACPA have been found in gingival crevicular fluid of patients with PD [54] and in sputum of subjects at risk for RA [22]. To our knowledge, local ACPA production in the gastrointestinal tract has not yet been investigated.

Conclusion

Although overall levels were low, the presence of IgG and IgA anti-CCP and IgM and IgA RF is independently of age, sex and smoking associated with lung mucosal inflammation (BR and CF) and may be associated with oral mucosal inflammation (PD). RA-AAB in the peripheral blood in the presence of mucosal inflammation, albeit not according to the diagnostic cut-off level, supports the hypothesis that formation of these autoantibodies may be induced at inflamed mucosal surfaces. To further determine whether mucosal inflammation functions as a site for induction of RA-AAB and precedes RA, longitudinal studies are necessary in which RA-AAB of specifically the IgA isotype should be assessed in inflamed mucosal tissues and/or in their inflammatory exudates.

Competing interests

The work of Leendert Trouw was supported by Pfizer, a NWO-ZONMW Vidi grant and a fellowship from Janssen Biologics.

Acknowledgements

The authors acknowledge the technical assistance of B. Doornbos-van der Meer, E.W. Levarht and S. de Boer. Prof. P.U. Dijkstra is acknowledged for his help with statistical analyses.

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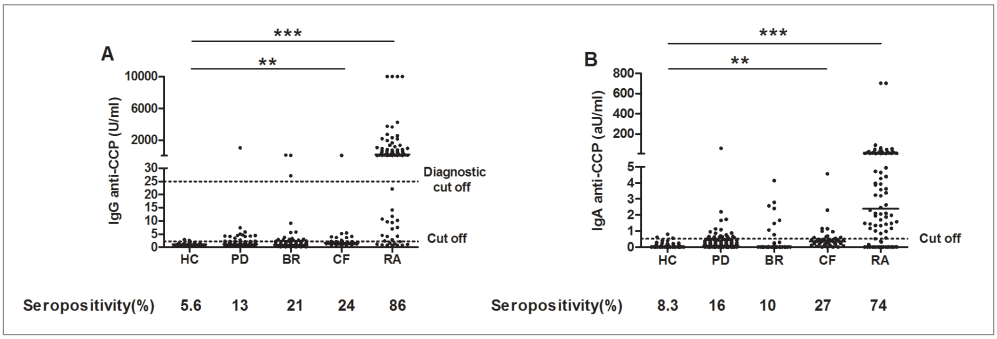
Tables and Figures

Table 1 Patient characteristics.

Patient group	Rheumatoid arthritis	Periodontitis	Bronchiectasis	Cystic fibrosis	Healthy controls	*p* value (vs. HC)
Subjects (n)	86	114	80	41	36	
Age, yr, median (IQR)	57 (48-64)	50 (45-57)	65 (56-71)	28 (21-36)	26 (24-46)	***RA, PD and BR
Female (%)	70	59	63	49	56	n.s.
Current smoker (%)	17	42	2.5	0	14	**PD, *for BR and CF
Ever smoker (%)	22	36	44	0	8.3	**PD, ***BR
Never smoker (%)	61	22	54	100	78	***PD, *BR, **CF
PISA (cm^2), median (IQR)	n.a.	14 (9.0-19)	n.a.	n.a.	n.a.	
$\%FEV_1$, median (IQR)	n.a.	n.a.	81 (60-97)	54 (36-80)	n.a.	
Exacerbations (n), median (IQR)	n.a.	n.a.	4 (3-6)	2 (1-3)	n.a.	
DAS28, median (IQR)	2.2 (1.7-2.8)	n.a.	n.a.	n.a.	n.a.	
CRP (mg/L), median (IQR)	1.9 (1.0-6.0)	1.0 (0.6-2.4)	5 (2.0-13)	6.0 (4.0-14)	0.4 (0.3-1.5)	***RA, BR and CF
No periodontitis (%)	31	0	n.a.	n.a.	100	
Moderate periodontitis (%)	41	0	n.a.	n.a.	0	
Severe periodontitis (%)	28	100	n.a.	n.a.	0	
Porpyromonas gingivalis positive (%)	14	43	n.a.	n.a.	0	
MTX (%)	71					
aTNFα (%)	10					
SASP (%)	3.5					
MTX + aTNFα (%)	3.5					
MTX + SASP (%)	4.7					
Other (%)	3.5					
None (%)	3.5					

*aTNFα: anti-TNFα inhibitors, CRP: C-reactive protein, DAS28: Disease Activity Score 28 tender and swollen joint count, exacerbations based on the number of antibiotic courses 12 months before inclusion, %FEV1: percentage predicted forced expiratory volume, MTX: methotrexate, n.a.: not assessed, n.s.: not significant, PISA: periodontal inflamed surface area, SASP: sulfasalazine *p < 0.05, **p < 0.01, ***p < 0.0001, Kruskal–Wallis one-way analysis of variance with Dunn's multiple- comparisons post-test or Fisher's exact test with two-tailed p value.*

Fig. 1 Serum immunoglobulin G (IgG) **(A)** and IgA anti-cyclic citrullinated peptide (anti-CCP) **(B)** levels in healthy controls (HC) and in patients with periodontitis (PD), bronchiectasis (BR), cystic fibrosis (CF) and rheumatoid arthritis (RA).



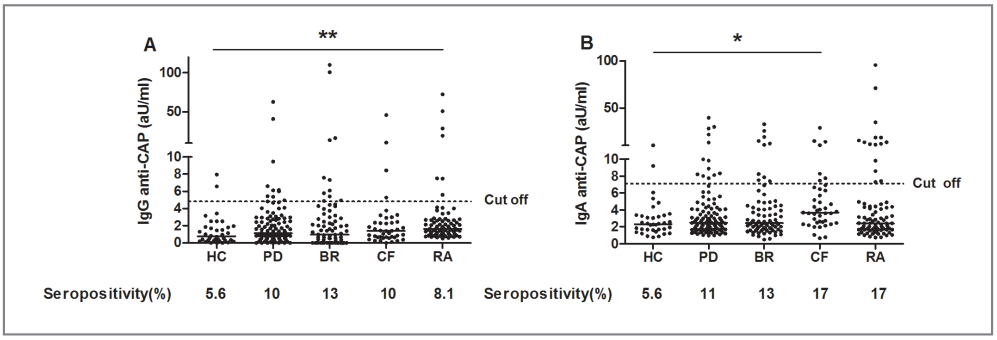
Cut-off values are indicated: diagnostic cut-off (25 U/ml) and >2 SD above the mean of HC for IgG anti-CCP and >2 SD above the mean of HC for IgA anti-CCP. Seropositivity (%) is indicated for cut-off based on >2 SD above the mean of HC. Bar indicates the median.
***p < 0.01, ***p < 0.0001, Kruskal–Wallis one-way analysis of variance with Dunn's multiple-comparisons post-test compared with HC if overall p < 0.05.*

Table 2 Percentages of seropositivity for anti-carbamylated antibodies and various citrullinated peptides and their native arginine counterparts according to cut-off levels of >2 SD above the mean of healthy controls.

Patient group	Healthy controls	Periodontitis	Bronchiectasis	Cystic fibrosis	Rheumatoid arthritis
anti-CarP IgG (% pos.)	0	3.5	3.8	7.3	48
Peptides IgG (% pos.)					
Cit. Fibrinogen-1	2.8	0.9	1.3	0	55
Arg. Fibrinogen-1	*0*	*0.9*	*1.3*	*2.4*	*0*
Cit. Fibrinogen-2	0	4.4	2.5	0	71
Arg. Fibrinogen-2	2.8	3.5	3.8	4.9	2.3
Cit. α-enolase	0	0.9	2.5	0	38
Arg. α-enolase	*2.8*	*1.8*	*6.3*	*4.9*	*1.2*
Cit. Vimentin	0	1.8	0	0	48
Arg. Vimentin	*5.6*	*5.3*	*1.3*	*7.3*	*1.2*
Peptides IgA (% pos.)					
Cit. Fibrinogen-1	0	0.9	0	0	8.1
Arg. Fibrinogen-1	*2.8*	*0.9*	*2.5*	*0*	*0*
Cit. Fibrinogen-2	2.8	2.6	0	0	19
Arg. Fibrinogen-2	*0*	*7.9*	*2.5*	*9.8*	*4.7*
Cit. α-enolase	0	2.6	0	0	7
Arg. α-enolase	*8.3*	*2.6*	*6.3*	*7.3*	*4.7*
Cit. Vimentin	0	0	0	0	4.7
Arg. Vimentin	*5.6*	*4.4*	*2.5*	*4.9*	*2.3*

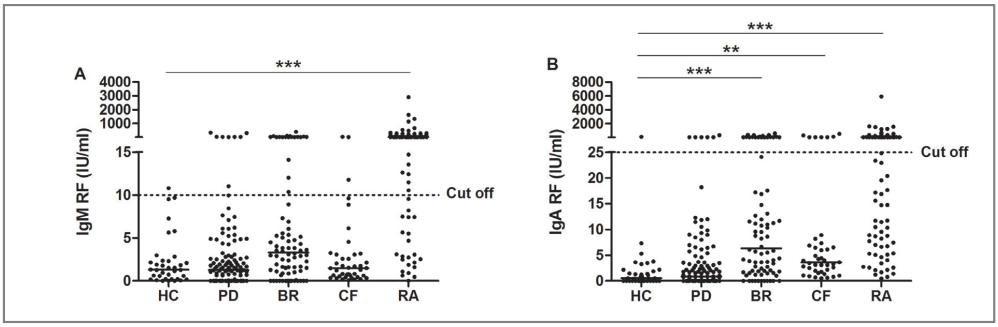
Arg.: arginine, Anti-CarP: anti-carbamylated protein, Cit.: citrulline, Ig.: immunoglobulin, % pos.: percentage positive.

Fig. 2 Serum immunoglobulin G (IgG) **(A)** and IgA anti-cyclic arginine peptide (anti-CAP) **(B)** levels in healthy controls (HC) and in patients with periodontitis (PD), bronchiectasis (BR), cystic fibrosis (CF) and rheumatoid arthritis (RA).



CAP represents the native counterpart of CCP. Cut off values are indicated: >2 SD above the mean of HC. Bar indicates the median.
**p < 0.05, **p < 0.01, Kruskal–Wallis one-way analysis of variance with Dunn's multiple-comparisons post-test compared with HC if overall p < 0.05.*

Fig. 3 Serum immunoglobulin M rheumatoid factor (IgM RF) **(A)** and IgA RF **(B)** levels in healthy controls (HC) and in patients with periodontitis (PD), bronchiectasis (BR), cystic fibrosis (CF) and rheumatoid arthritis (RA).



Cut off values are indicated: 10 IU/ml for IgM RF and 25 IU/ml for IgA RF. Bar indicates the median.
***p < 0.01, *** p < 0.0001, Kruskal–Wallis one-way analysis of variance with Dunn's multiple-comparisons post-test compared with HC if overall p < 0.05.*

CHAPTER

05

The peptidylarginine deiminase gene is a conserved feature of *Porphyromonas gingivalis*

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Scientific Reports, 2015 Sep 25;5:13936. doi: 10.1038/srep13936

Abstract

Periodontitis is an infective process that ultimately leads to the destruction of the soft and hard tissues that support the teeth (the periodontium). Periodontitis has been proposed as a candidate risk factor for rheumatoid arthritis (RA). *Porphyromonas gingivalis*, a major periodontal pathogen, is the only known prokaryote expressing a peptidyl arginine deiminase (PAD) enzyme necessary for protein citrullination. Antibodies to citrullinated proteins (anti-citrullinated protein antibodies, ACPA) are highly specific for RA and precede disease onset. Objective of this study was to assess *P. gingivalis* PAD (PPAD) gene expression and citrullination patterns in representative samples of *P. gingivalis* clinical isolates derived from periodontitis patients with and without RA and in related microbes of the *Porphyromonas* genus. Our findings indicate that PPAD is omnipresent in *P. gingivalis*, but absent in related species. No significant differences were found in the composition and expression of the PPAD gene of *P. gingivalis* regardless of the presence of RA or periodontal disease phenotypes. From this study it can be concluded that if *P. gingivalis* plays a role in RA, it is unlikely to originate from a variation in PPAD gene expression.

Introduction

Periodontitis is an infective process that ultimately leads to the destruction of the soft and hard tissues that support the teeth (the periodontium). Periodontitis has been proposed as a candidate risk factor for rheumatoid arthritis (RA) [1]. One of the biologically plausible causal mechanisms accounting for the association between periodontitis and RA could be induction of RA-related autoimmunity at inflamed mucosal sites, e.g., the periodontium [2].

Antibodies against citrullinated proteins (ACPA) are highly specific (98%) for RA [3] and can precede the clinical onset of RA [4]. Citrullination is a post-translational modification catalyzed by a family of enzymes called peptidylarginine deiminases (PAD) [5]. In this reaction, an arginine residue within a protein is converted into the non-coded amino acid citrulline. This modification leads to a loss of positive charge, reduction in hydrogen-bonding ability and subsequently in conformational and functional changes of the protein.

Porphyromonas gingivalis is a major periodontal pathogen involved in destructive periodontal disease [6] and is the only known prokaryote expressing a PAD enzyme [7]. *P. gingivalis* PAD (PPAD) is both a secreted and a cell or membrane vesicle associated enzyme [7]. In contrast to human PADs, PPAD is able to modify free arginine and is not dependent on calcium [7, 8]. Citrullination by PPAD enhances the survivability and increases the fitness of *P. gingivalis* due to several immune defense mechanisms. Additionally, a side effect of citrullination is ammonia production, which has a negative effect on neutrophil function and is protective during the acidic cleansing cycles of the mouth [7, 8]. PPAD is regarded as a virulence factor because citrullination by PPAD interferes with complement activity [9], inactivates epidermal growth factors [10] and contributes to infection of gingival fibroblasts and induction of the prostaglandin E2 synthesis [11]. Moreover, PPAD has been reported to be able to generate citrullinated forms of various arginine-containing proteins and peptides [8], among which are human fibrinogen and human α-enolase, two candidate autoantigens in RA [12].

A role of PPAD in autoimmunity is conceivable, considering that citrullinated host peptides generated by *P. gingivalis* are likely to expose epitopes previously hidden to the immune system, which may trigger an immune response in a genetically susceptible host [13]. In fact, cross reactivity has been shown for human antibodies against recombinant CEP-1, an immunodominant epitope of human α-enolase, with *P. gingivalis* enolase [14]. Moreover, there is strong animal experimental evidence supporting the theory that PPAD is the key player linking periodontitis and arthritis [15, 16].

Whether expression of PPAD is ubiquitous in *P. gingivalis* and whether there are different forms of the gene among *P. gingivalis* isolates from clinically different donors is currently unknown. Among oral bacteria, citrullination of endogenous proteins has only been shown in the *P. gingivalis* wild type strain W83 and four clinical isolates from patients with periodontitis without RA [12]. Related species such as *Porphyromonas endodontalis*, indigenous to the oral cavity, and *Porphyromonas asaccharolytica*, commonly found in the gastrointestinal tract, have not been tested for citrullination capacity.

The aim of this study was to assess expression of the PPAD-encoding gene in representative samples of *P. gingivalis* clinical isolates from patients with and without RA, as well as in related species of the genus *Porphyromonas* and in the periodontal pathogens *Prevotella intermedia* and *Fusobacterium nucleatum*. Additionally, variation in gene composition was analyzed using a combination of primer sets for the whole gene and for a region including the active site of the gene, by restriction enzyme analysis of the PCR products with three different restriction enzymes, and by whole gene sequencing.

Functional analysis of PPAD was carried out by assessment of endogenous citrullination patterns.

Methods

PPAD

Bacterial strains and culture conditions

Twelve *P. gingivalis* strains were isolated from 12 consecutive patients with RA and periodontitis, participants of an observational study on periodontitis and RA [17]. Eighty *P. gingivalis* strains were isolated from 80 consecutive subjects without RA (non-RA) with various periodontal diagnoses (periodontitis, n = 75; peri-implantitis, n = 2; gingivitis, n = 1 or a healthy periodontium (healthy carriers) n = 2), recruited for the control group of the same observational study [17]. Some general characteristics of the subjects from whom *P. gingivalis* was isolated are listed in Table 1, page 78. These clinical isolates, the *P. gingivalis* reference strains ATCC 33277 and W83, *P. asaccharolytica* (clinical isolate), *P. endodontalis* (clinical isolate), *F. nucleatum* (ATCC 25586) and *P. intermedia* (clinical isolate) were anaerobically grown on blood agar plates (Oxoid no. 2) supplemented with sheep blood (5% v/v), hemin (5 mg/L) and menadione (1 mg/L) and incubated in 80% N2, 10% H2 and 10% CO2, at 37°C [6, 17].

DNA extraction

Colonies from a blood agar plate were suspended in 500 µL Tris-EDTA buffer and bacterial DNA was isolated utilizing a Precellys®24 Technology tissue homogenizer (Bertin Technologies) (3 times per 30 sec. at 5000 rpm with 30-sec. breaks in between). Afterwards, samples were boiled for 10 min. at 95°C and centrifuged at 16100 g, 4°C for 10 min. Supernatants were collected and stored at -20°C. For whole genome sequencing, total DNA was extracted from 7 *P. gingivalis* strains using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories) following the manufacturer's instructions.

PPAD PCR

PCR was performed on the PPAD gene using Phusion DNA Polymerase (Thermoscientific) and two sets of primers. The first pair, P1F and P1R, covered the whole gene and the second pair, P2F and P2R, covered a short region around the active site (Cys351). The sequence of P1F was 5'-GGGGAGCT-CATGAAAAAGCTTTTACAGGCTAAAGC-3' while the sequence of P1R was 5'-GGGCTC-GAGTTTGAGAATTTTCATTGTCTCACGG-3'. The sequence of P2F was instead 5'-CTGATTCT-GAACAACAGGGT-3', while the sequence of P2R was 5'-TAAAGCTACCGGAACCATTG-3'. The samples were denatured at 98°C for 10 seconds, annealed at 56°C for 20 seconds and extended at 72°C for 2 minutes for a total of 33 cycles. Analysis was then performed using gel electrophoresis on a 1% agarose gel, immersed in SB buffer (10 mM NaOH; 36 mM boric acid, pH 8.0) and subjected to 120V for 30 minutes.

Restriction enzyme analysis

DNA samples for restriction enzyme analysis were cleaved with the four-nucleotide cutters *Sau*3AI, *Taqα*I or *Dpn*I following the instructions of the supplier (New England Biolabs) (incubation for 90 minutes at 37°C for *Sau*3AI and *Dpn*I and at 65 °C for *Taqα*I, followed by heat inactivation for 20 minutes at 80°C for *Taqα*I and *Dpn*I and at 65°C for *Sau*3AI). *Sau*3AI and *Taqα*I recognize the same sequence (GATC) but cut at different positions while *Dpn*I recognizes TCGA.
Whole genome sequencing and data analysis
DNA was extracted from a representative random sample of 7 *P. gingivalis* isolates that had been obtained from two RA patients with severe periodontitis, two RA patients with moderate periodontitis, two non-RA patients with severe periodontitis and one healthy carrier. The isolates originated from

unrelated individuals. The DNA concentration and purity were controlled by a Qubit® 2.0 Fluorometer using the dsDNA HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). The DNA library was prepared using the Nextera XT -v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's instructions and then run on a Miseq (Illumina) for generating paired-end 300 bp reads. De novo assembly was performed with CLC Genomes Workbench v7.0.4 (Qiagen, Hilden, Germany) after quality trimming (Qs ≥ 20) with optimal word size [18]. PPAD gene sequences were derived from the 7 assembled genomes and from 5 *P. gingivalis* genomes retrieved from GenBank (accession: NC_002950, NC_010729, NC_015571, CP007756 and AJZS01). DNA and amino acid sequences of 12 PPAD genes were aligned using the MAFFA v7 web server (http://mafft.cbrc.jp/alignment/software/). The PPAD gene sequences of strains 20655, 20658, MDS-16, MDS-45, MDS-56, MDS-85 and MDS-140 have been deposited at DDBJ/EMBL/GenBank under the accession numbers KP862650-KP862656.

Endogenous protein citrullination patters

Bacterial strains and culture conditions
The 12 *P. gingivalis* isolates from patients with RA and 12 randomly selected *P. gingivalis* isolates from non-RA subjects, and individual clinical isolates of *P. asaccharolytica*, *P. endodontalis* and *F. nucleatum* were analyzed for endogenous protein citrullination patterns. The isolated bacterial strains were anaerobically grown on blood agar plates (Oxoid no. 2), which were supplemented with sheep blood (5% v/v), hemin (5 mg/L) and menadione (1 mg/L) and incubated in 80% N2, 10% H2 and 10% CO2 at 37 °C.

Bacterial cell lysate preparation

Four-day old colonies of monocultures of the selected bacterial strains were suspended in sterile phosphate buffered saline (PBS) with protease inhibitors (Complete Mini Protease Inhibitor Cocktail Tablets, Roche Diagnostics, 1 tablet for 7 ml PBS). After washing and centrifugation cycles (3x 5 min., 14489 g, 4 °C) the bacterial pellets were resuspended in lysis buffer containing non-denaturing detergent (Noninet P-40, Sigma-Aldrich, Inc.) and sonicated on ice for 15 min. (Bioruptor® Standard sonication device, Diagenode s.a.). Protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Pierce Protein Biology Products).

SDS-PAGE and gel staining

Bacterial cell lysates were prepared with 2x SDS sample buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8) and 0.02% bromophenol blue) and boiled for 5 min. Per sample, 15 µg of protein was loaded onto a 12.5% SDS-PAGE gel (Criterion Tris-HCl, Bio-Rad Laboratories, Inc.) and resolved by running at 200V and 15 Watt constant for 1.5 hours. Gels were stained using Coomassie® staining (SimplyBlue™ SafeStain, Life Technologies Corporation) or transferred to a PVDF membrane (Immobilon® EMD Millipore Corporation, Billerica).
Western Blot
Citrulline-containing proteins were detected by Western blotting with a polyclonal IgG antibody (Anti-Citrulline Modified Detection Kit, Upstate, EMD Millipore Corporation) according to the manufacturer's instructions. In addition, detection of citrulline containing proteins was done with a monoclonal IgM antibody (F95) against a deca-citrullinated peptide (U2005-0033, UAB Research Foundation, Birmingham AL) using the following protocol: after blocking for one hour using a 1:1 dilution of Odyssey® Blocking Buffer (LI-COR Biosciences) and PBS, incubation with F95 in the same blocking buffer (final dilution 1:2000) with 0.1% Tween®20 (Sigma-Aldrich Co. LLC.) was done overnight at 4 ºC. IRDye® 800 conjugated goat anti-mouse IgM (Rockland Immunochemicals Inc., Gilbertsville) (1:10000) in Odyssey® Blocking Buffer and

PBS (1:4) with 0.1% Tween®20 (Sigma-Aldrich Co. LLC.) was used as secondary antibody for one hour at room temperature. In vitro citrullinated human fibrinogen (341578, Calbiochem, distributed by VWR international) by rabbit PAD (P1584, Sigma-Aldrich Co. LLC.) was used as positive control [19]. Non-specific binding of the secondary antibody was excluded by omitting the primary antibody. Protein bands were detected by the Odyssey system (LI-COR Biosciences). For graphical reproduction of the gels, the signal and size of the protein bands were analyzed using Image Studio Version 2.0.38 (LI-COR Biosciences) with the same image display settings per gel. *F. nucleatum* was considered as negative control [12] and, if present, the signal of the detected bands was corrected for the mean signal of *F. nucleatum*. The sizes of detected bands were plotted in a graph using GraphPad Prism 5 (GraphPad Software Inc.).

Results

PPAD gene is a conserved feature of P. gingivalis

The PPAD gene, consisting of 1668 base pairs, was detected by PCR in all 92 investigated *P. gingivalis* strains, but not in any of the other bacterial species tested (Fig. 1A, page 79). The same holds true for the region encoding the active site of PPAD, consisting of 328 base pairs (Fig. 1B, page 79). Cleavage of the PCR-amplified PPAD genes with three different restriction endonucleases and subsequent separation of the fragments by gel electrophoresis revealed no differences in the respective banding patterns for all 92 investigated *P. gingivalis* strains (shown for *Sau*3AI in Fig. 1C, page 79). Furthermore, no differences in the whole PPAD gene or in the active site-encoding regions of PPAD were observed between *P. gingivalis* isolates from RA patients or *P. gingivalis* isolates from non-RA patients (shown for the whole PPAD gene in Fig. 1D, page 79).

Conservation of PPAD gene sequence

Alignment of the PPAD gene sequences of the *P. gingivalis* strains revealed that the PPAD gene is highly conserved among all analyzed strains. At the DNA and amino acid level, no mutations were found in the signal peptide region and also the active site Cys351 residue is strictly conserved. Overall, the PPAD protein of each strain analyzed has no more than five different amino acids compared to the PPAD proteins of the reference strains W83 or ATCC 33277. In addition, none of the mutations is an insertion, deletion or leads to proteins truncations. However, allelic differences were detected in the PPAD gene sequences, especially for the clinical isolates 20655 (derived from a non-RA patient with periodontitis) and MDS-85 (derived from an RA patient with periodontitis), which displayed 23 and 18 single nucleotide mutations respectively compared to the reference strain W83. Table 2, page 78, summarizes the number of nucleotides in the PPAD genes and amino acid residues in the PPAD proteins that differentiate each strain from any other. Interestingly, the highest number of identified mutations is 25, which separates the PPAD genes from isolates 20655 and 20658 (both derived from non-RA patients with severe periodontitis), and from isolates 20655 (derived from a non-RA patient with severe periodontitis) and MDS-140 (derived from a healthy carrier). Notably, besides their low numbers, the majority of these mutations were synonymous. Taken together, these results show a very high level of PPAD conservation in the investigated *P. gingivalis* isolates.

Endogenous protein citrullination patterns

To determine possible differences in the protein citrullination activities of different PPAD enzymes both the AMC detection

method and the anti-citrulline F95 monoclonal antibody were employed. Both assays showed that the patterns of citrullinated proteins of *P. gingivalis* isolates from patients with RA were not detectably different when compared to the pattern of citrullinated proteins from *P. gingivalis* isolates from non-RA patients. Fig. 2 (panels A, C, E, page 80) shows the Coomassie-stained gel and Western blots for 6 representative *P. gingivalis* isolates of each group, including a graphical representation of the respective citrullination patterns (panels B and D, page 80). After correction for conjugate controls, *P. asaccharolytica* and *P. endodontalis* showed no protein bands with the AMC detection method. However, some citrullinated protein bands were observed for these species when the F95 antibody was applied. Neither of the two detection methods revealed citrullinated proteins in samples of *F. nucleatum* (Fig. 2, panels A and C, page 80).

Discussion

This is the first study assessing PPAD expression in a large sample of clinical *P. gingivalis* isolates obtained from patients with or without RA. Our findings indicate that PPAD is omnipresent in *P. gingivalis*, but absent from *P. endodontalis* and *P. asaccharolytica* as well as from the other periodontal pathogens studied.

Our present observations support the view that PPAD may represent one of few, if not the only prokaryotic peptidylarginine deiminase. Of note, our analyses show that the PPAD gene is highly conserved in *P. gingivalis*. Consequently, the encoded PPAD enzymes share 98.9-100% amino acid sequence identity. This may suggest that PPAD contributes to the ability of *P. gingivalis* to colonize and thrive in its human host. Notably, some mutations in PPAD are missense and it may be of interest to analyze the citrullination levels of the respective PPAD isotypes, in order to see whether these mutations influence the enzymatic activity. Similarly, the mammalian PAD enzyme is also highly conserved with 70–95% identical amino acids sequences [5]; hinting at the importance of protein citrullination for both mammals and *P. gingivalis*, although we found no indications that the PPAD is evolutionarily related to the mammalian PAD enzymes [20].

Concerning PPAD, no differences were noted in the PPAD genes among *P. gingivalis* isolates from patients with or without RA. Also, no differences in PPAD genes were noted among *P. gingivalis* isolates from patients with different stages of periodontal disease or periodontal health. Therefore, we assume that there are no different PPAD variants in *P. gingivalis*. Functional analysis of PPAD further substantiated this assumption. No differences in endogenous citrullination patterns were seen between *P. gingivalis* isolates from RA and non-RA patients, as determined with two different anti-citrulline antibodies. Some differences were observed in the citrullination patterns detected with the two antibodies against citrullinated proteins, which can probably be attributed to the monoclonal (F95) or polyclonal (AMC) nature and the isotypes of these antibodies (IgM and IgG, respectively). Another difference between the antibodies is the chemical modification of the citrulline residues in the AMC detection method to ensure detection of citrulline-containing proteins regardless of neighboring amino acid sequences.

Based on the observations in this study, we conclude that PPAD is apparently omnipresent in *P. gingivalis* but absent from *P. asacharolytica* and *P. endodontalis*, two related species of the genus *Porphyromonas*. There are no significant differences in the PPAD gene regardless of RA or periodontal disease phenotypes. Therefore, from this study it can be concluded that if *P. gingivalis* plays a role in RA, it is unlikely to originate from a variation in PPAD gene expression.

An important future goal to strive for will be a detailed characterization of the function of the PPAD protein and its post-translational modifications. The production of recom-

binant PPAD in *Escherichia coli* has been studied in order to investigate its protein function. The catalytic mechanism was identified and showed different enzyme activities based on an N-terminal truncation of the protein [21]. This finding is in accordance with a recent study by Konig et al. which showed that non-cleaved PPAD is autocitrullinated and has decreased activity [22]. Additionally, Konig et al. concluded that autocitrullination of PPAD is not the underlying mechanism linking *P. gingivalis* with RA because it does not occur in *P. gingivalis* cells and patient antibodies were directed specifically against non-citrullinated PPAD. Conversely, another recent study showed a peptidyl-citrulline specific antibody response in patients and concluded that PPAD autocitrullination is still a potential mechanism for breaching autoimmunity in RA patients [20]. Besides these theories mainly focusing on cleavage and autocitrullination of PPAD, it will be crucial to investigate the overall citrullination of bacterial and host proteins by PPAD in especially the in vivo situation, as well as the interaction of the human PADs with bacterial proteins, as proposed by Quirke et al. [20]. In conclusion, it is more likely that a difference in post-translational modification of PPAD might play an important role in RA, rather than a difference in the PPAD gene.

Acknowledgements

The authors acknowledge B. Doornbos-van der Meer and J. Bijzet, Department of Rheumatology of the University Medical Center Groningen, for their technical assistance and help in preparing the figures respectively.

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Tables and Figures

Table 1 General characteristics of patients from whom individual *P. gingivalis* strains were isolated.

Patients	Number	Median age in years (IQR)	Current smoker, percentage	Female, percentage
RA	12	64 (56-71)	25	75
non-RA	80	51 (42-60)	27	54
Characteristics of patients with RA*				
Median disease duration in years (IQR)			3 (2-9)	
Median DAS28 (IQR)			2.3 (1.6-4.0)	
Median C-reactive protein in mg/L (IQR)			3 (3-14)	
anti-CCP seropositive, percentage			92	
IgM RF seropositive, percentage			92	
MTX monotherapy, percentage			92	

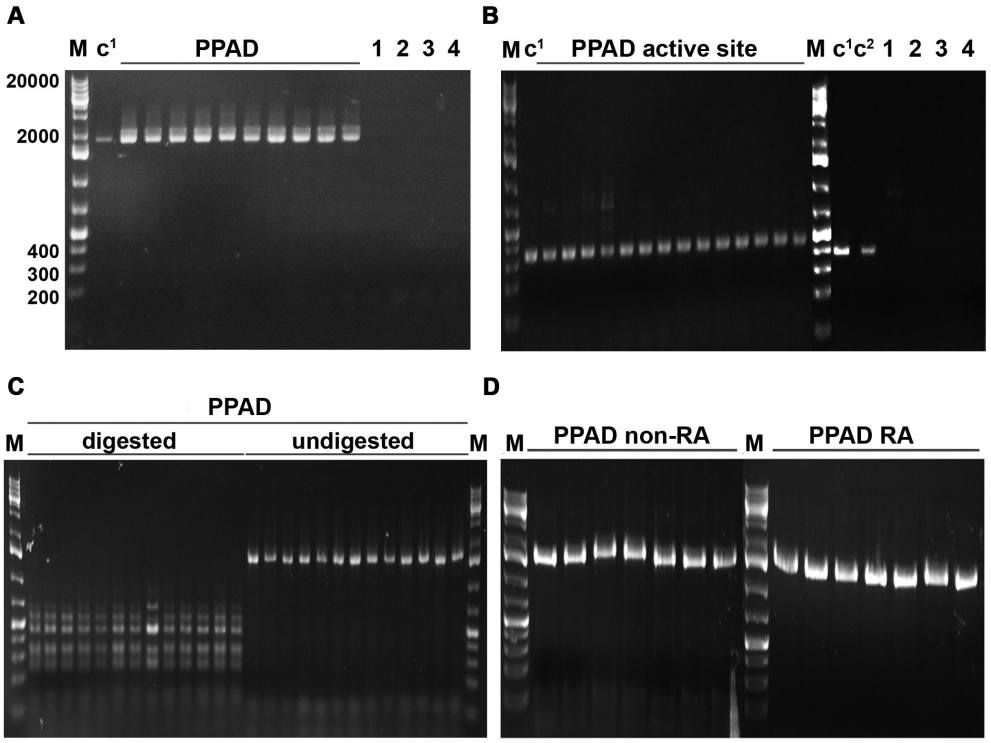
*RA: rheumatoid arthritis, non-RA: without rheumatoid arthritis, IQR: interquartile range, DAS28: disease activity score 28 tender and swollen joint count, anti-CCP anti-cyclic citrullinated protein antibody, IgM RF: Immunoglobulin M rheumatoid factor, MTX: methotrexate, *for details see reference [17].*

Table 2 Representation of the numbers of different nucleotides in PPAD genes and numbers of amino acid substitutions in the corresponding PPAD proteins.

	MDS-45	MDS-85	MDS-16	MDS-56	20655	20658	MDS-140	W50	HG66	TDC60	W83	ATCC33277
MDS-45	0	10	12	9	19	16	14	13	15	11	12	15
MDS-85	*5*	0	14	15	21	18	20	19	19	13	18	19
MDS-16	*3*	*4*	0	11	19	13	12	13	13	11	12	13
MDS-56	*2*	*5*	*3*	0	20	17	11	10	12	12	9	12
20655	*6*	*7*	*7*	*6*	0	25	25	24	22	24	23	22
20658	*3*	*4*	*2*	*3*	*5*	0	18	17	15	15	16	15
MDS-140	*4*	*5*	*3*	*4*	*8*	*3*	0	13	15	17	12	15
W50	*2*	*5*	*3*	*2*	*6*	*3*	*4*	0	12	16	1	12
HG66	*1*	*4*	*2*	*1*	*5*	*2*	*3*	*1*	0	16	11	0
TDC60	*3*	*6*	*2*	*3*	*7*	*2*	*3*	*3*	*2*	0	15	16
W83	*1*	*4*	*2*	*1*	*5*	*2*	*3*	*1*	*0*	*2*	0	11
ATCC33277	*1*	*4*	*2*	*1*	*5*	*2*	*3*	*1*	*0*	*2*	*0*	0

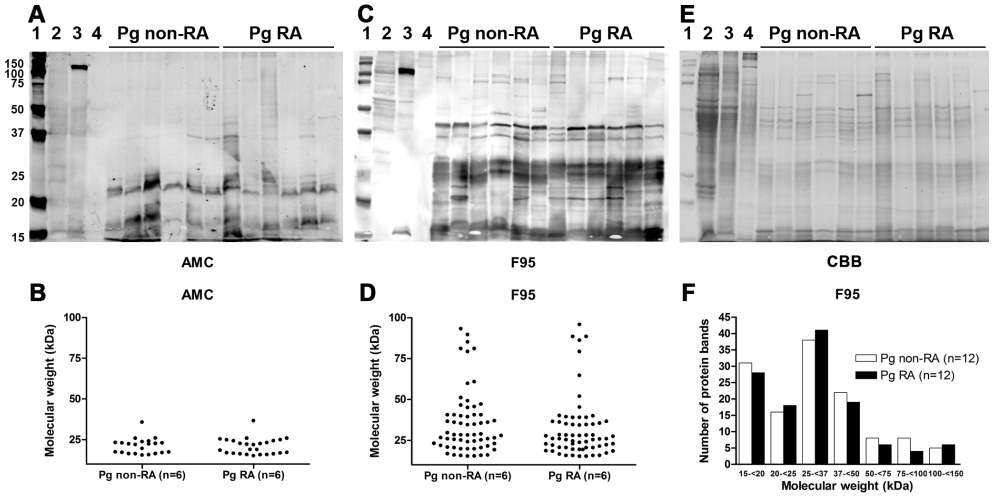
Top right, number of different nucleotides; bottom left, number of amino acid substitutions (italic). PPAD gene sequences from P. gingivalis isolates obtained from two RA patients with severe periodontitis (MDS-45, MDS-85), two RA patients with moderate periodontitis (MDS-16, MDS-56), two non-RA patients with severe periodontitis (20655, 20658) and one healthy carrier (MDS-140). Additional PPAD gene sequences were retrieved from GenBank (W50, HG66, TDC60, W83, ATCC 33277).

Fig. 1 PPAD gene composition analyzed by PCR and restriction enzyme analysis of the PCR products.
(A) PCR products of PPAD obtained with whole-gene primers (1668 base pairs) using 10 representative *P. gingivalis* isolates of patients without RA. No PPAD genes are detectable in other *Porphyromonas* species or other selected periodontal pathogens. **(B)** PCR products of PPAD obtained with active site region primers (328 base pairs) of 14 representative *P. gingivalis* isolates from patients without RA. No PPAD genes are detectable in other *Porphyromonas* species or other selected periodontal pathogens. **(C)** Restriction enzyme analysis with *Sau*3AI of PPAD PCR products obtained with whole-gene primers of 13 representative *P. gingivalis* isolates from patients without RA. **(D)** PPAD PCR products obtained with whole-gene primers



M: marker displayed as number of base pairs (GeneRuler™ 1 kb Plus DNA ladder), C1: positive control (PPAD of P. gingivalis W83), C2: positive control (PPAD of P. gingivalis ATCC 33277), 1: P. intermedia, 2: P. asaccharolytica, 3: P. endodontalis, 4: F. nucleatum, digested: PPAD PCR products digested with Sau3AI, undigested: PPAD PCR products of the same 13 P. gingivalis isolates not incubated with Sau3AI, RA: with rheumatoid arthritis, non-RA: without rheumatoid arthritis.

Fig.2 Patterns of citrullinated proteins of *P. gingivalis* isolates from patients with or without RA.
(A, C and E) Western blots and Coomassie staining of bacterial cell lysates of 12 representative *P. gingivalis* isolates of patients with or without RA (both n = 6); citrullinated protein patterns as detected with the AMC detection method (AMC) **(A)**, citrullinated protein patterns as detected with the F95 anti-citrulline antibody (F95) **(C)** and Coomassie staining **(E)**. **(B and D)** Graphical representation of the Western blots shown in panels A and C; citrullinated protein patterns as detected with the AMC detection method (AMC) **(B)** and citrullinated protein patterns as detected with the F95 anti-citrulline antibody (F95) **(D)**. **(F)** Graphical representation of citrullinated protein patterns as detected by Western blots using the F95 anti-citrulline antibody (F95) against bacterial cell lysates of 24 representative *P. gingivalis* isolates of patients with or without RA (both n = 12).



1: Molecular weight marker in kilo Dalton (kDa), 2: P. asaccharolytica, 3: P. endodontalis, 4: F. nucleatum, Pg non-RA: P. gingivalis isolates from subjects without RA, Pg RA: P. gingivalis isolates of patients with RA. The Western blots were analyzed with the same image display settings. The strong positive staining at circa 120 kDa in P. endodontalis (3) both with the AMC and the F95 detection method (panels A and C) is due to non-specific binding of the secondary antibody.

CHAPTER

06

General Discussion

Periodontitis and rheumatoid arthritis: what do we know?

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Journal of Periodontology 2015 Sep;86(9):1013-9. doi: 10.1902/ jop.2015.150088. Epub 2015 May 13.

Introduction

In the field of rheumatology there is currently much attention for possible causality between periodontitis and rheumatoid arthritis (RA). Systemic inflammatory and infectious challenges have long been considered to be involved in triggering rheumatoid factor (RF), the first important biomarker for diagnosis and prediction of RA. Later, another auto-antibody system, anti-citrullined protein antibodies (ACPA), was found to be more specific for RA. These antibodies can be present before the disease becomes symptomatic [1]. Why ACPA and RF are induced as well as their role in RA development is still unclear. The relation between periodontitis and RA is not well understood. On one hand, systemic manifestation of increased 'total inflammatory burden' through periodontitis has been documented. On the other hand, infection with *Porphyromonas gingivalis* specifically has been suggested to play a role a because of its unique capacity of protein citrullination [2, 3].
Because causality is ultimately tested in longitudinal cohort studies that do currently not exist for periodontitis and RA, in this commentary the Bradford Hill [4] criteria are applied on existing literature to assess causality as most likely interpretation of this association.

1. Strength and consistency

Quantitative studies on the association between periodontitis and RA performed thus far are mostly case-control studies with a relatively low sample size. Nineteen of these studies, representing participants from different ethnic backgrounds, were included in a recent systematic review [5]. It was concluded that patients with RA, compared to those without RA, have a significantly higher incidence of periodontitis and a higher number of missing teeth. A recent case-control study explored the degree to which the association is affected by shared genetic and/or environmental factors [6]. After multivariable adjustments, including positivity for Human Leucocyte Antigen (HLA)-DRB1 shared epitope (SE) alleles, ever smoking, age, sex, race/ethnicity, body mass index, self-reported diabetes mellitus, marital status, presence of oral dryness, and education), the incidence of periodontitis remained significantly higher in ACPA seropositive RA patients than in controls (odds ratio 1.6). The association between periodontitis and RA is thus relatively consistent; however the strength of the association is uncertain.

2. Biological plausibility

Certain genetic, hormonal, infectious and environmental risk factors, such as smoking, can contribute to a susceptibility background against which RA can develop. In susceptible individuals, deregulation of the immune system occurs and can present itself with formation of autoantibodies such as RF and ACPA, which can be present before the disease becomes symptomatic [1]. Whether and why progression to the symptomatic phase occurs is unknown, but theoretically a 'second hit' could be necessary. In this 'two hit' model, the 'first hit' is the induction of ACPA formation, the 'second hit' is the induction of arthritis and expression of citrullinated antigens in the inflamed joint [7, 8] (Fig. 1, page 93).

ACPA production may be induced in inflamed periodontium

It has been hypothesized that initiation of ACPA production occurs at inflamed mucosal surfaces of lungs and periodontium [9]. Inflamed periodontium contains citrullinated proteins [10] and ACPA have been found in the inflammatory exudates [11]. Independent of smoking status, periodontitis patients and patients with lung mucosal inflammation (e.g., bronchiectasis) without RA have hi-

gher serum ACPA levels compared to healthy controls, although lower than in RA patients [12-15].
In addition, it has been suggested that ACPA production is induced by the periodontal pathogen *Porphyromonas gingivalis (P. gingivalis)*, being unique in expressing a variant of the deiminating enzyme necessary for protein citrullination, peptidyl arginine deiminase (PAD) [2, 3]. *P. gingivalis* PAD (PPAD) is able to citrullinate endogenous as well as human proteins, thereby creating antigens that have been presumed to initiate the ACPA response in RA [16].
ACPA in RA include antibodies against citrullinated histones [17]. Histone citrullination is a common event during neutrophil activation and neutrophil death induced by different pathways, including apoptosis and neutrophil extracellular trap (NET) formation. NETs are increased in RA and are a source of citrullinated autoantigens [18]. Neutrophils from patients with periodontitis have been shown to be hyperreactive in terms of baseline, unstimulated generation and release of extracellular reactive oxygen [19]. As NET release is known to be dependent upon production of extracellular reactive oxygen, periodontal disease may be associated with excessive production of NETs, i.e., in a process triggered initially by the response of neutrophils to plaque bacteria. High and concentrated levels of NET associated molecules could lead to a localized chronic inflammatory response, potentially followed by an autoimmune response in genetically prone persons [20].
Recently, Romero et al. [21] identified a different citrullination pattern in PAD expressing RA synovial fluid cells (neutrophils and monocytes), which they termed cellular hypercitrullination because of the broad spectrum of citrullination across the entire range of proteins. Cellular hypercitrullination is induced by two immune-mediated membranolytic pathways, mediated by perforin and the membrane attack complex both leading to calcium influx. These data are supported by Neeli et al. [22], who showed that in human neutrophils PAD4 induces histone citrullination in the presence of calcium ionophore. Up to now, it is unknown whether cellular hypercitrullination is present in inflamed periodontium.

Mutual exacerbation of inflammatory responses

Another interpretation of the 'two-hit' model is that of mutual exacerbation: periodontitis serves as 'first hit', while an (unknown) arthritrogenic hit induces RA ('second hit') that leads, in susceptible individuals, to mutual exacerbation of the inflammatory responses mediating self-perpetuating tissue destruction in both the joint and the periodontium [8] (Fig. 1, page 93).
Neutrophils are the major cell type involved in periodontal inflammatory responses [20]. Besides providing a source of citrullinated autoantigens, neutrophils can cause tissue destruction through release of degradative enzymes (e.g., matrix metalloproteinases) and cytotoxic substances such as extracellular reactive oxygen. Neutrophils indirectly mediate destructive effects by chemotactic recruitment of T-helper 17 (Th17) cells. Th17 cells selectively produce the pro-inflammatory cytokine IL-17 which is crucial for host defense against extracellular pathogens [23]. Uncontrolled Th17 activity has been implicated in joint inflammation and bone destruction in RA, both at onset and in established disease [24, 25]. Presence of IL-17 and Th17 cells in human periodontitis may be associated with disease severity, possibly after activation of innate immune cells by *P. gingivalis* [26]. How this response regulates inflammation-mediated bone destruction has not been fully elucidated (see for illustration of possible mechanisms Hajishengallis et al. [27]).
In both periodontitis and RA, osteoclasts are predominantly activated by mechanisms dependent on upregulation of receptor activator of nuclear factor κB ligand (RANKL), a member of the tumor necrosis factor (TNF)

cytokine family that is also implicated in RA [28]. Mechanisms that underlie chronic joint inflammation in RA have not been clarified, although there is some evidence for both a T-cell dependent autoimmune mechanism and a more progressive fibroblast mediated chronic inflammation [29]. Th17 cells are recognized as effective B-cell helpers for antibody responses in inflammatory conditions [27]. B-cells constitute, along with T-cells, a major source of membrane-bound and secreted RANKL in the periodontal lesions (see for illustration Hajishengallis et al. [27]).

3. Temporal relationship

In both interpretations of the 'two-hit model', periodontitis precedes RA. Commonly, more advanced forms of periodontitis are present at disease onset in patients with new-onset RA [30, 31]. A recent nationwide, population-based case-control study using longitudinal administrative data found an association between a history of periodontitis and newly diagnosed RA in Taiwan (odds ratio 1.2) [32].

4. Specificity regarding *Porphyromonas gingivalis*

The PPAD gene is highly conserved, ubiquitous in *P. gingivalis* and absent in *P. gingivalis* related species (chapter 5). We found no indications that RA patients carry a different PPAD *P. gingivalis* variant than patients without RA (chapter 5). Studies on oral colonization by *P. gingivalis* have shown no difference in subgingival *P. gingivalis* distribution in patients with or without RA independent of periodontal status, detection techniques and RA disease duration [6, 30, 31, 33]. However, DNA of periodontal pathogens has been detected in synovial fluid of RA patients [34]. Of five different periodontal pathogens assessed, only DNA of *P. gingivalis* was more frequently detected in synovial fluid of RA patients than in synovial fluid of non-RA controls [35].

According to the hypothesis that *P. gingivalis* contributes to ACPA production, the association of *P. gingivalis* presence and serum ACPA levels has been assessed. In new-onset RA patients, subgingival presence of *P. gingivalis* was not correlated with ACPA levels as determined using the diagnostic anti-cyclic citrullinated peptide 2 (anti-CCP2) test [30]. In established RA patients, presence of subgingival *P. gingivalis* was not of influence on ACPA levels, but increased reactivity against several citrullinated peptides of fibrinogen, fillagrin, clusterin, histone 2B and apolipoprotein was found when subgingival *P. gingivalis* was present [6, 33].
In periodontitis patients without RA, we found no differences in ACPA levels between *P. gingivalis* positive and –negative periodontitis patients [15], in contrast to Lappin et al. [12] who found that serum ACPA levels in periodontitis patients carrying subgingival *P. gingivalis* were higher compared to periodontitis patients without subgingival *P. gingivalis*. The relatively low patient numbers in both studies and different detection techniques of *P. gingivalis* and ACPA may account for this discrepancy.
Levels of serum anti-*P. gingivalis* antibodies showed no differences in a large cohort of RA patients and a cohort of osteoarthritis control patients [6]. A study in Japanese patients with RA showed higher anti-*P. gingivalis* antibody levels compared to age-, sex-, smoking status-, and periodontal condition-balanced healthy controls [36]. Independent of *P. gingivalis* distribution, we observed a more robust antibody response against *P. gingivalis* in established RA patients with severe periodontitis than in non-RA controls with the same periodontal status [33]. The role of the antibody response in periodontitis is not fully understood, but it may be not protective [27]. No differences in anti-*P. gingivalis* antibody levels were found between ACPA and/or RF seropositive arthralgia patients, who developed or did not develop RA within 2 years follow-up [37].
In established RA patients a weak correlation

between ACPA levels and anti-*P. gingivalis* antibody levels has been found [6, 33]. Nevertheless, anti-*P. gingivalis* antibody levels were found to be significantly higher in children with ACPA positive juvenile idiopathic arthritis compared to children with ACPA negative juvenile idiopathic arthritis, while no differences were noted for anti-*Prevotella intermedia* anti-*Fusobacterium nucleatum* antibody levels [38].

5. Dose–response relationship

A dose–response pattern in the association between severity of periodontitis and RA development was found in a Taiwanese population based on longitudinal administrative data [32]. Also, a large case-control study by Mikuls et al. [6] revealed that presence of periodontitis was associated with increased swollen joint counts, greater RA disease-activity according to the 28-joint Disease-activity Score (DAS28). We showed an association between severity of periodontitis and severity of RA [33]; RA patients with severe periodontitis had significantly higher DAS28 scores than RA patients with no or moderate periodontitis.

Experimental evidence in humans on the effect of periodontal treatment on RA disease-activity is limited. Only four studies met the inclusion criteria according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and were included in the recent meta-analysis of Kaur et al. [39] on the effect of non-surgical periodontal therapy on RA disease activity measured by clinical (DAS28) and laboratory parameters (ESR, TNF-α and CRP levels). These studies generally had a small sample size and a relatively short follow up period (up to 6 months). Microbiology was not assessed; except for one study that measured serum anti-*P. gingivalis* antibodies. Although these studies are considered preliminary and indicate the need for further large-scale intervention studies, they reported a beneficial effect of periodontal therapy on laboratory RA parameters and clinical symptoms of RA.

6. Experimental evidence

The most widely studied model of RA is collagen-induced arthritis (CIA) in genetically susceptible mice. Balb/c mice with pre-existing periodontitis, induced by oral inoculations of *P. gingivalis*, developed more severe CIA at a faster rate compared to CIA mice without periodontitis [40]. Micro-CT analysis of joint and periodontal bone loss provided evidence for a bidirectional relationship between periodontitis and arthritis; mice with CIA only showed alveolar bone loss, whereas mice with periodontitis only showed bone loss within radiocarpal joints [40]. The severity of adjuvant arthritis in Dark Agouti rats was increased when there was a pre-existing extra-synovial chronic inflammatory lesion induced by subcutaneous sponges impregnated with heat-killed *P. gingivalis* [41]. No evidence of arthritis development occurred in Dark Agouti rats with a *P. gingivalis* extra synovial inflammatory lesion only, however, using computer assisted morphometric analysis, periodontal bone loss after adjuvant arthritis induction was seen in Lewis rats as measured on defleshed jaws [42].

Experimental evidence for involvement of periodontitis in pathogenesis of RA via cellular immunity comes from a murine model of T-cell–dependent experimental arthritis. Periodontitis induced by oral inoculations with *P. gingivalis* and *Prevotella nigrescens* significantly aggravated severity of CIA characterized by increased arthritic bone erosion in DBA/1 mice via induction of an antigen specific Th17 response [43]. A model in which experimental periodontitis and arthritis were co-induced in an inflammation-prone mouse strain using oral inoculations with *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* in pristane-induced arthritis showed that co-induction in control mice did not alter the course of both periodontitis and arthritis. It was concluded that the interaction between periodontitis and arthritis in

mice involves a shared hyper-inflammatory genotype and functional interferences in innate and adaptive immune responses [44]. Experimental evidence for PPAD as key player in the link between periodontitis and RA comes from aggravation of CIA in DBA/1 mice, which appeared dependent on the expression of PPAD [45]. Increased serum ACPA levels were measured after infection with wild type *P. gingivalis* W83 as compared to mice infected with *P. gingivalis* W83 with PPAD deletion. Moreover, at the site of infection with wild type strain higher levels of citrullinated proteins were found as compared to the site of infection with the PPAD knock-out strain. These results were confirmed by Gully et al. [46] using another murine model for experimental periodontitis and another *P. gingivalis* strain (W50); in BALB/c mice, the extent of CIA was significantly reduced in animals exposed to prior induction of periodontal disease through oral inoculation of a PPAD knock-out *P. gingivalis* W50 strain versus prior periodontal infection with the *P. gingivalis* W50 wild type stain. Furthermore, serum ACPA tended to be lower in mice prior infected with PPAD-deficient *P. gingivalis* compared to ACPA levels in CIA mice with periodontitis induced by the wild type strain.

7. Coherence

Animal models have shown that pre-existing periodontal infection (first hit) leads to exacerbation of arthritis after an arthritogenic hit (second hit) [40, 41]. In the same animal models, a bidirectional relationship between experimental periodontitis and experimental arthritis existed, e.g., experimental arthritis leads to alveolar bone loss, and experimental periodontitis leads to joint inflammation [40-42]. This is in concordance with observations that newly diagnosed and early RA patients have more frequently periodontitis and more periodontal attachment loss compared to controls [30-32]. In human periodontitis patients joint inflammation has not been systematically assessed.

Mutual exacerbation of the inflammatory responses in animal experiments, where both diseases co-existed, was shown to involve Th17 mediated immunity and to be dependent on a shared hyperinflammatory genotype [43, 44]. The correlation of RA disease-activity with the severity of periodontitis in humans [33] can probably be contributed to mutual exacerbation of inflammatory responses of both diseases. The importance of Th17 mediated immunity is increasingly acknowledged in human RA and periodontitis [24, 26] while gene polymorphisms within the IL-1 gene cluster are associated with cytokine levels in patients with periodontitis and in patients with RA, but not in healthy controls [47]. The latter supports the hypothesis of a shared genetic background for cytokine profiles [47]. IL-10 gene polymorphisms are also suggested to contribute to susceptibility for both RA and periodontitis. Three of several polymorphisms of IL-10 have been studied in some detail regarding to RA susceptibility. Meta-analysis of twenty-two relevant studies on these IL-10 polymorphisms, all located in putative regulatory regions of the gene promoter, suggest that these IL-10 polymorphisms contribute to susceptibility of RA in European, Asian, and Black populations [48]. Recently, an IL-10 polymorphism, also located in the genetic region upstream of IL-10, was validated as a candidate gene in aggressive periodontitis in European patients [49].

Animal experiments have shown that there is an important role for PPAD in protein citrullination, ACPA formation and development and aggravation of experimental arthritis [45, 46]. There are, however, no differences in PPAD gene and endogenous citrullination patterns of *P. gingivalis* isolated from patients with or without RA (chapter 5). Also, oral colonization by *P. gingivalis* is not different in RA patients compared to patients without RA, independent of periodontal status, detection techniques and RA disease duration [6, 30, 31, 33]. At this moment the relevance of PPAD in priming autoimmunity and RA development in humans is not clear.

8. Analogy

Infections have been shown to be highly associated with the onset of systemic lupus erythematosus (SLE), a chronic destructive autoimmune disease characterized by immune dysregulation and hyperproduction of different autoantibodies. Particularly Epstein-Barr virus (EBV), parvovirus B19, retrovirus, and cytomegalovirus (CMV) infections might play a pathogenetic role, however, the etiopathogenesis of SLE is far from being completely elucidated [50]. Viral (e.g., parvovirus B19, EBV and CMV) and microbial (e.g., *Campylobacter*) infections play a role in acute arthritis, but the role of infection in the development of RA needs further prospective controlled studies [51]. Approximately 10–20% of patients with early RA have serological evidence of recent infection, however, no single infectious agent is predominant, which indicates that total infectious exposure can represent a risk factor that could trigger RA [51].

Summary

From an epidemiological point of view, RA patients have a higher incidence of periodontal disease than subjects without RA. In addition, there is a dose–response pattern in the association between the severity of periodontitis and RA disease-activity.
There are indications that periodontitis precedes RA, but there is yet no evidence available to show that *P. gingivalis* plays a direct role in this temporal relationship. The role of the unique characteristic of citrullination by *P. gingivalis* remains unexplained. In animal models however, periodontal pathogens and PPAD play a distinct role in development and aggravation of experimental arthritis.
Although the role of periodontal pathogens in RA remains speculative, a causative role for periodontitis as a chronic inflammatory disease caused by infectious agents in RA seems biologically plausible. Considering the great variety in disease manifestation of both periodontitis and RA, a causal relationship, if existing, may only be present between certain forms of periodontitis and RA.

Future perspectives

More evidence in humans is needed to rate the association between periodontitis and RA in susceptible individuals. It has to be mentioned that when systemic effects are subtle, determining cause-and-effect mechanisms is complicated. Therefore, it is worthwhile to assess the influence of periodontitis on arthritis development in prospective follow-up studies with distinct and well described patient groups with a minimum of confounding factors. Given the complex etiology of periodontitis and RA, periodontitis patients and patients at risk for developing RA should be assessed at the microbiological level, based on the presence of oral dysbiotic microbial communities [52], and should be assessed for genetic factors that may predispose to or protect from disease.

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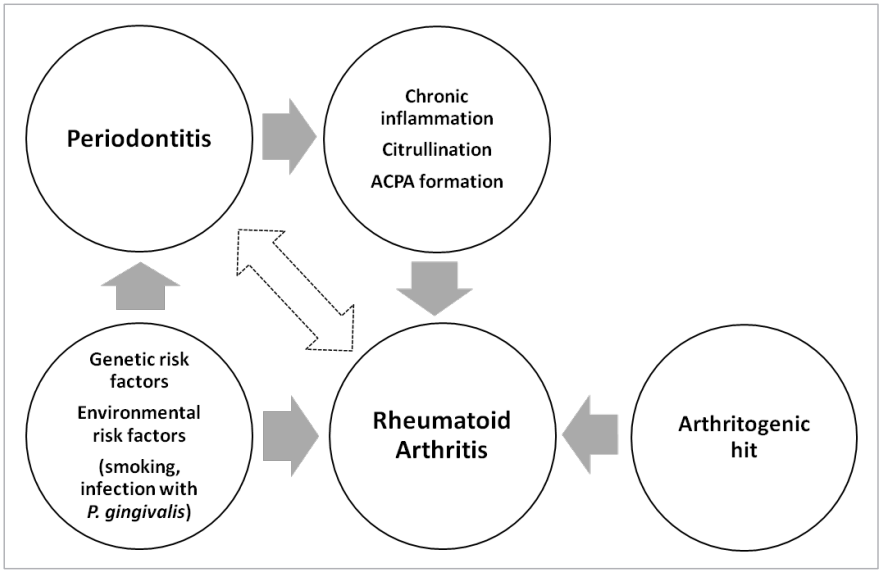
Figures

Fig. 1 Hypothetical two hit model proposed for contribution of periodontitis to RA.

Periodontitis and RA share certain genetic and environmental risk factors including smoking and infection with P. gingivalis. The named risk factors contribute to a susceptibility background against which periodontitis and/or RA can develop. In this 'two hit' model, the 'first hit' is induction of anti-citrullinated protein antibodies (ACPA), possibly in the inflamed periodontium, and the 'second hit' is induction of arthritis and expression of citrullinated antigens in the inflamed joint [7]. Another possible sequence regarding the 'two-hit' model is that of mutual exacerbation: periodontitis as a chronic inflammation serves as 'first hit', while an (unknown) arthritogenic hit ('second hit') induces RA, which leads, in susceptible individuals, to mutual exacerbation of inflammatory responses mediating self-perpetuating tissue destruction (including bone loss) in both joints and periodontium [8] (white arrow).



Summary

Periodontitis is a chronic inflammatory disease that leads to destruction of the soft and hard tissues supporting the teeth (the periodontium) and, if left untreated, may ultimately result in loss of teeth. Periodontitis has also been shown to affect systemic health. Prolonged inflammatory response as a result of infection, like periodontitis, may play a role in the initiation, progression, and perpetuation of chronic autoimmune disease like rheumatoid arthritis (RA).
In the past decade the interest in the epidemiological and pathological relationships between periodontitis and RA has been rising, driven in part by interest in the role of citrullination and attendant autoantibody responses as a disease-defining feature of RA, and the recognition that oral bacteria and inflammation may play important roles.

Chapter 1 discusses possible interactions, particularly related to the periodontal pathogen *Porphyromonas gingivalis*, which could explain the observed association between these two prevalent diseases. RA and periodontitis are both chronic inflammatory disorders characterized by deregulation of the host inflammatory response and shared risk factors. Increased secretion of pro-inflammatory mediators results in soft and hard tissue destruction of the synovium and periodontium respectively.
Systemic inflammatory and infectious challenges have long been considered to be involved in triggering rheumatoid factor (RF), i.e., autoantibodies to the constant domain of IgG. RF was the first important biomarker for diagnosis and prediction of RA. Later, another auto-antibody system, anti-citrullined protein antibodies (ACPA), was found to be more specific for RA. ACPA and RF act as diagnostic markers for RA as they can be detected in serum before clinical signs and symptoms of the disease are apparent and their serum levels strongly correlate with disease severity. Why ACPA and RF are induced as well as their role in RA development is still unclear.
The breakdown of immune tolerance to citrullinated proteins requires susceptible individuals, such as carriers of HLA-DRB1 shared epitope alleles that bind selectively to citrullinated sequences and may influence antigen presentation in ways that lead to ACPA production. *P. gingivalis*, a key periodontal pathogen, is the only known prokaryote that expresses a peptidyl arginine deiminase (PAD) enzyme necessary for protein citrullination. This unique citrullination by *P. gingivalis* of bacterial and host proteins could generate neoepitopes to which immune tolerance does not exist, and consequently lead to the generation of anti-citrullinated autoantibodies.

Despite the suggested role of *P. gingivalis* in the disease association between RA and periodontitis, colonization of *P. gingivalis* in the oral cavity of RA patients has been scarcely considered. To explore whether the association between periodontitis and RA is dependent on *P. gingivalis*, we compared in **Chapter 2** subgingival colonization by *P. gingivalis* and serum antibodies responsive to *P. gingivalis* in 95 established RA patients with a control group of non-RA subjects matched for age, gender, number of teeth, body mass index, and smoking and periodontal status. Furthermore, we compared the prevalence of periodontitis in this RA population with a non-RA population of the same geographic area. A higher prevalence of severe periodontitis was observed in RA patients compared to non-RA controls (27% versus 12%, $p < 0.001$). Moreover, severity of periodontitis was related to severity of RA. Furthermore, RA patients with severe periodontitis had a more robust antibody response against *P. gingivalis* than non-RA controls, although subgingival occurrence of *P. gingivalis* was not different. In RA patients with severe

periodontitis, there were no correlations between anti-*P. gingivalis* titers and IgM RF and ACPA. The strong correlation between ACPA levels in serum and ACPA levels in the inflammatory exudate of the periodontium (gingivocrevicular fluid, GCF) of RA patients ($n = 45$, $\rho = 0.89$, $p < 0.0001$) is suggestive of diffusion of ACPA from plasma to GCF. Within the limitations of the method used in this study, we found no indication for local ACPA production in the periodontium. In this study we confirmed the epidemiological association between periodontitis and RA, however, it was not related to subgingival presence of *P. gingivalis*. Furthermore, we found a dose response relation between the severity of periodontitis and disease activity of RA.

In **Chapter 3** we investigated whether infection with *P. gingivalis* is prognostic for RA, by measuring the antibody response against *P. gingivalis* in baseline serum samples of patients participating in a prospective follow-up study on RA development. The cohort comprised 289 adult arthralgia patients seropositive for IgM RF and/or ACPA. The occurrence of arthralgia in people with these autoantibodies probably represents a late stage in the preclinical development of RA. Influence of anti-*P. gingivalis* positivity on RA development was analyzed using a multivariate Cox proportional hazards model with time until RA development as dependent variable and age, gender, HLA-DRB1 SE carriage, smoking, number of tender joints, and CRP-, ACPA- and IgM RF-positivity at inclusion as independent variables. Within the follow-up (median 30 months), 33% (n = 94) of the seropositive arthralgia patients (SAP) had developed RA according to 2010 American College of Rheumatology/European League against Rheumatism criteria. SAP who developed RA did not have elevated anti-*P. gingivalis* antibody levels at baseline compared with SAP who did not develop RA within the follow-up period. When using cutoff values for anti-*P. gingivalis* positivity, the proportion of IgA and IgG anti-*P. gingivalis*-positive patients was even higher in SAP who did not develop RA. A weak correlation of IgM anti-*P. gingivalis* with ACPA was found in SAP who developed RA ($p < 0.05$, $\rho = 0.23$). Multivariate analysis showed no influence of anti-*P. gingivalis* antibody levels, CRP levels, age, gender and smoking on RA development. Within the limitations of this study, we concluded that anti-*P. gingivalis* antibody levels are not prognostic for development of RA. Specificity and temporal relation regarding infection with *P. gingivalis* in RA development could not be established.

Temporal relation between the two diseases was further assessed in **Chapter 4**. In this chapter we investigated whether periodontitis provokes development of RA associated autoantibodies (RA-AAB). Next to inflammation of oral mucosal sites (e.g., periodontitis), it has been hypothesized that initiation of RA associated autoantibody formation can occur at inflamed mucosal surfaces of the lung. The aim of this study was to assess systemic presence of RA-AAB in patients without RA with oral or lung mucosal inflammation.
Presence of RA-AAB (IgA and IgG anti-cyclic citrullinated peptide 2 antibodies (anti-CCP), IgM and IgA RF, IgG anti-carbamylated protein antibodies (anti-CarP) and IgG and IgA anti-citrullinated peptide antibodies against fibrinogen, vimentin and α-enolase were determined in serum of non-RA patients with periodontitis (PD, n = 114), bronchiectasis (BR, n = 80) or cystic fibrosis (CF, n = 41) and periodontally healthy controls (HC, n = 36). Established RA patients (n = 86) served as a reference group. Cutoff for seropositivity was >2 SD above the mean of HC for anti-CCP and the diagnostic cut off was used for RF. Association of the diseases with RA-AAB seropositivity was assessed with a logistic regression model, adjusted for age, sex and smoking. Statistical analysis revealed that IgG anti-CCP seropositivity was associated with BR, whereas the association with PD was borderline significant ($p = 0.05$). IgA anti-CCP- and IgA RF seropositivity were associated with

CF. The association of IgM RF seropositivity with BR was borderline significant (p = 0.05). Apart from influence of smoking on IgA RF in RA patients, there was no influence of age, sex and smoking on the association of RA-AAB seropositivity with the diseases. Anti-CarP levels were only increased in RA patients. The same held true for IgG reactivity against all investigated citrullinated peptides. It was concluded that, although overall levels were low, RA-AAB seropositivity is associated lung mucosal inflammation (BR and CF) and may be associated with periodontitis. To further determine whether mucosal inflammation functions as a site for induction of RA-AAB and precedes RA, longitudinal studies are necessary in which RA-AAB of specifically the IgA isotype should be assessed in inflamed mucosal tissues and/or in their inflammatory exudates.

Whether there is a difference in the nature of the citrullinating enzyme of *P. gingivalis* (PPAD) in *P. gingivalis* isolates from RA patients or from non-RA patients was investigated in **Chapter 5**. In this study, expression of the PPAD-encoding gene was assessed in representative samples of *P. gingivalis* clinical isolates from patients with and without RA, as well as in related species of the genus *Porphyromonas*. Variation in the composition of the PPAD gene was assessed by whole genome sequencing and by polymerase chain reaction (PCR) using a combination of primer sets for the whole gene and for a region including the active site. In addition, restriction enzyme analysis of the PCR products, using three different restriction enzymes, was carried out. Functional analysis of PPAD was studied by assessment of endogenous citrullination patterns. We found that PPAD is omnipresent in *P. gingivalis*, but absent in related Porphyromonas species. Regarding PPAD, no dominant genetic variations or differences in endogenous citrullinated protein patterns were observed for *P. gingivalis* isolates from RA patients compared to *P. gingivalis* isolates from non-RA patients. From this study it can be concluded that if *P. gingivalis* plays a role in RA, it is unlikely to originate from a variation in PPAD gene expression.
Because causality is ultimately tested in longitudinal cohort studies which do currently not exist for periodontitis and RA, in **Chapter 6**, causality as most likely interpretation for the association between periodontitis and RA was assessed by applying the Bradford Hill criteria on existing literature, including our own investigations.
From an epidemiological point of view, RA patients have a significantly higher incidence of periodontal disease than subjects without RA. In addition, there is a clear dose–response pattern in the association between the severity of periodontitis and RA disease-activity. There are indications that periodontitis precedes RA, but there is yet no evidence available to show that *P. gingivalis* plays a direct role in this temporal relationship. The role of the unique characteristic of protein citrullination by *P. gingivalis* remains unexplained. In animal models however, the citrullinating enzyme of *P. gingivalis* play a distinct role in development and aggravation of experimental arthritis. Although the role of periodontal pathogens in RA remains speculative, a causative role for periodontitis as a chronic inflammatory disease caused by infectious agents in RA seems biologically plausible. Considering the great variety in disease manifestation of both periodontitis and RA, a causal relationship, if existing, may only be present between certain forms of periodontitis and RA.

At this moment, it can be concluded that there is strong experimental evidence for a role of *P. gingivalis* in the development of arthritis in animal models. This has not yet been shown in humans, although the majority of the Bradford Hill criteria for causation can be fulfilled in the disease association between periodontitis and rheumatoid arthritis.

Nederlandse samenvatting

Parodontitis en reumatoïde artritis; een zoektocht naar causualiteit en de rol van *Porphyromonas gingivalis*

Parodontitis is een chronische ontstekingsziekte met beschadiging van de zachte en harde weefsels die het steunweefsel van gebitselementen vormen (het parodontium) als gevolg. Als parodontitis niet behandeld wordt, kan het uiteindelijk leiden tot verlies van gebitselementen.
Parodontitis heeft ook invloed op de algemene gezondheid. Parodontitis, als voorbeeld van een chronische onstekingsreactie veroorzaakt door een infectie, zou een rol kunnen spelen in de inductie, progressie en het in stand houden van chronische auto-immuunziekten zoals reumatoïde artritis (RA).
In het laatste decennium is er een groeiende interesse ontstaan voor epidemiologische en pathologische relaties tussen parodontitis en RA. Deze interesse is mede gewekt door de posttranslationele eiwit-modificatie citrullinatie en de daaropvolgende productie van auto-antistoffen, die kenmerkend zijn voor RA. Infectie en bepaalde micro-organismen zouden hierin een belangrijke rol kunnen spelen.

In **hoofdstuk 1** worden mogelijke interacties besproken die de associatie tussen deze veel voorkomende ziekten zouden kunnen verklaren. Daarbij wordt vooral ingegaan op de mogelijke rol van de parodontale pathogeen *Porphyromonas gingivalis*.
RA and parodontitis zijn beide chronische ontstekingsziekten die worden gekenmerkt door een verstoorde immuunrespons. Verhoogde secretie van pro-inflammatoire ontstekingsmediatoren resulteren in beschadiging van de zachte en harde weefels van het synovium en het parodontium. Ook delen beide aandoening risicofactoren, zoals genetische predispositie en roken.
Sinds lange tijd wordt verondersteld dat systemische ontsteking en infectie een rol spelen in het uitlokken van productie van de reumafactor (RF). Met de reumafactor worden auto-antistoffen tegen het constante gedeelte van IgG bedoeld. RF was de eerste belangrijke biomarker voor de diagnose en het voorspellen van het verloop van RA. Later vond men een ander, meer specifiek auto-antistof systeem voor RA; antistoffen tegen gecitrullineerde eiwitten (ACPA). Citrullinatie is een postranslationele modificatie die veranderingen in eiwitconformatie kan veroorzaken. ACPA en RF zijn diagnostische markers voor RA; ze kunnen al in de pre-klinische fase van de ziekte aanwezig zijn en ze zijn sterk gecorreleerd met ziekte-activiteit. Waarom ze worden geproduceerd en wat hun precieze rol is in de ontwikkeling van RA is niet duidelijk.
In een vatbaar persoon kan tolerantie tegen eigen gecitrullineerde eiwitten verloren gaan. Vatbare personen zijn bijvoorbeeld dragers van de HLA-DRB1 allelen die een gedeeld epitoop (SE) coderen, wat zou bijdragen aan antigeenpresentatie van gecitrullineerde eiwitten en leiden tot ACPA productie. *P. gingivalis*, een parodontale pathogeen die geassocieerd is met ernstige vormen van parodontitis, is de enige bacterie waarvan bekend is dat het een peptidylarginine deiminase (PAD) enzym bezit, een enzym dat nodig is voor eiwitcitrullinatie. Doordat eiwitcitrullinatie uniek is voor *P. gingivalis* en doordat citrullinatie door *P. gingivalis* PAD enigszins verschilt van citrullinatie door humaan PAD, zou citrullinatie van bacteriële of humane eiwitten door *P. gingivalis* nieuwe epitopen kunnen genereren waartegen geen tolerantie bestaat en zo kan leiden tot de productie van ACPA.

P. gingivalis wordt dus verondersteld een belangrijke rol te spelen in de associatie tussen parodontitis en RA. Er is echter opvallend weinig onderzoek gedaan naar de orale kolonisatie van *P. gingvalis* in RA patiënten. Om

uit te zoeken of de associatie tussen parodontitis en RA afhankelijk is van *P. gingivalis*, onderzochten we in **hoofdstuk 2** subgingivale kolonisatie met *P. gingivalis* en de antistoftiter tegen *P. gingivalis* in het serum van 95 patiënten met bestaande RA. We vergeleken dit met een controle groep van personen zonder RA (non-RA) die vergelijkbaar was wat betreft leeftijd, geslacht, aantal gebitselementen, body mass index (BMI), rookgedrag en parodontale status. Verder vergeleken we de prevalentie van parodontitis in deze RA patiënten met een controlepopulatie van 420 personen uit dezelfde geografische regio, die vergelijkbaar was wat betreft leeftijd, geslacht en sociaal economische status. We vonden een hogere prevalentie van ernstige parodontitis in RA patiënten vergeleken met de non-RA controles (27% versus 12%, $p < 0.001$). Bovendien vonden we dat de mate van parodontitis gerelateerd was aan de mate van RA ziekte-activiteit. Er was geen verschil in IgM RF of ACPA titers tussen de RA patiënten met verschillende mate van parodontitis. Wel hadden RA patiënten met ernstige parodontitis een meer uitgesproken antistofrespons tegen *P. gingivalis* dan de non-RA controles met ernstige parodontitis. Het subgingivaal voorkomen van *P. gingivalis* was niet verschillend in beide groepen. In RA patiënten detecteerden we ACPA in de gingivocreviculaire vloeistof (GCF), een ontstekingsexcudaat van het parodontium. ACPA titers in serum en GCF van deze patiënten vertoonden een sterke correlatie ($\rho = 0.89$, $p < 0.0001$), suggestief voor diffusie van ACPA vanuit het plasma. Deze studie gaf ons geen aanwijzingen voor lokale ACPA productie in het parodontium.

In **hoofdstuk 3** onderzochten we of infectie met *P. gingivalis* prognostisch is voor het ontwikkelen van RA, door *P. gingivalis* antistoftiters te meten in serum van patiënten met een verhoogd risico op het ontwikkelen van RA die hier prospectief voor gevolgd werden (n = 289). Antistoftiters werden gemeten in baseline serum monsters van patiënten die seropositief waren voor IgM RF of ACPA en gewrichtsklachten hadden (seropositieve arthralgia patiënten, SAP). De aanwezigheid van gewrichtsklachten in deze patiënten representeert waarschijnlijk een late fase van het pre-klinische stadium van RA. Invloed van anti-P.gingivalis seropositiviteit op het ontwikkelen van RA werd geanalyseerd met behulp van een multivariaat Cox regressie model met de duur tot de diagnose RA als afhankelijke variabele, en leeftijd, geslacht, aanwezigheid van HLA-DRB1-SE, roken, aantal pijnlijke gewrichten, en CRP, ACPA en IgM RF positiviteit ten tijde van de inclusie als andere variabelen. Tijdens de follow-up periode (mediaan 30 maanden) ontwikkelde 33% (n = 94) van de SAP RA volgens de American College of Rheumatology/European League against Rheumatism criteria van 2010. SAP die RA ontwikkelden hadden geen hogere anti-*P. gingivalis* titers op baseline vergeleken met SAP die geen RA ontwikkelden gedurende de follow-up periode. Bij gebruik van (arbitraire) waarden voor anti-*P. gingivalis* seropositiviteit was het aandeel IgA and IgG anti-*P. gingivalis*-positieve patienten zelfs hoger in SAP die geen RA ontwikkelden. In SAP die RA ontwikkelden vonden we een zwakke correlatie van IgM anti-*P. gingivalis* met ACPA ($p < 0.05$, $\rho = 0.23$). Multivariate analyse liet geen invloed zien van anti-*P. gingivalis*, CRP, leeftijd geslacht en roken op het ontwikkelen van RA. We concludeerden uit deze studie dat anti-*P. gingivalis* titers niet prognostisch zijn voor het ontwikkelen van RA.

Of parodontitis voorafgaat aan het ontwikkelen van RA werd verder onderzocht in **hoofdstuk 4**. We onderzochten of parodontitis aanzet tot productie van auto-antistoffen die geassocieerd zijn met RA (RA geassocieerde auto-antistoffen, RA-AAB). Behalve ontsteking van de orale mucosa (zoals parodontitis) is ook verondersteld dat ontsteking van long mucosa zou kunnen aanzetten tot de productie van RA-AAB. Doel van deze studie was te onderzoeken RA-AAB aanwezig zijn in serum van patiënten zonder RA, maar met ontste-

king van orale- of long mucosa.
Aanwezigheid van RA-AAB (IgA en IgG anti-cyclisch gecitrullineerd peptide-2 (anti-CCP), IgM en IgA RF, IgG anti-gecarbamyleerd eiwit (anti-CarP) en IgG en IgA anti-gecitrullineerde antistoffen tegen peptides van fibrinogeen, vimentine en α-enolase werd bepaald in serum van patiënten met parodontitis (PD, n = 114), bronchiectasis (BR, n = 80) of cystic fibrosis (CF, n = 41), allen zonder RA. Parodontaal gezonde controle personen (HC, n = 36) en patiënten met bestaande RA (n = 86) fungeerden als referentiegroepen. Voor RF werden diagnostische waarden voor seropositiviteit aangehouden. Seropositiviteit voor de andere RA-AAB werd gedefinieerd als meer dan 2 standaard deviaties boven de gemiddelde waarde van de HC. Associatie van mucosale ontsteking met RA-AAB seropositiviteit werd bepaald met behulp van een logistisch regressiemodel gecorrigeerd voor leeftijd, geslacht en roken.
Statistische analyse liet zien dat IgG anti-CCP was geassocieerd met BR, en bijna significant met PD (p = 0.05). IgA anti-CCP- en IgA RF waren geassocieerd met CF. IgM RF was bijna significant geassocieerd met BR (p = 0.05). Leeftijd, geslacht en roken waren niet van invloed op de associatie van RA-AAB met de mucosale ontstekingsziekten. Seropositiviteit voor anti-CarP, evenals seropositiviteit voor alle onderzochte gecitrullineerde peptides, was alleen aanwezig in RA patiënten. We concludeerden dat seropositiviteit voor RA-AAB geassocieerd is met ontsteking van long mucosa (BR en CF) en mogelijk met ontsteking van orale mucosa (PD), alhoewel de titers over het algemeen laag waren. Longitudinaal onderzoek is nodig om te bepalen of mucosale ontsteking RA-AAB productie induceert en voorafgaat aan de ontwikkeling van RA. Daarin zou lokale aanwezigheid van RA-AAB van met name het IgA isotype moeten worden onderzocht in het ontstoken mucosale weefsel of in het ontstekingsexcudaat.

In **hoofdstuk 5** werd expressie van het PPAD gen geanalyseerd in *P. gingivalis* isolaten uit patiënten met RA (n = 12) of zonder RA (n = 80). Functionele analyse van PPAD bestond uit het beoordelen van citrullinatiepatronen van endogene eiwitten. Daarnaast werd aanwezigheid van het PPAD gen en endogene eiwitcitrullinatie patronen onderzocht in twee aan *P. gingivalis* verwante species; *Porphyromonas endodontalis* en *Porphyromonas asaccharolytica*. Variatie in gencompositie werd onderzocht door middel van sequentieanalyse en door polymerase ketting reactie (PCR) met verschillende primer sets; een voor het gehele PPAD gen en een voor het actieve gedeelte van het PPAD gen. Verder werden restrictie patronen van de PCR producten onderzocht die verkregen werden na digestie met drie verschillen restrictie-enzymen.
PPAD was aanwezig in alle isolaten van *P. gingivalis*, maar afwezig in de verwante *Porphyromonas* species. Er werden geen dominante genetische variaties in PPAD of verschillen in gecitrullineerde endogene eiwitpatronen gevonden tussen *P. gingivalis* isolaten uit RA patiënten en isolaten uit patiënten zonder RA. Uit deze studie concludeerden we dat als *P. gingivalis* een rol spelt in RA dit waarschijnlijk niet komt door een variatie in het PPAD gen.

Causualteit wordt bij uitstek getest in longitudinale prospectieve cohort studies die op dit moment niet bestaan voor parodontitis en RA. Daarom werd in **hoofdstuk 6** de thans bestaande literatuur, inclusief ons eigen onderzoek, getoetst voor causualiteit als meest voor de hand liggende verklaring voor de associatie tussen parodontitis en RA aan de hand van de Bradford Hill criteria.
Vanuit epidemiologisch oogpunt komt parodontitis vaker voor in RA patiënten dan in patiënten zonder RA. De relatie tussen parodontitis en RA is dosisafhankelijk, dat wil zeggen, de ernst van parodontits hangt samen met de ziekteactiviteit van RA. Er zijn aanwijzingen dat parodontitis voorafgaat aan de ontwikkeling van RA, maar het is nog

niet duidelijk of *P. gingivalis* met zijn unieke mogelijkheid om eiwitten te citrullineren hier een rol in speelt. PPAD, het enzym van *P. gingivalis* dat nodig is voor citrullinatie, heeft in dierstudies wel laten zien dat het een rol speelt in het ontwikkelen en het verergeren van experimentele artritis. Een causale rol voor parodontitis als chronische infectieuze ontstekingsziekte in de ontwikkeling van RA lijkt biologisch gezien aannemelijk, hoewel de rol van parodontale pathogenen in mensen nog speculatief is. Aangezien er een grote variatie bestaat in ziektemanifestatie van zowel parodontitis als RA, zou een causaal verband mogelijk alleen aanwezig zijn tussen bepaalde vormen van parodontitis en RA.

Op dit moment concluderen we dat er sterk bewijs is voor een rol van *P. gingivalis* in de ontwikkeling van artritis in diermodellen. Dit is nog niet vastgesteld in mensen. Desalniettemin kan in de associatie tussen parodontitis en RA aan het aan het merendeel van de Bradford Hill criteria voor causualiteit worden voldaan.

Acknowledgements

Because this PhD thesis was by no means a solo achievement, I want to greatly acknowledge everyone for their contribution, and for giving me such great pleasure in working with them over the past years. I will name only few of them:

Thank you Frank, for bringing me into the field of periodontology and giving me the opportunity to start this research project

Thank you to the whole department of Rheumatology and Clinical Immunology of the UMCG, for not distinguishing between 'self' and 'non-self'

Thank you Arie Jan, for being –in every respect- the best professor I could ever wish for

Thank you Arjan, for your continuous accessibility and enormous willingness to help

Thank you Hannie, you are a great friend

Thank you Liesbeth, for being the best doctor a patient could wish for

Thank you Jan Maarten, for the humor that accompanied our 'serious meetings'

Thank you Koen, Giorgio and Tim, working with you will be continued!

Finally Lambert, I know you don't want to be mentioned, but without you, there is only no-thingness

Curriculum vitae

I graduated in dentistry from the University of Groningen in 2004. From 2005 until 2006, I worked as a dentist in three general dental practices. In 2006, I started my training in periodontology at the Catholic University of Leuven, and completed my master's degree in 2009. While I was working and training in Belgium, I became interested in research and was co-author of three peer-reviewed publications. In 2009, I returned to Groningen and started my PhD on the potential role of Porphyromonas gingivalis in the association between periodontitis and rheumatoid arthritis. This turned into a fruitful collaboration between the departments of Rheumatology and Clinical Immunology-, Medical Microbiology-, Oral and Maxillofacial Surgery-, and Dentistry and Oral Hygiene of the University Medical Center Groningen, and the departments of Rheumatology of the VU University Medical Center Amsterdam (Dr. D. van Schaardenburg)-, Leiden University Medical Center (Dr. L.A. Trouw)-, and the Martini Hospital Groningen (Dr. M. Bijl). In addition to this research project, I worked as a periodontist in the Clinic for Periodontology Groningen until 2013. Since 2014, I work together with Heleen Botterman-Foo in the Clinic for Periodontology Emmen.

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**Equal contribution*

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