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-citrullinated 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
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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, ρ = 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

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, ρ = 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.
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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.