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STUDIES ON THE RELATIONSHIP BETWEEN PERIODONTITIS AND RHEUMATOID ARTHRITIS

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Cover image: A patient with established rheumatoid arthritis holding an extracted tooth. Photographed by Kaja Eriksson (a written consent for publication was obtained from the patient).

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Studies on the relationship between Periodontitis and Rheumatoid Arthritis

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Let the dataset change your mindset

-Professor ***Hans Rosling***
(1948 - 2017)

To my family, with love

Forever and for always

ABSTRACT

Periodontitis and rheumatoid arthritis (RA) are both widespread multifactorial diseases, characterized by chronic inflammation leading to tissue and bone destruction around teeth or joints, respectively. An association between periodontitis and RA has been proposed, suggesting that the periodontal pathogen *Porphyromonas gingivalis* (*P. gingivalis*) may be involved in the generation of anti-citrullinated protein antibodies (ACPAs) in RA. Previous findings indicate that patients with RA may have increased prevalence/incidence and severity of periodontitis; however, more studies are needed to confirm this link, elucidate the potential mechanisms involved, and ascertain the temporal relationship between periodontitis and RA. Hence, the general aim of this thesis was to study the relationship between periodontitis and RA using population-based, clinical, immunohistochemical and experimental animal studies.

Study I investigated the prevalence of periodontitis with special focus on ACPA status in the Swedish Epidemiological Investigation of RA (EIRA), a well-characterized population-based RA case-control cohort. Linking the EIRA registry with the Dental Health Registry (DHR) demonstrated no differences in the prevalence of periodontal diseases (gingivitis, periodontitis, or peri-implantitis) between RA patients and matched healthy controls. Additionally, among subjects with RA, we detected no differences in prevalence of periodontitis based on ACPA or rheumatoid factor (RF) antibody status.

Study II explored the effect of pre-existing periodontitis on the development and the immune/inflammatory response of experimental arthritis in an animal model. Eight weeks prior to induction of pristane-induced arthritis, we induced experimental periodontitis in arthritis-susceptible Dark Agouti rats using ligatures in combination with swabs containing periodontal bacteria. After monitoring the progression and severity of both periodontitis and arthritis for another 7 weeks, we compared the results to animals with experimental arthritis only and to healthy animals without arthritis or periodontitis. We detected increased levels of antibodies against citrullinated *P. gingivalis* peptidylarginine deiminase (PPAD) proteins in rats with both pre-existing periodontitis and pristane-induced arthritis compared to arthritic animals without pre-existing periodontitis. However, the pre-existence of periodontitis did not affect the development or severity of pristane-induced arthritis in rats.

Study III examined the presence of citrullinated proteins and the expression of human peptidylarginine deiminase (PAD2 and PAD4) enzymes in relation to the presence of *P. gingivalis* in gingival tissue biopsies from patients with periodontitis and healthy controls. In gingival connective tissue, we detected citrullinated proteins in 80% of the subjects with periodontitis compared to in 25% of healthy controls. Analysis of the sections also showed increased expression of PAD2 and PAD4 in the gingival connective tissue of patients with periodontitis. We found no differences in the presence of *P. gingivalis* in the epithelium or connective tissue of gingival biopsies from patients with periodontitis and healthy controls without periodontitis. Moreover, we observed no correlations between the presence of *P. gingivalis* and citrullinated proteins or citrullinating enzymes in gingival tissue from patients with periodontitis and healthy controls.

Study IV was a clinical study that further explored the association between RA and the severity of periodontitis. This pilot study aimed to investigate the severity of periodontitis in relation to ACPA/RF status, immunological parameters associated with RA, and the presence of subgingival *P. gingivalis* DNA in patients with established RA. The findings showed that the majority of patients with RA had moderate/severe periodontitis (75%), while the others had no/mild periodontal disease. ACPA positivity was significantly more common in RA subjects with moderate/severe periodontitis compared to those with no/mild periodontal disease. We detected no differences in the presence of *P. gingivalis* in subgingival plaque samples based on periodontitis severity or ACPA status. Nor did we detect any between-group differences in RA disease duration or disease activity or type of RA treatment/medication. Cytokine profiling of serum, saliva, and gingival crevicular fluid samples showed that the levels of several inflammatory mediators were up-regulated in patients with moderate/severe periodontitis. Interestingly, the levels of a proliferation-inducing ligand (APRIL), implicated in B-cell survival and maturation, were significantly higher in both serum and saliva samples of patients with moderate/severe periodontitis compared to those with no/mild periodontal disease.

In conclusion, we found no evidence of an increased prevalence of periodontitis in patients with established RA relative to healthy controls, in the large population-based registry study. However, the clinical study showed that the more severe forms of periodontitis, defined as moderate/severe periodontitis, were frequent in subjects with established RA, and associated with ACPA positivity. Our findings also demonstrate that the presence of citrullinated proteins and expression of human citrullinating PAD enzymes are increased in gingival tissue of non-RA subjects with chronic periodontitis, and that the pre-existence of periodontitis can induce a systemic antibody response against citrullinated proteins derived from *P. gingivalis* PAD enzyme in animals with experimental arthritis. Taken together, the results indicate that periodontal infection could possibly contribute to the autoimmunity against citrullinated proteins associated with RA. Since ACPA status may be associated with periodontitis severity, future studies on the association between periodontitis and RA should focus on investigating the potential beneficial effects of periodontal treatment in patients with RA. Moreover, additional research into predisposing risk factors and molecular mechanisms connecting periodontitis with RA are also warranted.

LIST OF SCIENTIFIC PAPERS

I. **Eriksson K**, Nise L, Kats A, Luttropp E, Catrina AI, Askling J, Jansson L, Alfredsson L, Klareskog L, Lundberg K, Yucel-Lindberg T.
Prevalence of Periodontitis in Patients with Established Rheumatoid Arthritis: A Swedish Population Based Case-Control Study.
PLoS One. 2016;11:e0155956.

II. **Eriksson K***, Lönnblom E*, Tour G, Kats A, Mydel P, Georgsson P, Hultgren C, Kharlamova N, Norin U, Jönsson J, Lundmark A, Hellvard A, Lundberg K, Jansson L, Holmdahl R, Yucel-Lindberg T.
Effects by periodontitis on pristane-induced arthritis in rats.
J Transl Med. 2016;14:311.

III. Engström M, **Eriksson K**, Lee LK, Hermansson M, Nicholas AP, Lundberg K, Klareskog L, Catrina AI, Yucel-Lindberg T.
Increased levels of citrullinated proteins and expression of peptidylarginine deiminases in gingival tissue of periodontitis patients.
Manuscript

IV. **Eriksson K**, Fei G, Lundmark A, Benchimol D, Lee LK, Kats A, Saevarsdottir S, Catrina AI, Klareskog L, Lundberg K, Jansson L, Yucel-Lindberg T.
Periodontal health in patients with rheumatoid arthritis: a cross-sectional pilot study.
Manuscript

*These authors contributed equally to the work

CONTENTS

LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
Anti-CCP	Anti-cyclic citrullinated peptide
APRIL	A proliferation-inducing ligand
BMI	Body mass index
BOP	Bleeding on probing
CAIA	Collagen antibody-induced arthritis
CAL	Clinical attachment level
CEJ	Cementoenamel junction
CEP-1	Citrullinated α-enolase peptide 1
CIA	Collagen-induced arthritis
CPP3	Citrullinated PPAD peptide
CRP	C-reactive protein
DA	Dark Agouti
DAS28	Disease Activity Score including 28-joint count
DHR	Dental Health Registry
DMARD	Disease-modifying antirheumatic drug
EIRA	Epidemiological Investigation of Rheumatoid Arthritis
F95	Monoclonal anti-citrulline antibody
FAB	Fastidious anaerobe broth
F. nucleatum	*Fusobacterium nucleatum*
GCF	Gingival crevicular fluid
gDNA	genomic DNA
HAQ	Health assessment questionnaire
HLA-DRB1 SE	HLA-DRB1 shared epitope
IL	Interleukin
MMP	Matrix metalloproteinase

NSAID	Non-steroidal anti-inflammatory drug
PAD	Peptidyl arginine deiminase
P. gingivalis	*Porphyromonas gingivalis*
PIA	Pristane-induced arthritis
PPAD	*P. gingivalis* peptidyl arginine deiminase
PPD	Probing pocket depth
PPV	Positive predictive value
qPCR	quantitative Polymerase Chain Reaction
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RPP3	Uncitrullinated PPAD peptide
T. denticola	*Treponema denticola*
T. forsythia	*Tannerella forsythia*
TNF-α	Tumor necrosis factor alpha
VAS	Visual analog scale
α-1-AGP	alpha-1-acid glycoprotein

INTRODUCTION

Periodontitis and rheumatoid arthritis (RA) are both widespread chronic inflammatory diseases that destroy bones and supporting structures around teeth or joints, respectively, and can lead to permanent disability. These two inflammatory conditions share etiological and pathogenic features, such as similar cytokine profiles and smoking as a major risk factor. Previous studies have proposed an association between RA and periodontitis, although the strength and temporality of this association is still unclear. The following summary includes a brief background of periodontal disease and RA (including a short introduction to the anatomy of the periodontium and the joint), similarities between periodontitis and RA, the potential bacterial link, as well as an overview of previous studies conducted on the relationship between these two diseases.

THE PERIODONTIUM

The periodontium refers to the tissues supporting the teeth, and is also known as the tooth's "attachment apparatus" [1]. It involves the gingiva, the periodontal ligament, the root cementum as well as the alveolar bone, described in detail below (Figure 1) [1]. A periodontitis affected periodontium is characterized by an accumulation of inflammatory cells in the tissues adjacent to the tooth, a breakdown of the collagen fibers connecting the root cementum to the connective tissue and bone, a subsequent pocket formation and apical migration of the junctional epithelium, as well as resorption of the marginal alveolar bone [2].

Gingiva

The gingiva is the part of the oral mucosa that surrounds the alveolar process and the tooth's cervical parts, and can be divided into free or attached gingiva (Figure 1) [1]. Microscopically the free and attached gingiva consists of an outer layer of epithelium (oral epithelium, sulcular epithelium or junctional epithelium) and an inner layer of connective tissue (Figure 1) [1].

Gingival epithelium

The gingival epithelium located nearest to the oral cavity is called the oral epithelium (depicted as OE in Figure 1), and is mostly (90%) comprised of *keratin-producing cells*. Other cell types found in the oral epithelium are *melanocytes* (pigment-synthesizing cells), *Langerhans cells* (involved in an early immunological response to the antigens/pathogens trying to penetrate the epithelium), *Merkel´s cells* (believed to have sensory functions) and *inflammatory cells* [1]. The sulcular epithelium (depicted as SE in Figure 1), located at the groove between the top of the gingiva and the enamel, is an extension of the oral epithelium and is not in contact with the tooth's surface. Unlike the oral epithelium, this sulcular epithelium is non-keratinized and mostly consists of cube-like cells [1]. The junctional epithelium (depicted as JE in Figure 1) is the contact point between the tooth and the gingiva [1]. It mainly consists of a basal/suprabasal cell layer, but also contains *hemidesmosomes*

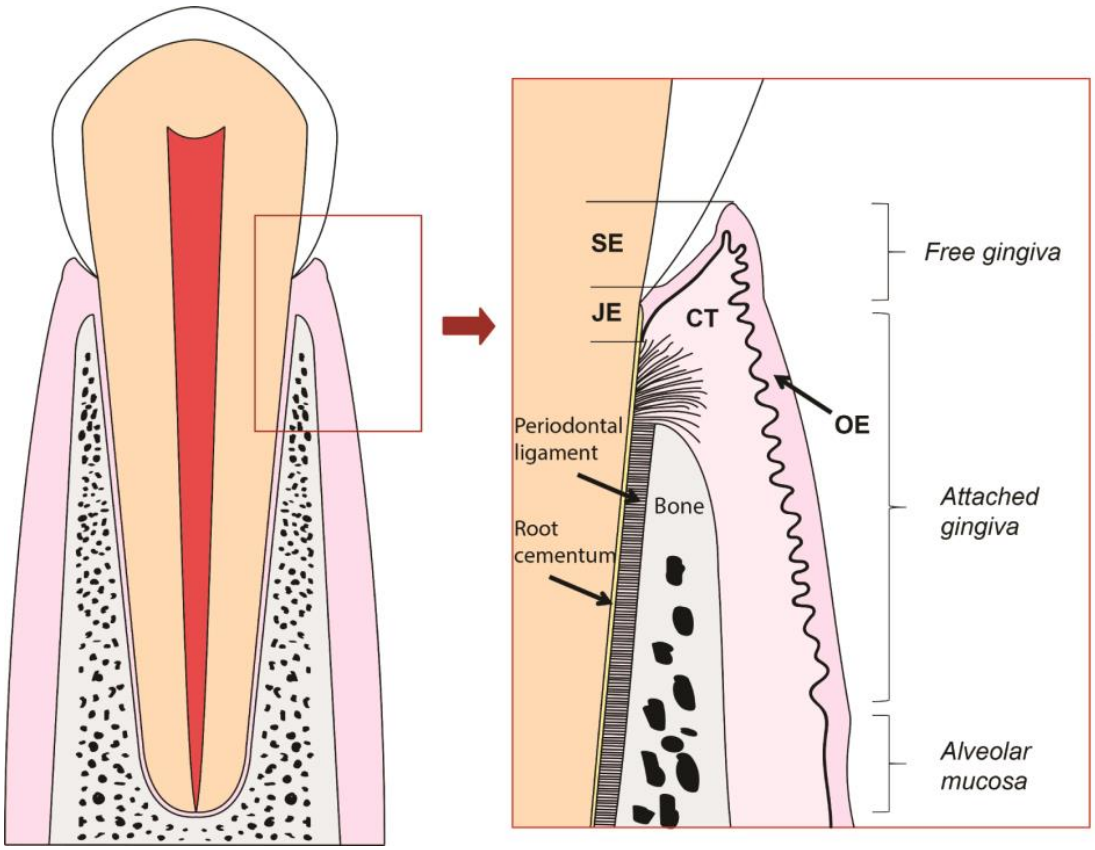

Figure 1.
The tissues supporting the teeth include the gingiva, the periodontal ligament, the root cementum and alveolar bone. In periodontally healthy individuals, the free gingiva covers the tooth enamel and is normally coral pink. The attached gingiva firmly covers the alveolar bone and the root cementum via connective tissue fibers. It is also coral pink in healthy individuals, and extends from the cementoenamel junction in an apical direction towards the loosely bound alveolar mucosa. Microscopically the gingiva consists of an outer epithelial layer and an inner layer of connective tissue.
OE, oral epithelium; SE, sulcular epithelium; JE, junctional epithelium; CT, connective tissue.
Illustration by Igor Kraszewski and Kaja Eriksson.

physically attaching the epithelium to the tooth. Moreover, *white blood cells* can also present in the wide intercellular space of this epithelium, as they travel towards the gingival sulcus [1]. In periodontitis the junctional epithelium proliferates downwards along the surface of the root, extending finger-like projection of the pocket epithelium (which is not attached to the root surface) into the connective tissue [2].

Gingival connective tissue

The connective gingival tissue (depicted as CT in Figure 1) represents the largest part of the gingiva [1]. It predominantly consists of collagen fibers (60%), followed by blood vessels and nerves (35%) as well as fibroblasts (5%). Four types of cells are commonly found in the connective gingival tissue: *fibroblasts* which are involved in the production of collagen fibers and synthesis of the tissue matrix, *mast cells* that create some parts of the tissue matrix and produce vasoactive substances affecting blood flow in the tissue, *macrophages* which have both a phagocytic and synthesizing role in the connective tissue as well as *inflammatory cells* (including *lymphocytes*, *neutrophils* and *plasma cells*). Both *macrophages* and *inflammatory*

cells are involved in the hosts immune-inflammatory defense system against periodontal pathogens [3]. *Lymphocytes* (*T*- and *B-cells*) for example can provide an immune defense against these pathogens by producing antibodies and inflammatory mediators that neutralize extracellular infectious agents, and activate *neutrophils* and *macrophages* that can digest the bacteria and produce toxic products neutralizing intracellular infectious agents [2, 4].

Periodontal ligament

The periodontal ligament (Figure 1) is the tissue that connects the tooth to the alveolar bone, through bundles of collagen fibers attaching to the root cementum and the alveolar wall socket [1]. In addition, the periodontal ligament contains cells involved in the formation of cementum and bone, *cementoblasts* and *osteoblasts*, as well as *fibroblasts*, *epithelial cells*, *nerve fibers* and bone resorbing *osteoclasts*. *Blood* and *lymphatic vessels* in the periodontal ligament supply the tissue with nutrients and remove by-products following metabolism [3]. The periodontal ligament is fundamental for tooth mobility including the absorbance and distribution of forces elicited during loading/eating etc [1].

Root cementum

The root surface is cover by the root cementum, a thin mineralized layer attaching the periodontal ligament to the root [1]. It consists of collagen fibers surrounded by an organic matrix, and is a relatively porous tissue. If root cement gets exposed to the oral cavity, toxins from the dental plaque can penetrate the cementum, preventing the periodontal ligament from attaching [3]. During mechanical periodontal treatment, the root cementrum is removed and the exposed root surface may result in sensitivity of the tooth [3]. These symptoms are often reversible as new cementum is constantly being synthesized by *cementoblasts* [3].

Alveolar bone

In periodontally healthy individuals the alveolar bone is normally located approximately 2 – 3 mm apically of the cementoenamel junction (CEJ), following its outline [3]. It consists of an outer layer of compact bone, and a more porous inner layer containing bone marrow [3]. Cells important for bone remodeling (*osteoblasts*, *osteoclasts* and *osteocytes*) are present in the alveolar bone as it is constantly being remodeled to adjust to the mechanical loading and to repair minor damages (e.g. microfractures) [3]. In periodontitis, however, local stimuli from microbial and host-derived factors, including different inflammatory mediators, stimulate the bone resorbing *osteoclast* ultimately leading to the loss of alveolar bone [5, 6].

THE JOINT

A joint is a junction or a connecting point at which two bones come together [7]. The joints main functions include holding the skeleton together and allowing movement. Joints can be classified according to their mobility (immovable/synarthrotic, slightly movable/amphiathrotic or freely movable/diarthrotic) or the type of material they consist of that bind the bones together (fibrous, cartilaginous or synovial) [7]. Most of the joints in the

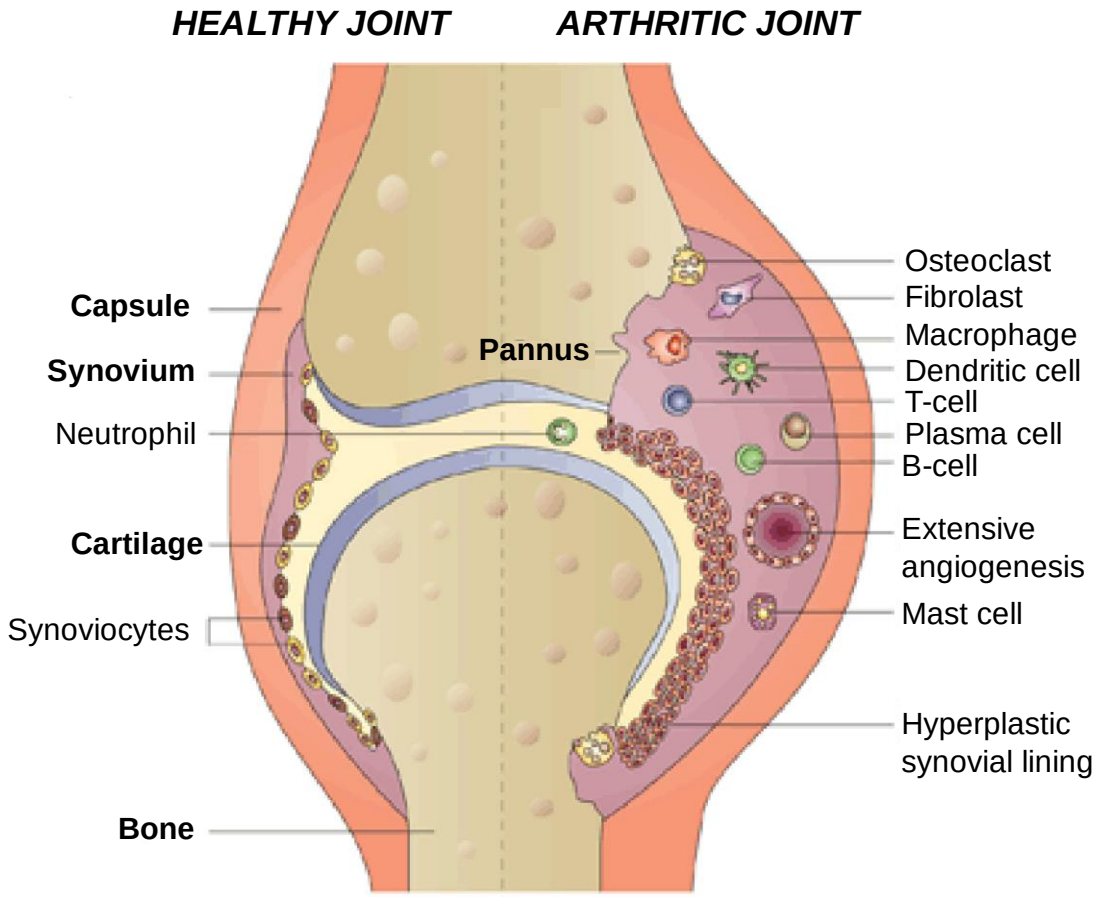

Figure 2.
The synovial joint is composed of two adjacent bones covered with a layer of cartilage, separated by a joint space and surrounded by the synovial membrane and joint capsule. The synovial membrane consists of a thin layer of synoviocytes (macrophage and fibroblast derived). Only a few, if any, mononuclear cells are interspersed in the sublining connective tissue layer, which has considerable vascularity. An arthritic joint, e.g. in RA, is initially characterized by an inflammatory response of the synovial membrane ('synovitis') that is conveyed by a transendothelial influx and/or local activation of a variety of mononuclear cells, such as T-cells, B-cells, plasma cells, dendritic cells, macrophages, mast cells, as well as by new vessel formation. The lining layer becomes hyperplastic and the synovial membrane expands and forms villi. The bone destructive portion of the synovial membrane is termed 'pannus', and the destructive cellular element is the osteoclast; destruction mostly starts at the cartilage–bone–synovial membrane junction. Bone repair by osteoblasts usually does not occur in active RA. Polymorphonuclear leukocytes are found in high numbers in the joint fluid, but very rarely are seen in the synovial membrane, suggesting very rapid transgression from blood to the joint space. The neutrophils' enzymes, together with enzymes secreted by synoviocytes and chondrocytes, lead to cartilage degradation. *Adapted by permission from Macmillan publishers Ltd: Nature Reviews Drug Discovery (Smolen and Steiner 2003)[9], copyright (2003).*

body are synovial joints, which are diarthrotic [7]. These consist of an outer joint capsule (a layer of ligaments) and an inner synovial membrane, some also including elastic fibrocartilage pads which act like cushions protecting the bone during movement (Figure 2). Some synovial joints, such as elbows and knees, also have bursae (fluid-filled sack-like cavities) situated nearby between muscles/tendons and bone to facilitate gliding and movement [7].

In a healthy synovial joint the inner layer of the capsule, the synovial membrane, secretes synovial fluid thereby lubricating the joint and supplying nutrition to the capsule [7, 8]. The synovial membrane is composed of a vascularized loose connective tissue, where the layer closest to the cartilage and bone is made up of *macrophage-* and *fibroblast-derived synoviocytes* (Figure 2) [9]. During an inflammatory stage, such as RA, the synovial membrane is infiltrated by various types of immune cells including *T-*, *B-* and *plasma cells* (from the adaptive immune system), as well as *mast cells*, *dendritic cells* and *macrophages* (from the innate immune system) (Figure 2) [10]. Moreover, the *macrophage-like* and *fibroblast-like synoviocytes* produce cytokines and proteases, collectively contributing to the inflammation and cartilage and bone destruction in RA [8]. During this inflammatory process activated *fibroblasts*, *T-* and *B-cells* (which express the receptor activator of nuclear factor κ B ligand, RANKL) interact with *macrophages*, *monocytes* and *dendritic cells* (which express the receptor RANK) to promote the generation of bone resorbing *osteoclast* [10]. Meanwhile matrix metalloproteinases (MMPs) together with additional metalloproteinase-enzymes (e.g. membrane-type metalloproteinases, MT-MMPs) contribute to cartilage destruction [10, 11].

PERIODONTAL DISEASE

Periodontal disease includes both reversible and irreversible conditions, where the milder reversible forms comprise inflammation of the gingival tissue (gingivitis), and the irreversible chronic inflammation (periodontitis or peri-implantitis) causes destruction in connective tissue and loss of alveolar bone [6].

Gingivitis

The reversible form of periodontal disease, gingivitis, does not include any destruction of the connective tissue or bone [1, 12]. In gingivitis, the inflammation of the gingival tissue can be plaque (bacteria) or non-plaque induced (e.g. viral infections, allergies), with the plaque induced form being the most common [3, 12]. The diagnosis is based on clinical features including swelling, redness and bleeding of the gingival tissue [1]. This condition can be reversed by treatment and optimization of oral hygiene [1].

Periodontitis

Periodontitis, a major cause of tooth loss in adults, is one of the world's most prevalent chronic infectious inflammatory diseases, affecting up to 25 - 40% of the population [13-15]. The most severe form of the disease, ultimately resulting in the loss of teeth, affects 5 - 15% of the global population [13, 14]. Diagnosis of periodontitis is based on outcomes from intra-oral and radiographic examination [1, 12], including assessment of bleeding on probing (BOP), probing pocket depth (PPD), clinical attachment level (CAL), alveolar bone level and tooth mobility [16]. Chronic periodontitis is classified according to extent (localized or generalized) and severity (slight, moderate or severe) of the disease, based on number of sites with PPD and/or CAL (Table 1) [12, 16]. Different classifications for periodontal disease

Table 1. International consensus criteria for classification of chronic periodontitis

Classification according to		Case definition	
Armitage 1999 [12] "Development of a Classification System for Periodontal Diseases and Conditions"	**Extent**	***Localized***	≤ 30% of the sites affected
		Generalized	> 30% of the sites affected
	Severity	***Slight***	1 - 2mm CAL
		Moderate	3 - 4mm CAL
		Severe	≥ 5mm CAL
Page and Eke 2007 [16] "Case Definitions for Use in Population-Based Surveillance of Periodontitis"		***No/mild periodontitis***	Not fulfilling criteria for moderate or severe disease
		Moderate periodontitis	≥ 2 interproximal sites with CAL ≥ 4mm (not on the same tooth) or ≥ 2 interproximal sites with PPD ≥ 4mm (not on the same tooth)
		Severe periodontitis	≥ 2 interproximal sites with CAL ≥ 6mm (not on the same tooth) and ≥ 1 interproximal sites with PPD ≥ 5mm (not on the same tooth)

CAL, clinical attachment level; PPD, periodontal pocket depth.

have been recommended over the years and the classifications currently in use are based on the International Consensus Report on Chronic Periodontitis [12, 16].

Chronic periodontitis is initiated by the presence of a microbial biofilm (dental plaque), which accumulates around the gingival margin [1, 17]. The first microbial species to colonize usually include the Gram-positive *Actinomyces* and *Streptococcus* [17]. As the plaque continues to accumulate the environment becomes more anaerobic and the biofilm extends deeper into the gingival sulcus [1, 17]. In severe periodontitis, this biofilm includes high proportions of Gram-negative anaerobic periodontal pathogens [*Treponema denticola* (*T. denticola*), *Tannerella forsythia* (*T. forsythia*) and *Porphyromonas gingivalis* (*P. gingivalis*)] also referred to as the red complex, additional organisms believed to facilitate the red complex colonization (e.g. *Fusobacterium nucleatum*) as well as other periodontitis associated pathogens such as *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) [1, 18, 19]. In susceptible individuals, the bacterial dysbiosis may lead to a chronic inflammation and the development of periodontitis [19]. The progression and the disease susceptibility is dependent upon several factors including genetic and environmental risk factors (e.g. smoking) and systemic health status that influence the host inflammatory response (Figure 3) [19, 20]. Periodontal pathogens from the oral biofilm can trigger the inflammatory response resulting in a release of various cytokines and inflammatory mediators including interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α), prostaglandin E_2 (PGE_2), and MMPs, collectively contributing to the destruction of tooth-supporting structures [6, 20]. Currently, the main therapy for periodontal treatment is to control the oral biofilm by improving oral hygiene and removing plaque and

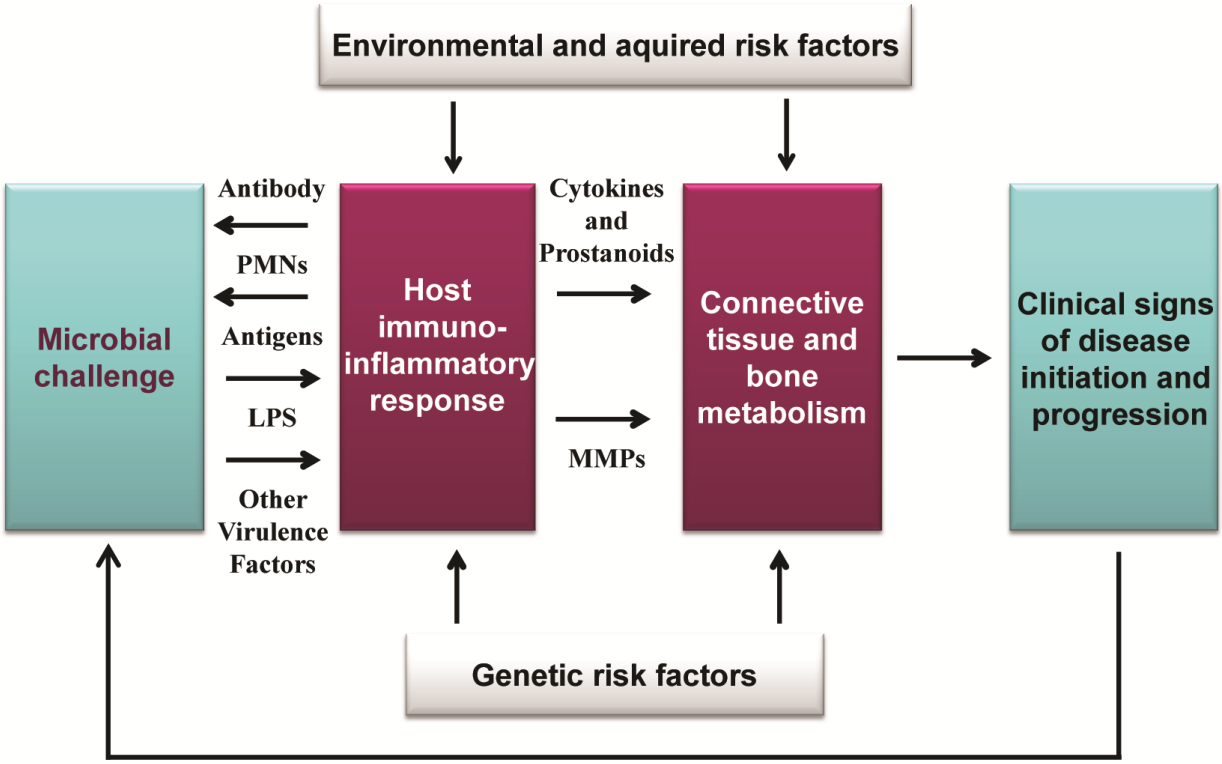

Figure 3.
Model of the pathogenesis of periodontitis. This schematic model shows the complex interaction between mechanism involved in the pathogenesis of periodontitis, including an initial microbial challange, a host immune-inflammatory response, as well as genetisk, environmental and aquired risk factors.
PMNs, polymorphonuclear leucocytes; LPS, lipopolysaccharide; MMPs, matrix metalloproteinases.
Adapted from Page and Kornman 1997 [20], reprinted with premission from John Wiley and Sons.

dental calculus [21]. Up to date, there are no clinically available biomarkers for periodontitis, but understanding how oral pathogens misdirect the host immune response could potentially reveal new therapeutic targets for the disease [21, 22]. Periodontitis may also affect the general health, for example poor glycemic control has been reported in diabetic patients with periodontitis [23]. In addition, patients with periodontitis have increased prevalence and/or risk of systemic diseases including cardiovascular disease, diabetes and RA [23-26].

Peri-implantitis

Peri-implantitis is defined as an inflammatory process around a functional implant, associated with both soft-tissue inflammation and loss of supporting bone [27]. The diagnosis is made based on results from a clinical and radiographic examination, including manifestations of swelling, redness, BOP and radiographic loss of bone usually in a shape of a crater around the implant [28]. The prevalence of peri-implantitis differs between studies but has been estimated to affect approximately 20 - 56% of patients after 1 - 10 years post-implant placement [27, 28]. Peri-implatitis is a multifactorial disease where both surgical-, prosthetic- and patient-related factors may contribute to its development and severity [27]. This disease shares several similarities with periodontitis including risk indicators such as smoking, diabetes and poor oral hygiene [28, 29]. Peri-implantitis, like periodontitis, is initiated by an accumulation of dental plaque containing a large amount of Gram-negative anaerobic

bacteria (e.g. *P. gingivalis, A. actinomycetemcomitans*, *T. denticola* and *T. forsythia*), adjacent to the implant [30]. However, also *Staphylococcus aureus*, a Gram-positive species not associated with periodontitis, has been implicated in the pathogenesis of peri-implant disease [30]. The plaque accumulation/bacterial infection adjacent to the implant can trigger an inflammatory response in the surrounding tissues, resulting in loss of supporting bone (past the normal bone remodeling) [27, 30]. If peri-implantitis is left untreated this inflammatory disease may ultimately bring about the loss of the implant [27, 30]. Similar to periodontitis, the treatment of peri-implantitis aims to control the infection trough improvement of the oral hygiene, mechanical elimination of plaque and dental calculus, and detoxification of the surface of the implant [28, 30].

RHEUMATOID ARTHRITIS

RA is a painful and disabling systemic chronic inflammatory autoimmune disease [31, 32], affecting almost 1% of the world population, predominately women [32-34]. It is associated with long term morbidity and early mortality despite antirheumatic treatment [32]. This chronic inflammatory condition can lead to joint destruction and has also been associated with systemic complications (pulmonary, ocular, neurological) as well as cardiovascular comorbidity and type 1 diabetes [35-39]. In addition, an association between periodontitis, and RA has also been suggested [40]. The etiology of RA is still unknown but is suggested to be a combination of genetic, environmental, hormonal and infectious risk factors [32, 40, 41]. RA is characterized by synovial joint inflammation and pannus (hypertrophied synovium) formation leading to irreversible destruction of cartilage and underlying bone (Figure 2) [38]. The joint inflammation is associated with an infiltration of inflammatory cells (such as *T*- and *B-cells*, *macrophags* etc) into the synovium as well as a hyperplastic expansion of the synovial membrane/synovial cells (*fibroblast-like* and *macrophage-like synoviocytes*) resulting in production of cytokines and proteases [8, 10, 42]. The overproduction of pro-inflammatory cytokines (e.g. TNF-α) is central to RA pathogenesis, driving joint destruction by stimulating *synovial fibroblasts* and *chondrocytes* (cartilage cells) to secrete collagen degrading enzymes, as well as by activating *osteoclast* differentiation, ultimately leading to bone and cartilage destruction [35, 43]. In addition, a recent study suggests that anti-citrullinated protein antibodies (ACPAs) may also contribute to joint destruction in RA [44]. The diagnosis of RA is made according to the American College of Rheumatologys (ACR) classification criteria using the combination of results from physical examination (swelling/arthritis in joints, duration of symptoms) and blood tests [presence of ACPAs and/or rheumatoid factor (RF) antibodies, abnormal acute phase reactants such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)], as well as radiographic changes [45, 46]. The patient's functional status is traditionally assessed by a self-administered Health Assessment Questionnaire (HAQ), while the disease activity in RA is assessed by a Disease Activity Index, for example the Disease Activity Score including 28-joint count (DAS28) [31, 47]. The DAS28 score is widely used in clinical assessment of RA disease activity, as

well as in research and clinical trials [31, 47, 48]. It is calculated based on the physical examination of the number of swollen and tender joints (hands, arms and knees), the patient´s global assessment/general health measured on a visual analog scale (VAS, 0 - 100) and measurements of ESR or CRP (DAS28 CRP) [47]. The disease course in RA can exhibit two different patterns; a chronic-persistent affecting 20 - 44% [49-52], or a relapsing-remitting affecting 7 - 79% of patients with RA [49, 51, 53-55]. Remission without RA-medication is possible, although sustained remission beyond 1 year is uncommon in patients with RA [54, 56].

The management of RA includes medication, physical therapy and surgery, and aims to relieve symptoms, slow down joint damage and achieve remission [57]. Analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) reduce joint pain and stiffness [57]. The cornerstone in treatment of RA is, however, the use of disease-modifying antirheumatic drugs (DMARDs), which reduce the joint inflammation and limit or prevent joint damage [57-59]. Non-biological DMARDs (such as methotrexate, sulfasalazine, hydroxychloroquine and leflunomide) and biological DMARDs (such as TNF-α and IL-6 inhibitors and *T*- and *B-cell* target therapies), alone or in combination, are demonstrated highly effective in placebo-controlled trials [57, 58, 60]. Moreover, glucocorticoids can also be used in combination with DMARDs to "bridge the patient" until the onset of the therapeutic effect of DMARDs, and have also been recommended if the disease activity flares in patients with established RA that are already on DMARD/biological DMARD therapy [59, 61]. However, many of these medications have side effects including nausea, hypertension, osteoporosis, increased risk of infection and peptic ulcers [57, 62]. The specificity and regulation of the underlying autoimmune reactions in RA have for long been unclear, preventing a deeper understanding of disease processes and the development of new therapies. The discovery of ACPAs, however, has given new opportunities and biological tools to design novel models of etiology and therapy strategies in RA.

The ACPA antibodies are specific serological markers for RA, detected in approximately 70% of patients [19, 63]. These antibodies were first described in 1964, as anti-perinuclear factor [64] and anti-keratin antibodies [19, 65, 66] with the ability to recognize citrullinated peptides [67], and have since 2010 been included as one of the diagnostic criteria for the disease [46]. Presence of ACPAs, measured by a diagnostic test based on reactivity against a synthetic cyclic citrullinated peptide (anti-CCP), can be detected up to 10 - 15 years before clinical onset of RA [68, 69], making them a useful diagnostic test for the detection of early disease. In addition, ACPA-positive RA is associated with a more severe disease course/radiological destruction [70-72], as well as major environmental and genetic risk factors for RA [smoking and HLA-DRB1 shared epitope (SE)] [32, 73], compared to ACPA-negative disease subset. Due to the differences in genetic and environmental risk factors between ACPA-positive and ACPA-negative RA, different pathogenic pathways may underlie ACPA-positive and ACPA-negative disease subset [74]. Some of the most well characterized ACPAs in RA are citrullinated fibrinogen, vimentin, collagen and α-enolase [74].

The RF antibodies, another diagnostic criteria for RA, were the first antibodies to be associated with the disease [46, 75]. This polyclonal antibody is primarily directed against the Fc region of immunoglobulin G (IgG), but can react with other antigens and is detectable also in other immunoglobulin subclasses (IgA, IgD, IgE, IgM) [76]. Even though RF is not specific for RA and can be found in a variety of other diseases [77], it is present in approximately 60 - 80% of RA patients (usually measured as IgM-RF) [76, 78, 79] and can be detected up to 5 - 15 years before disease onset [32, 80]. Moreover, most RA patients that are positive for ACPAs are also positive for RF [34], and the risk of RF-positive RA has been associated with both genetic and environmental risk factors (HLA-DRB1 SE and smoking) [32, 74, 81]. It has been suggested that RF by itself does not contribute to RA disease progression [82], as the association with joint destruction has been attributed to ACPA rather than RF [34, 82]. Nevertheless, RF is still used in classification criteria for RA, and a study from 2015 demonstrated an association between RF and increased disease activity in RA patients, in contrast to ACPAs [79].

SIMILARITIES BETWEEN PERIODONTITIS AND RA

Periodontitis and RA are both chronic inflammatory diseases that can lead to permanent disability [83-85]. These two diseases share numerous features including tissue and bone destruction; production of inflammatory mediators including cytokines, prostaglandins and degradation enzymes (i.e. MMPs) and common risk factors such as smoking [86, 87]. The destruction of soft and hard tissue is a result of a large number of signal molecules released by inflammatory cells in the synovia/gingiva which contribute, directly or indirectly, to the degradation of tissue and bone [83-85]. Both diseases have similar cytokine profiles characterized by high levels of inflammatory mediators (such as PGE_2, TNF-α, IL-6 and IL-1β) and degradation enzymes (e.g. MMP1, MMP9 and MMP13), and decreased levels of anti-inflammatory mediators (such as IL-10 and TGF-β) [85, 88-92]. In addition, a well-established major risk factor for both periodontitis and RA is cigarette smoking. Higher frequency of bone loss, attachment loss and edentulism is exhibited in smokers, compared with non-smokers [93]. In RA, cigarette smoking has been reported to increases the risk of seropositive disease (both ACPA and RF) in genetically predisposed individuals, and has also demonstrated a dose-dependent effect regarding the risk of ACPA-positive disease [32, 81, 94]. Moreover, a well-established genetic risk factor for RA, HLA-DRB1 SE alleles, has also been implicated as a risk factor for periodontitis [95-97]. Additionally, antibodies associated with RA (both ACPA and RF) have been detected in non-RA patients with periodontitis [87, 98-100]. For example, individuals with periodontitis have higher serum ACPA-levels compared to healthy controls [99]. Furthermore, increased levels of citrullinated proteins have been reported in inflamed periodontal tissue [98, 101], and expression of ACPAs has been demonstrated in gingival crevicular fluid (GCF) and saliva of patients with RA [98, 102]. Similarly, RF has been reported in gingiva, subgingival plaques and in serum of patients with periodontitis [87].

BACTERIAL LINK BETWEEN PERIODONTITIS AND RA

In 2004, a hypothesis of a possible pathogenic connection between periodontitis and RA was proposed, implicating the involvement of the periodontal pathogen *P. gingivalis* in the pathogenesis of RA, through the process of citrullination [24, 87]. Citrullination is an enzyme-mediated post-translational modification of the amino acid arginine in a protein into the non-standard amino acid citrulline (Figure 4) [74]. This process generates citrullinated proteins containing epitopes against which ACPAs can be raised [103]. Citrullination is driven by the enzyme peptidyl arginine deiminase (PAD), and the activity of this enzyme is dependent on high calcium concentrations [74]. To date, *P. gingivalis* is the only known pathogen with the ability to express a citrullinating PAD enzyme (*P. gingivalis* PAD, PPAD), equivalent to the PAD enzymes (PAD1 - 4 and PAD6) in mammals [87]. By the process of citrullination, PPAD could potentially initiate a local breakdown of tolerance to citrullinated proteins generated in gingiva, followed by epitope spreading to host-citrullinated proteins in the joint [24]. This mechanism (together with human PAD enzymes) could sustain the production of ACPAs, which can precede the development of RA, thereby implicating PPAD in the pathogenesis of RA [24]. In addition to the *P. gingivalis* link hypothesis, most recently Konig *et al.* suggested that another periodontal bacterium, *A. actinomycetemcomitans* could be associated with the RA related autoimmunity, due to its ability to trigger (trough leukotoxin A) an activation of citrullinating enzymes in host neutrophils [104]. Moreover, the same study also reported that, in patients with RA, the leukotoxic strain of *A. actinomycetemcomitans* was associated with both ACPA as well as RF antibodies [104]. Because these data are very recent, the association between RA and the leukotoxic strain of *A. actinomycetemcomitans* was not investigated in this thesis.

PREVIOUSLY PUBLISHED STUDIES ON THE RELATIONSHIP BETWEEN PERIODONTITIS AND RA

Incidence/prevalence and disease activity of RA in patients with periodontitis

Previous studies, most of them with relatively low sample size, have proposed a correlation between RA and periodontitis [105-107], including increased incidence and prevalence of RA in patients with this periodontal disease [24, 108-112]. For example, in a study by Hashimoto *et al.* periodontitis correlated with the incidence of RA in 72 individuals with arthralgia [24]. Furthermore, in a study including 32 American patients with new-onset RA, high prevalence of periodontitis was reported at disease onset [108]. Regarding studies with large number of patients, in one of the largest studies to date (13 779 Taiwanese patients with newly diagnosed RA and 137 790 controls), a weak association (OR = 1.16, 95% CI: 1.12 – 1.20) was reported between periodontitis and incident RA based on administrative data from the National Health Insurance Program [109]. Importantly however, authors concluded that this weak association was further limited due to lack of information about the subjects smoking habits, a well-known risk factor for RA and periodontitis [109]. Presence of periodontitis has also been associated with RA disease activity [105, 111, 113-115]. In addition, a dose-

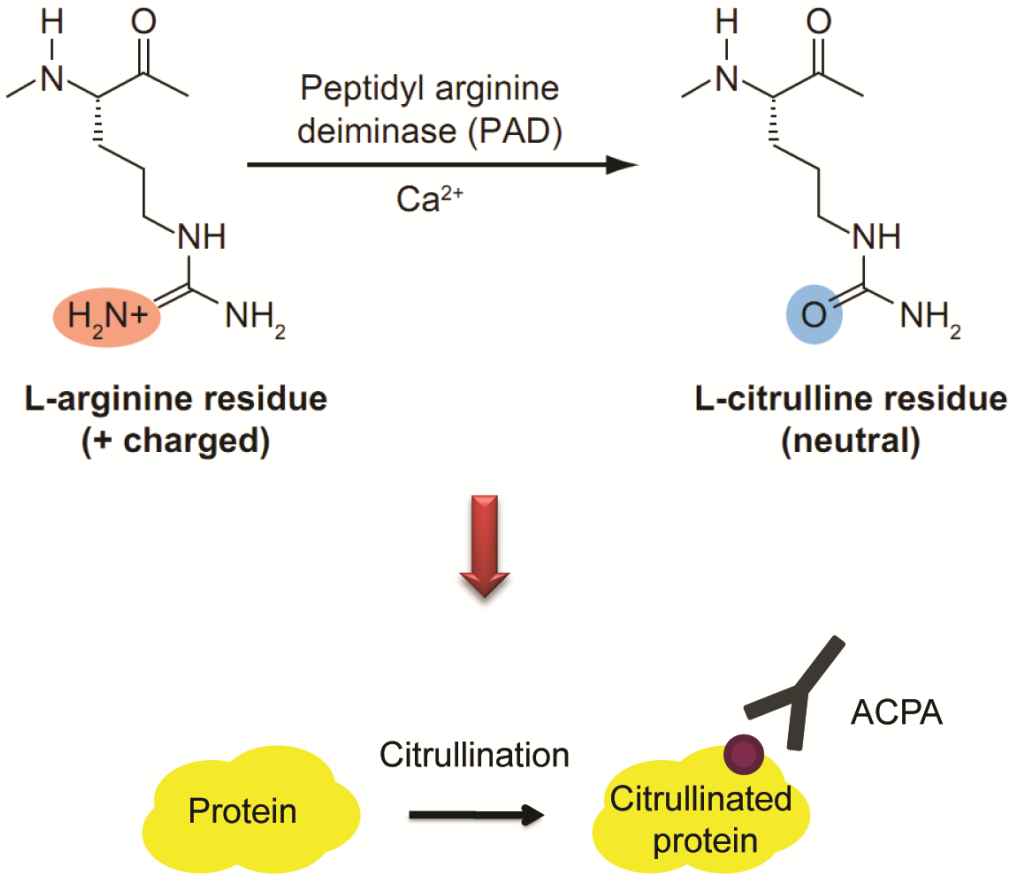

Figure 4.
The process of citrullination of proteins, driven by the calcium dependent petidyl arginine deiminase (PAD) enzyme. Deimination of peptidylarginine to peptidylcitrulline is a post-translational process, also known as citrullination. The enzymatic conversion results in the loss of one positive charge for every arginine residue converted to a neutral citrulline. This causes changes in intra- and intermolecular interactions, which could lead to altered protein folding, enhanced degradation by proteases, and exposure of cryptic epitopes. RA specific antibodies (ACPAs) can recognise and bind to citrullinated proteins. The production of citrullinated proteins can thereby contribute to the autoimmune reponse in RA.
ACPA, anti-citrullinated protein antibody.
Adapted and reproduced from Klareskog et al 2008 [74], with premission fromAnnual Reviews.

response pattern between periodontitis severity and RA disease activity has been reported. For example, higher RA disease activity, as indicated by higher DAS28 score, has been described in RA patients with periodontitis [111, 113, 114, 116]. In addition, subjects with severe periodontitis demonstrated highest DAS28 scores compared to moderate or periodontitis-free individuals [111]. Furthermore, in an Australian population (including 65 RA cases and 65 controls), increased periodontal bone loss and deeper pockets were associated with indicators of RA disease activity, such as higher number of swollen joints, and levels of CRP and ESR in patients with RA [117]. Conversely, there are also studies reporting no association between levels of ESR or CRP and periodontitis [106, 118]. A meta-analysis performed in 2013 by Kaur *et al.* reported no statistical difference in ESR titers, and only low level of evidence for elevated CRP and the association with periodontitis in RA subjects [106].

Prevalence/severity of periodontitis in patient with RA

In accordance with reports that patients with periodontitis have increased risk for RA/severe RA, as discussed previously, it has also been published that patients with RA have increased risk of periodontitis/severe form of periodontitis. Numerous studies have shown that patients with RA have substantially increased frequency of periodontitis/severe periodontitis as compared to healthy individuals [40, 87, 108, 111, 113-115, 117, 119-127]. For example, in an American study (including 103 RA cases and 4358 controls) patients with RA were more likely to be edentulous and have periodontitis compared to controls [124]. In a Finnish population, poorer periodontal parameters were reported in patients with chronic active RA as well as in a population with early untreated RA, when compared to non-RA controls [127]. Furthermore, in an Indian population, the odds of periodontitis were 4.28 (95% CI: 2.35 – 7.38, $p < 0.001$) times higher in non-smoking DMARD-naive RA participants as compared to healthy controls (prevalence 64.8% versus 28%, $p < 0.001$) [113]. Additionally, in the same population, periodontitis was associated with higher titers of ACPA among RA cases [113]. Similarly, in studies comparing the periodontal health of patients with RA to that of non-inflammatory arthritis (osteoarthritis), it was demonstrated that periodontitis is more common in both RA and ACPA-positive RA subjects compared to patients with osteoarthritis [114, 116, 128, 129]. Moreover, a review article by Kaur *et al.* indicated that more severe periodontitis including greater CAL and increased tooth loss is associated with RA, although the authors concluded that larger population-based studies were needed to confirm this link [106].

Studies reporting no association between periodontitis and RA

In contrast to the studies reporting an association between periodontitis and RA, there are also reports, including both large population-based [130, 131] and clinical studies [118, 132-138], demonstrating no differences in prevalence of periodontitis between RA patients and healthy controls [130, 131, 133, 134]. In addition, even less severe periodontal tissue destruction in subjects with RA has been described [135, 137, 138]. For instance, in a longitudinal study conducted over a time period of 20 years (9702 patients, 138 with established RA and 433 with incident RA) subjects with periodontal disease displayed numerically higher prevalent/incident RA than subjects without periodontitis, although most ORs were non-statistically significant [131]. Furthermore, the largest prospective study to date, conducted among 81 132 American women including 292 with incident RA, reported no association between severe periodontitis (as estimated by history of periodontal surgery and/or tooth loss) and risk of RA [130]. Additionally, one of the earliest clinical case-control studies, comparing periodontal parameters among Swedish subjects with RA (n = 161) and matched controls (n = 204), showed even less frequent severe periodontal breakdown in patients with RA [135].

The conflicting reports on the relationship between periodontitis and RA, described above, may depend on ethnic differences between study populations and/or substantial differences in disease classification criteria for periodontitis. In 2013, Linden *et al*. concluded that studies do not provide support for a link between periodontitis and RA since very few studies meet a

stringent threshold for periodontitis [139]. Although most of the previous studies today suggest that an association between periodontitis and RA exists [108-117, 120-124, 128], even more recent review articles conclude that studies with sufficient sample sizes are still needed to ascertain the temporal relationship between these two diseases [107, 140]. Moreover, in a recently published review by Chapple *et al*. the authors state that even though patients with RA seem to have an increased prevalence of periodontitis, they deemed the certainty of this association as low [141].

Effects of periodontal treatment on RA

Results from previous clinical studies indicate that non-surgical periodontal treatment may have beneficial effects on RA disease severity [105, 142-144]. Scaling and root planning in RA patients with chronic periodontitis have resulted in significant reduction of RA disease activity and serum levels of TNF-α [143-151], in some cases regardless of RA medications [148, 152]. Furthermore, periodontal treatment has been reported to significantly reduce ACPA levels in non-RA patients with periodontitis [99], however, these results have not yet been demonstrated in subjects with RA [106, 143, 144]. Likewise, no statistical difference in RF-levels has been demonstrated following non-surgical periodontal therapy [143, 150], indicating no effect of periodontal therapy on serological markers for RA. In recently published review articles it was highlighted that there is a need for additional randomized controls trials and large-scale longitudinal intervention studies investigating the effect of periodontal therapy on RA disease activity [105, 142].

Effects of RA medication on periodontitis

Studies indicate that treatment of RA with biological agents, such as anti-TNF-α and IL-6 inhibitors, may have beneficial effect on periodontitis [88]. Anti-TNF-α therapy (adalimumab, infliximab) can significantly decrease clinical parameters of periodontitis severity (BOP, gingival index, PPD and CAL) without additional periodontal treatment [153-156]. Attachment loss significantly decreased in patients evaluated before and after infliximab-therapy [153]. The decrease in the severity of periodontitis might be explained by the fact that anti-TNF-α therapy also reduces the levels of inflammatory cytokines (IL-1β, IL-6, IL8, MCP-1 and TNF-α) in serum and GCF samples of patients with RA [154, 155, 157]. Furthermore, one study reported similar results after IL-6 receptor (IL-6R) inhibition therapy showing reduction in gingival index, BOP, PPD, CAL, serum IL-6 and MMP3 in RA subjects, without additional periodontal therapy [158]. However, the positive effect of biological agents on the periodontal condition has not been successfully replicated in all studies [148]. In patients with active RA for example, PPD was not affected after 6 weeks of anti-TNF-α therapy, even though the gingival inflammation (BOP) was reduced [156]. Moreover, in 40 patients with moderate/severe RA, anti-TNF-α therapy (without any additional periodontal treatment) had no effect on periodontitis [148].

Previous studies investigating the effect of periodontal treatment on RA disease activity and vice versa the effect of RA medication on periodontitis include low sample size and relatively

short observation time (up to 6 months follow-up) [88, 105, 153]. Thus, the effect of periodontal therapy on RA disease activity [105, 142] as well as the therapeutic efficiency of RA biological therapy (inhibition of pro-inflammatory cytokines) on periodontitis require more extensive studies including larger cohorts in order to better assess their efficacy [88].

Experimental periodontitis and arthritis – results from animal studies

In addition to human studies, animal studies have also been performed to investigate the relationship between periodontitis and arthritis. Chronic oral infections with periodontal pathogens have been demonstrated to affect arthritis development in animal models [105, 159-161]. For example, infection with *P. gingivalis* aggravated experimental arthritis in mice and rats, as indicated by more rapid onset of disease, increased bone loss/cartilage destruction and paw swelling [84, 162-166]. Moreover, rats with experimental arthritis have shown increased periodontal bone loss and tooth mobility, without being induced with experimental periodontitis [165, 167].

P. gingivalis is currently the most common oral bacterium used in animal models investigating the effect of oral pathogen infection on arthritis development [84, 159, 161-164]. The ability of *P. gingivalis* to exacerbate arthritis seems to be dependent on PPAD expression, as the extent of experimental arthritis is reduced in animals infected with a PPAD-deficient strain before arthritis-induction [162, 163]. Moreover, PPAD may be associated with the serum levels of ACPAs in mice [162, 163]. Gully *et al*. demonstrated a tendency towards higher ACPA serum levels in animals that were infected with wild-type *P. gingivalis*, as compared to a PAD-deficient *P. gingivalis* strain [162].

There are, however, animal studies that do not report an increase in arthritis severity by periodontitis co-induction [159, 163]. Mice infected with *P. gingivalis* or *A. actinomycetemcomitans* showed no differences in arthritis severity parameters [159]. Furthermore, infection with *Prevotella intermedia* and heat-killed *P. gingivalis* failed to cause aggravation of experimental arthritis in mice [163]. A possible explanation for this lack of effect on arthritis severity may be that the systemic effect of certain strains of periodontal pathogens or types of experimental periodontitis is lower than that of experimental arthritis, thus insufficient for modulating arthritis severity [159, 163]. Another explanation may be that live bacteria, associated with some active bacterial component, are needed in order to have a systemic effect on the host and trigger an autoimmune response [163].

Studies investigating the *P. gingivalis* bacterial link hypothesis

In support of the *P. gingivalis*-hypothesis linking periodontal pathogens to the pathogenesis of RA, antibodies against *P. gingivalis* as well as *P. gingivalis* DNA has been detected in serum, synovial fluid and synovial tissue of patients with RA [168-171]. Anti-*P. gingivalis* antibodies have also been associated with the presence of ACPAs [114, 172, 173]. In addition, it has been demonstrated that PPAD is capable of citrullinating RA-specific antigens such as human fibrinogen and α-enolase, the two major citrullinated autoantigens in RA [24, 174]. Because antibodies to human citrullinated α-enolase peptide 1 (CEP-1) can cross-react with the *P. gingivalis* citrullinated version of the same peptide, a bacterial infection could

potentially be involved in breaking immune tolerance and leading to autoantibody generation in susceptible individuals [175]. Moreover, PPAD itself could be the citrullinated antigen that breaks the immune tolerance, as previous data has showed that PPAD polypeptide chain can undergo auto-citrullination [160, 176, 177]. Interestingly, antibody response against citrullinated PPAD is elevated in sera from patients with RA and ACPA-positive RA, in contrast to PPADs uncitrullinated form [176, 177]. In animal studies, *P. gingivalis* ability to aggravate experimental arthritis has been attributed to PPAD [162]. Mice infected with PPAD deficient strain of *P. gingivalis* demonstrated significantly reduced extent of arthritis [162]. However, the results from studies investigating the *P. gingivalis* bacterial link between periodontitis and RA are inconsistent. Some reports demonstrate no association between the presence of *P. gingivalis* and RA, and no correlation between anti-*P. gingivalis* antibodies and RA severity/ACPA status in patients with RA [112, 168, 178-180].

Summary of previously published studies

Periodontitis and RA share several features including pathogenic processes and environmental risk factors such as smoking [88, 106]. Epidemiological studies suggest that an association exists based on increased incidence of periodontitis in patient with RA and a dose-response pattern between periodontitis severity and RA disease activity [105]. However, available data is controversial, possibly due to the heterogeneity in the definition of periodontitis [139]. Although periodontal pathogens (such as *P. gingivalis*) might be a link in the association between periodontitis and RA, not all studies show this association and there is a lack of evidence for a direct role in the temporal relationship [105, 179, 180]. The results of intervention studies, indicating that non-surgical periodontal treatment can improve RA disease activity in individuals with comorbid disease are still preliminary due to small number of subjects and short follow-up time [105, 142, 145, 149, 181]. Larger studies with well-defined populations, well-defined clinical outcomes and longer follow-up time are required to explore the biochemical processes, effect of therapy as well as the clinical relationship between these two diseases [68, 88, 106, 140, 181]. A causative role of periodontitis or periodontal bacteria in RA pathogenesis still remains speculative [105, 140].

AIMS OF THE THESIS

General aim

The general aim of this thesis was to study the relationship between periodontitis and RA.

Specific aims

Study I

The aim of *Study I* was to investigate the prevalence of periodontitis in patients with RA, with special focus on seropositivity, both ACPA and RF, in the Swedish population-based Epidemiological Investigation of Rheumatoid Arthritis (EIRA) case-control study, by linking EIRA with the National Dental Health Registry (DHR).

Study II

Study II aimed to explore the effects of pre-existing periodontitis, induced by periodontal pathogens in combination with ligatures, on the development and the immune/inflammatory response of experimental arthritis.

Study III

In *Study III* the objective was to investigate the presence of citrullinated proteins and the protein expression of the citrullinating enzymes PAD2 and PAD4, in relation to *P. gingivalis* in gingival biopsies from patients with and without periodontitis.

Study IV

Study IV aimed to investigate the severity of periodontitis in patients with established RA, in relation to antibody status (ACPA and RF), medication, clinical and immunological parameters associated with RA, and the presence of *P. gingivalis*.

MATERIALS AND METHODS

The following chapter will present an overview of the methods used to obtain the results in *Studies I - IV*, included in this thesis. A brief information about the study design, recruitment of participants and experimental techniques will be outlined. For more detailed information about the participants, protocols, chemicals and substances used, please refer to the Materials and Methods sections of *Studies I - IV*.

ETHICAL CONSIDERATIONS

All of the studies in this thesis followed the ethical principles for medical research as stated in the Declaration of Helsinki, and were conducted in accordance with current legislation in Sweden. Ethical permits were obtained from the Regional Ethical Review Board in Stockholm (*Studies I, III* and *IV*) or the Stockholm North Animal Ethics Committee (*Study II*), before initiation of each study. In studies involving human subjects (*Studies I, III* and *IV*) a written informed consent for participation was obtained from all subjects prior to inclusion.

HUMAN STUDY POPULATIONS (*Studies I*, *III* and *IV*)

The *Studies I, III* and *IV* contain data, samples and gingival biopsies from human subjects. In the first study we investigated the prevalence of periodontitis in patients with RA and matched healthy controls in relation to autoantibody status, by linking data from two Swedish registries. *Study III* investigated the potential for citrullination (possibly contributing to autoimmunity) in gingival tissue from patients with periodontitis and healthy controls, by studying the expression of citrullinated proteins and citrullinating enzymes in gingival biopsies. A clinical evaluation of periodontitis severity in RA patients, in relation to autoantibody status, was performed in *Study IV*. A schematic illustration of the study designs of the above mentioned studies are demonstrated in Figure 5.

EIRA and DHR registries (*Study I*)

In *Study I* the prevalence of periodontitis was investigated in 2740 subjects with RA and 3942 matched healthy control included in the Swedish population-based case-control study EIRA. The EIRA registry was initiated in May 1996 and contains the participants' genetic, environmental and serological data (ACPA and RF status). The participants included in *Study I* were enrolled until October 2009. At the time of admission into the EIRA registry, all patients with RA were newly diagnosed by rheumatologists according to the 1987 American College of Rheumatology (ACR) Criteria for RA. The healthy controls were recruited from the Swedish national population registry, and were matched to RA cases based on age, gender and residential area.

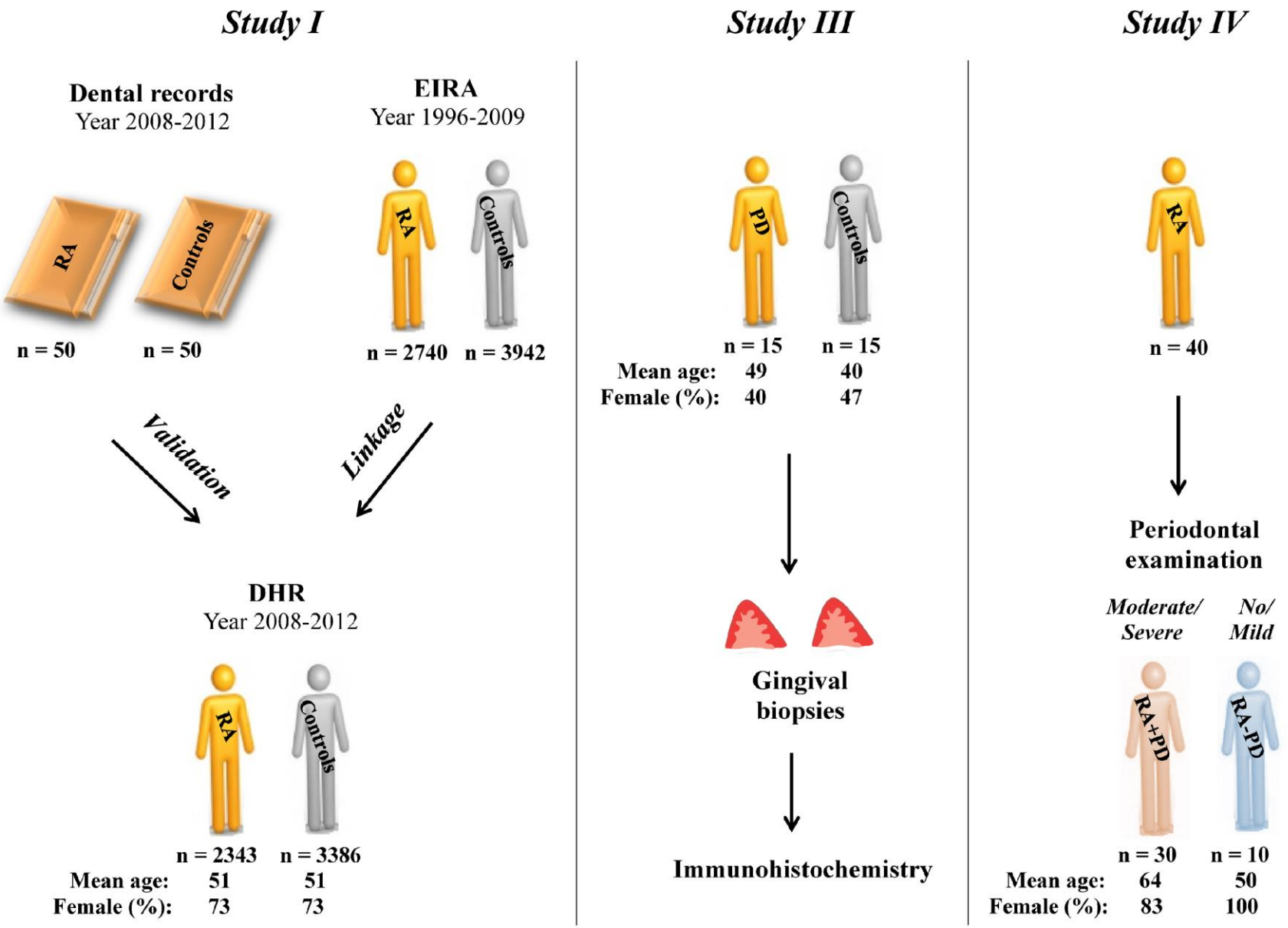

Figure 5.
Schematic overview of the study populations included in *Studies I, III* and *IV*.
EIRA, Epidemiological Investigation of Rheumatoid Arthritis case-control study; DHR, Dental Health Registry; RA, rheumatoid arthritis; PD, periodontitis; n, numbers.

Information about the prevalence of periodontal diagnoses among the participants was obtained by linking the EIRA registry to the Swedish DHR through the participants' social security numbers. The DHR contains information about dental diagnostic- and treatment codes for all Swedish citizens over the age of 20 who have visited the dentist at least one since the initiation of the registry (July 2008). All diagnoses in the DHR have been registered by a dentist or a dental hygienist. In *Study I* we focused our attention on specific periodontal diagnostic codes including: increased risk of periodontitis (2041), increased risk of peri-implantitis (2051), gingivitis (3041), periodontitis (3043) and peri-implantitis (3044). We also analyzed the prevalence of periodontal treatment codes registered in combination with the diagnostic codes for periodontitis (3043) and increased risk of periodontitis (2041). The investigated treatment codes were: minor non-surgical treatment of periodontal disease, major non-surgical treatment of periodontal disease including several deep pockets and/or furcation involvement, as well as the codes for surgical treatment. We identified 86% of the EIRA study population, both RA cases and controls who had visited the dentist after July 2008, in the DHR. There were no significant differences between the identified RA cases and controls regarding gender, age or ACPA/RF antibody status.

Dental records (*Study I*)

At the time of *Study I*, the DHR was a new and not yet validated registry. Therefore, in addition to the linkage analysis, we decided to validate the periodontal diagnostic codes and confirm the diagnoses by using information from the patients dental records. At the time of enrollment into EIRA, the RA cases and controls had completed a questionnaire covering a broad range of topics, including one question related to periodontal disease. The subjects answering "Yes" to the periodontitis-related question were asked to participate in the validation of the DHR by giving their permission to examine their dental records. The request was sent to 151 RA subjects and 155 controls, of which 100 subjects in total (50 RA cases and 50 controls) gave their informed consent to participate. Periodontal diagnosis was based on oral radiographs and all other information in the dental records, which were reviewed by two independent dentists. Criteria from the International Consensus Report on Chronic Periodontitis [182] were used to define and diagnose periodontitis.

Gingival biopsies (*Study III*)

In *Study III*, gingival biopsies were collected from patients with and without periodontitis (15 in each group) in order to study the expression of citrullinated proteins and human citrullinating enzymes (PAD2 and PAD4), in relation to *P. gingivalis*, in the inflamed and healthy gingival tissues. The mean age of the participants with and without periodontitis was 49 and 40 years, respectively (Figure 5). All patients in the periodontitis group demonstrated radiographic bone loss and probing pocket depth of ≥5 mm at the site of the collected biopsy. Patients included in the control group without periodontitis had no signs

of radiographic bone loss and probing pocket depth ≤3 mm. All participants were systemically healthy and did not take anti-inflammatory drugs.

Clinical assessment and samples collection in patients with RA (*Study IV*)

A clinical evaluation of the periodontal health of subjects with RA was conducted in *Study IV*. The population consisted of 40 patients, aged 29 - 80, fulfilling the 2010 ACR criteria for RA (Figure 5). The gender distribution, including a majority of female participants, was representative for RA given that this disease is known to be more common in women.

All participants underwent a full-mouth dental and periodontal examination, performed by the same dentist. Patients were divided into two groups based on their periodontal diagnosis (no/mild of moderate/severe periodontitis) (Figure 5). The examining dentist did not have information about the participant's periodontal or rheumatological status beforehand. Periodontitis in *Study IV* was diagnosed following the standardized clinical definition of periodontitis intended for use in population-based studies [16].

Various parameters related to RA disease activity were analyzed in patients with no/mild and moderate/severe periodontitis, including the self-assessed health (HAQ) score, the DAS28 score, autoantibody status (ACPA and RF), CRP levels as well as a number of inflammatory mediators. The presence of subgingival *P. gingivalis* was investigated in relation to periodontal diagnosis and ACPA antibody status.

Samples were collected during the dental examination in order to investigate the presence of RA related autoantibodies, CRP levels, the presence of *P. gingivalis* and the expression of inflammatory mediators. The collected samples included blood, saliva, GCF and subgingival plaque, which were all stored at -80°C until analysis. For information about the sampling procedure, handling and preparation of different samples the reader is referred to the Methods section in the manuscript (*Study IV*).

ANIMAL STUDY (*Study II*)

To investigate the effects of pre-existing periodontitis on arthritis, an experimental animal model was used in order to achieve the immune systems complex interactions within the body. An *in vivo* model was chosen because the immune systems complexity cannot be reproduced *in vitro* in a cell culture environment, and there were no *in-silico* models that could replace the *in vivo* model.

Animals

Study II included 25 inbred adult male Dark Agouti (DA) rats, weighting between 165 and 220 g. This rat stain has previously been used in arthritis research as it is highly susceptible to both acute and chronic types of arthritis. All animals were bred and housed under pathogen free conditions in individually ventilated cages at the Medical Inflammation Research facility, Karolinska Institutet, and received water and food *at*

libitum. The duration of *Study II* was set to 15 weeks to include both active and chronic arthritis. At the beginning of the study the animals were randomly divided into one of the following three groups: (1) a group consisting of both experimental periodontitis and arthritis (PA, n = 9), (2) a group with only experimental arthritis (A, n = 12) and (3) a small control group (Healthy, n = 4) used mainly to monitor the normal physiological changes in the animals over time (Figure 6).

Experimental periodontitis and arthritis

Experimental periodontitis was induced during a course of 8 weeks, prior to arthritis induction, to facilitate a severe chronic periodontal disease. The animals were anesthetized using isoflurane and silk ligatures that promote bacterial adhesion were placed and knotted at the cervical part of the two upper second molars of each rat in the PA group, to induce the development of experimental periodontitis. The ligatures were checked every 10 days and replaced if loosened. To further facilitate the progression of periodontitis and to ensure the presence of the periodontal pathogen *P. gingivalis* (potentially implicated in the association between periodontitis and RA), weekly bacterial swabs were administered containing the pathogens *P. gingivalis* and *F. nucleatum* (known to promote the colonization of *P. gingivalis*). The rats without experimental periodontitis (the A and the healthy control group) received swabs with only the vehicle (4% Gantrez) that was used to reconstitute the bacteria in the PA group. The presence and progression of periodontitis was clinically monitored in all animals every 10 days throughout the course of the study, and assessed radiographically at endpoint (Figure 6).

After 8 weeks, a well-established model of arthritis, the pristane-induced arthritis (PIA), was used to induce the disease in both the PA and the A group (Figure 6). The PIA model closely mimics human RA by triggering a chronic relapsing form of arthritis that additionally fulfills many criteria for RA including presence of RF, symmetrical involvement of peripheral joints as well as cartilage and bone destruction. PIA was administered by a single intradermal pristane-injection at the dorsal side of the rats' tail, and clinical manifestations of arthritis were visible 2 - 3 weeks post injection. The development and severity of arthritis was monitored continuously by two independent examiners, using a previously describes visual scoring system [183] which assesses the inflammation in wrists, ankles, knuckles and toes. Weight changes were also recorded as an objective measure of arthritis disease activity.

Sample collection in the animal study

Every 10 days blood samples were collected from the tail of each rat, during anesthesia with isoflurane, to monitor the levels of acute phase proteins such as α-1-acid glycoprotein (α-1-AGP). At the end of the 15 week long experiment additional blood samples were collected by cardiac puncture to investigate the presence of antibodies against arginine gingipain B (RgpB) which is a virulence factor of *P. gingivalis* essential for PPAD citrullination, antibodies against citrullinated peptides, as well as the levels of inflammatory markers and cytokines.

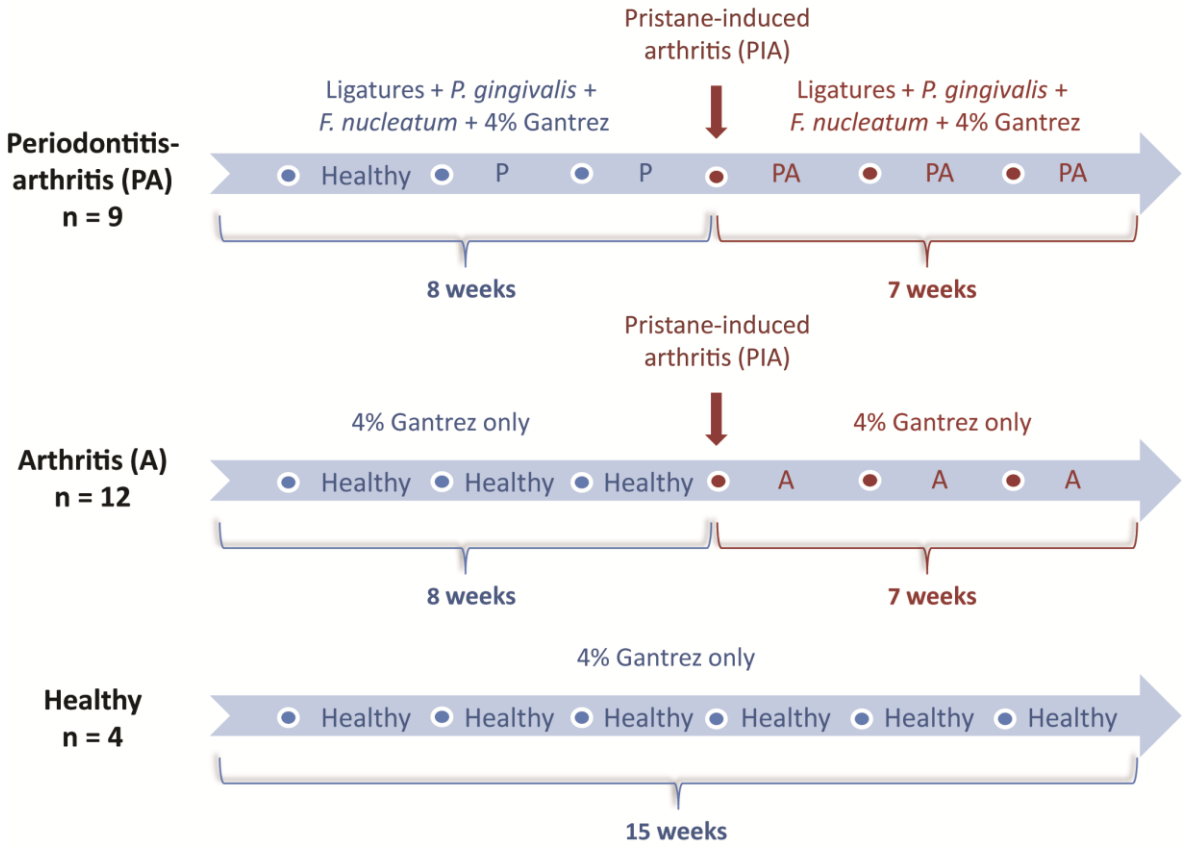

Figure 6.
A schematic illustration and timeline of the experimental protocol. Briefly, in the PA group, the experimental periodontitis was induced by tying silk ligatures around the upper second molars of DA rats and administering oral bacterial swabs (with *P. gingivalis* and *F. nucleatum* recostituted in 4% Gantrez medium). The periodontal status and tooth mobility were asssesed in all animals (PA, A and Heathy) every 10 days. In the PA group, additional bacterial swabs were also administered in-between the times for periodontal examination. Rats in the A and the Healthy control groups received swabs with only 4% Gantrez medium. At week 8, experimental arthritis (PIA) was induced in the PA and the A groups and arthritic changes were monitored in all animals until the end of the experiment (week 15).
PA, animals with experimental periodontitis and induced arthritis; A, arthritis induced animals without periodontitis; Healthy, animals without experimental periodontitis or arthritis, DA, Dark Agouti; PIA: pristane-induced arthritis.

The ligatures were recovered at the end of the study (week 15) to confirm the presence of *P. gingivalis* and *F. nucleatum* at the infected sites. One ligature per rat was transferred into FAB-medium and transported to the laboratory to culture the bacterial flora on anaerobic plates. The other ligature was snap-frozen in liquid nitrogen and transferred to -80°C, pending quantitative polymerase chain reaction (qPCR) analysis to detect specific bacterial DNA.

To assess the alveolar bone loss at endpoint (week 15), the heads of the animals were collected in 4% formaldehyde and stored at 4°C, for radiographic analysis. The front and hind paws were also collected and stored in the same way, to radiographically illustrate the bone loss and arthritis inflammatory changes in the rats. To detect the presence of bacterial

genomic DNA (gDNA) in the connective tissue and capsule of the joints, one paw per rat was frozen in liquid nitrogen and stored at -80°C for qPCR analysis. For details about the radiographic examination and the qPCR analysis the reader is referred to the Methods section of *Study II*.

ANALYSIS OF SAMPLES (*Studies II*, *III* and *IV*)

Quantitative polymerase chain reaction (qPCR) (*Studies II* and *IV*)

The presence of *P. gingivalis* in *Studies II* and *IV* was detected by qPCR analysis. First, gDNA containing the organisms genetic data, was isolated from the ligatures and subgingival plaque samples by using a QIAamp DNA Mini Kit (Qiagen, USA). During gDNA extraction the ligatures and bacterial plaque samples were resuspended in tissue assay buffer and lysed with proteinase K at 56°C, followed by washing steps with ethanol containing assay buffers as instructed by the manufacturer. The quantification and quality of the extracted gDNA was assessed spectrophotometrically with NanoVue spectrophotometer (GE Healthcare Bio-Science, Sweden) (*Study II*) or Quibit 2.0 fluorometer (Invitrogen, Life Technologies, USA) (*Study IV*).

The isolated gDNA was used in qPCR analysis to investigate the presence of bacteria in ligatures and joints from DA rats (*Study II*) and subgingival plaque samples from patients with RA (*Study IV*). In *Study II*, 40 ng of the target gDNA was assayed together with TaqMan Universal PCR Master Mix (2x) (Applied Biosystems, USA) and the specific bacterial primers and probe (Cybergene AB, Sweden), in a total reaction volume of 20 µl. In *Study IV*, the reaction volume of 20 µl comprised of 5 ng of template gDNA, TaqMan Universal PCR Master Mix (1x) together with the specific primers and probe (Eurofins Genomics, Ebersberg, Germany). The primers and probes used in these studies, targeting *P. gingivalis* and the universal 16S ribosomal RNA gene (16S), are described in detail in the manuscripts of *Study II* and *IV*. In both studies the 7500 Fast Real-Time qPCR System (Applied Biosystems, Foster City, CA, USA) was used and programmed as follows: initial incubation at 50°C for 2 min, a 10 min denaturation at 95°C followed by 40 PCR cycles of annealing at 95°C for 15 s and extension at 60°C for 1 min.

Analysis of CRP, α-1-AGP, RbpB, citrullinated/uncitrullinated PPAD peptides and inflammatory mediators (*Studies II* and *IV*)

To detect the levels of CRP and α-1-AGP, and the antibody levels against RgpB and citrullinated/uncitrullinated PPAD peptides (CPP3/RPP3), an enzyme-linked immunosorbent assay (ELISA) approach was applied. In *Study II*, plasma levels of CRP and α-1-AGP were detected by using prefabricated ELISA kits according to manufacturer instructions (Sigma-Aldrich, USA and Life Diagnostics Inc., USA, respectively). The samples, diluted 1:40 000, were incubated with the target specific antibodies (biotinylated rat CRP or anti-rat α-1-AGP) for 1 h or 45 min, respectively. Next the substrate-antibody complex solution was incubated with the enzyme horseradish peroxidase (HRP) conjugate

secondary antibody and finally the tetramethylbenzidine (TMB) reagent, which together form a colored product that can be detected spectrophotometrically. The absorbance of this colored substrate-antibody product was measured at 450 nm, and the plasma levels of CRP and α-1-AGP were calculated using mean absorbance from the standard curve. In *Study IV*, the CRP levels were measured in a similar matter by a commercially available ELISA kit (USCN Life Science, Wuhan, China). The saliva and GCF samples in *Study IV* were diluted 1:3 and 1:2, respectively, before being incubated with the CRP antibodies for 2 h, followed by incubation with HRP-conjugate secondary antibody and subsequently detected with TMB. The levels of antibodies against RgpB and citrullinated/uncitrullinated PPAD peptides investigated in *Study II*, were measured using an in-house ELISA [184]. Here the plasma samples were diluted 1:50 and 1:2, respectively, and incubated overnight in plates (MaxiSorp, Nunc) coated with RgpB (3.4 μg/ml) or CPP3/RPP3 protein (10 μg/ml). After a washing step with PBS-Tween (0.05%) the plates were incubated for 1 h with HRP-conjugated goat anti-rat IgG (Jackson Immuno Research Inc, USA) and the absorbance was measured at 450 nm.

Concentrations of cytokines and inflammatory mediators were investigated using the Bio-Plex 200 system and Bio-Plex immunoassay; specifically the Bio-Plex Pro™ Rat Cytokine 24-Plex and the Bio-Plex Pro™ Human Inflammation Panel 1, 37-Plex (Bio-Rad, Sweden), according to the manufacturer protocols. Detailed information about the investigated inflammatory mediators, including sensitivity levels, is provided in the Methods section of *Study II* and *IV*.

Immunohistological staining (*Study III*)

Immunohistochemical stainings were performed in *Study III* to detect the presence of citrullinated proteins and citrullinating enzymes (PAD2 and PAD4) in inflamed and healthy gingival tissues. The collected gingival biopsies were embedded in paraffin and sectioned (4 μm thickness). Sections of each biopsy were deparaffinised using xylene, rehydrated and stained with primary antibodies directed against citrullinated proteins (F95), PAD2 and PAD4 (Cosmo Bio, ROI002, Tokyo, Japan and Abcam, AB128086, Cambridge, UK, respectively) or against *P. gingivalis* using monoclonal antibodies produced by hybridoma HB-9968 (from ATCC). Isotype-matched control antibodies, mouse myeloma IgM (for F95), rabbit immunoglobulin fraction (for PAD2), mouse IgG1 (for PAD4) and IgG2b (for *P. gingivalis*), were used to stain corresponding negative control sections. Next, the sections were incubated with biotinylated secondary antibodies, goat anti-mouse IgM or goat anti-rabbit IgG (Vector Laboratories, USA), and the avidin-biotin complex (ABC-Elite solution) was used to enable the binding between the secondary antibody and peroxidase in the next step. The diaminobenzidine (DAB) substrate was added to the sections to obtain a brown colored product, visualizing the antigen localization. All sections were counterstained using Mayer´s hematoxylin to stain the cell nuclei blue and provide contrast, making visualization easier. To illustrate T-cell infiltration, sections of the gingival tissues were also stained with antibodies against the cluster of differentiation molecule CD3 (DAKO, A0452, Glostrup, Denmark). A visual

scoring system, with a 4 point scale (ranging between 0 – 3), was used to assess the grade of inflammation and the degree of positively stained cells in the gingival tissues. For details about the protocol and the scoring system please see under the Materials and Methods section in *Study III*.

STATISTICAL ANALYSES (*Studies I - IV*)

The Chi-square test or Fisher´s exact test was applied in *Studies I, III* and *IV*, to analyze the differences in dichotomous variables (e.g. nominal variables with only two categories such as gender, number of positively stained gingival samples or patients positive for *P. gingivalis*) between the groups. The Mann-Whitney *U* test and the two sample *t*-test were used when analyzing ordinal and continuous variables, such as differences in histological scoring, alveolar bone level or levels of inflammatory mediators etc (applied in *Studies II, III* and *IV*). A logistic regression analysis was performed in *Studies I* and *IV* to analyze the associations between RA related autoantibodies (ACPA/RF) and the prevalence and severity of periodontitis or presence of periodontal bacteria. The estimated odds ratios with 95% confidence interval were adjusted for residential area (*Study I*), gender, age and smoking habits (*Studies I* and *IV*). In all studies, *p* value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In the following section the results from studies included in this thesis will be highlighted and discussed in relation to previous literature. The full-length manuscripts of all included studies are available in the appendix. *Studies I* and *II* have been published in open access peer-reviewed journals and are reproduced in their published form. *Studies III* and *IV* are in manuscript form.

STUDY I

Prevalence of periodontal diagnostic codes in patients with RA and controls

The prevalence of periodontal disease, determined by periodontal diagnostic codes registered in the DHR by dentist and dental hygienists, was investigated in patients with RA and matched healthy controls included in the EIRA study. By performing a linkage analysis between the two Swedish registries, DHR and EIRA, we were able to investigate the prevalence of periodontal diagnoses in 2343 patients with RA and 3386 matched healthy controls. These subjects, representing 86% of the entire EIRA cohort (n = 6682), had visited the dentist at least once since the initiation of the DHR in 2008. Our results showed no differences in prevalence of gingivitis, periodontitis, peri-implantitis or increased risk of periodontitis/peri-implantitis between RA patients and controls (Table 2).

Table 2. Prevalence of periodontal diagnostic codes in RA cases and controls

Diagnosis	RA cases	Controls	*p* value[†]
Gingivitis	784 (33)	1193 (35)	NS
Increased risk of periodontitis	597 (25)	932 (28)	NS
Increased risk of peri-implantitis	17 (0.7)	20 (0.6)	NS
Periodontitis	762 (33)	1091 (32)	NS
Peri-implantitis	109 (4.7)	140 (4.1)	NS

Results are presented as number (%). RA, rheumatoid arthritis; NS, not significant.
[†]Statistical differences in prevalence of periodontal diagnostic codes between RA cases and controls. p value < 0.05 was considered statistically significant.

Our findings are in agreement with several other reports, showing no significant association between RA and prevalence/severity of periodontitis [118, 130, 132-137, 185]. For example in both an Indonesian (75 RA; 75 controls) and a Japanese population (80 RA; 38 controls), no differences in prevalence of periodontitis were reported between patients with established RA and healthy non-RA controls [133, 134]. Moreover, in the largest prospective study to date, including 81 132 female nurses of whom 292 developed RA, no association was found between incident RA and periodontal surgery and/or tooth loss [130]. On the contrary, several other studies have reported more prevalent and severe periodontitis in patients with RA, as compared to healthy controls [108-110, 112, 113, 115, 117, 119-122, 124, 125, 142]. For example, a weak but significant association was found between periodontitis and RA (OR =

1.17; 95% CI: 1.15 – 1.19, $p < 0.001$) in the largest registry study conducted to date, comprising of 1 025 340 Korean patients with different diseases (57 024 with RA out of which 46% had periodontitis) [112]. Similar ORs (OR = 1.16; 95% CI: 1.12 – 1.20) were reported in the second largest study, a Taiwanese linkage study including 13 779 patients with newly diagnosed RA in a total of 151 569 participants [109]. However, importantly, in these two large population based studies the authors were not able to adjust the analysis for smoking, the major confounding factor for periodontitis [112]. The inconsistent reports on the association between RA and periodontitis could be due to several factors such as ethnic differences between cohorts or differences in definition of periodontitis. In 2013 a review by Linden *et al.* concluded that there was a substantial heterogeneity of periodontitis definitions in previous studies and that very few reports met a stringent threshold for the disease [139]. The authors further stated that the previously conducted studies did not support a link between RA and periodontitis [139]. Although the definition of periodontitis still varies between different studies, recent systematic reviews and meta-analyses conclude that there is support for an association between RA and periodontitis [140, 186], and that the risk of periodontitis is increased in subjects with RA (relative risk = 1.13; 95% CI: 1.04 – 1.23, $p = 0.006$) [140].

Prevalence of non-surgical and surgical periodontal treatment in patients with RA and controls

In an effort to assess the severity of periodontitis in RA cases and controls, the prevalence of different non-surgical and surgical treatment codes were also investigated. The prevalence of the non-surgical periodontal treatment codes, recorded in combination with the diagnostic code for "periodontitis", showed no differences between RA patients and controls. The code for minor non-surgical treatment was prevalent in 59% of the RA cases and in 61% of the controls, whereas major non-surgical treatment was reported in 58% and 56% of the patients, respectively. In both RA cases and controls, 2% of the participants were treated surgically. Moreover, based on ACPA status in patients with RA, no significant differences were detected in any of the investigated treatment codes for periodontitis (minor- and major non-surgical, as well as surgical periodontal treatment).

Prevalence of periodontal diagnostic codes in relation to ACPA and RF status in patients with RA

ACPA and RF are two important serological markers in RA. They are both part of the classification criteria for the disease [46], and have been associated with RA disease activity and/or severity [70-72, 79]. Moreover, both ACPA and RF antibodies have also been reported in non-RA subjects with periodontitis [87, 99, 100]. In *Study I* we investigated the prevalence of periodontitis in relation to ACPA and RF status in patients with RA. Patients with ACPA-positive RA demonstrated no significant differences in the prevalence of any periodontal diagnostic codes (gingivitis, periodontitis, peri-implantitis, increased risk of periodontitis or increased risk of peri-implantitis) as compared to ACPA-negative RA (Table 3). In addition, there were no differences based on RF status (Table 3).

Table 3. Prevalence of periodontal diagnostic codes in RA cases, in relation to ACPA and RF status

Diagnosis	ACPA-positive	ACPA-negative	RF-positive	RF-negative	*p* value[†]
Gingivitis	487 (33)	291 (34)	508 (34)	270 (33)	NS
Increased risk of periodontitis	360 (25)	230 (27)	383 (25)	209 (25)	NS
Increased risk of peri-implantitis	10 (0.7)	7 (0.8)	12 (0.8)	5 (0.6)	NS
Periodontitis	487 (33)	268 (31)	498 (33)	259 (32)	NS
Peri-implantitis	72 (5.0)	37 (4.3)	76 (5.0)	33 (4.0)	NS

Results are presented as number (%). RA, rheumatoid arthritis; ACPA, anti-citrullinated protein antibody; RF, rheumatoid factor; NS, not significant.

[†]Statistical differences in prevalence of periodontal diagnostic codes between ACPA-positive and ACPA-negative or RF-positive and RF-negative RA cases. p value < 0.05 was considered statistically significant.

To our knowledge, the *Study I,* which includes 1469 ACPA-positive and 852 ACPA-negative subjects with RA, is the largest study investigating the prevalence of periodontitis in relation to ACPA status in patients with established RA. The results, showing no association between ACPA antibody status and periodontitis in subjects with established RA, are consistent with other reports [115, 187, 188]. Recently, among 264 Korean patients with established RA, no correlation was found between ACPA titers and periodontitis, as estimated by PPD or CAL [115]. Moreover, Swiss patients with established RA (n = 52) showed no differences in serum levels of ACPAs based on the prevalence of periodontitis [187]. Additionally, in a Dutch population, although ACPA positivity was significantly associated with RA, the association with periodontitis was only borderline significant [188]. Similar results have recently been reported also in individuals with pre-RA and early RA, showing no association between the presence of periodontitis and APCA or RF status [173]. Nevertheless, there are also studies reporting a significant association between ACPA antibody status and periodontitis [113, 114]. In a US population, the proportion of ACPA-positive RA patients with periodontitis was higher when compared to osteoarthritis controls (37% versus 26%), resulting in the OR of 1.59 (95% CI: 1.01 – 2.49, $p < 0.043$) [114]. RA patients with periodontitis have also been reported to have higher titer of both ACPA and RF as compared to RA subjects without periodontitis [113]. Moreover, RF-positive RA patients have been reported to have increased odds/OR for periodontitis and higher likelihood of being edentulous as compared to RF-negative RA [114, 124].

Age and smoking habits are associated with increased prevalence of periodontitis

Age and smoking are well known factors associated with the prevalence and severity of periodontitis [93, 189, 190]. *Study I* confirmed that both smoking (Table 4) and age increased the risk and prevalence of periodontitis both in healthy controls and in patients with RA. The prevalence of periodontitis increased significantly ($p < 0.01$) with age, but did not differ between RA cases and controls or based on gender or ACPA status. Moreover, logistic regression analysis showed that the risk of periodontitis increased with smoking habit after

adjusting for age, gender and residential area. In patients with RA, current smokers showed the highest risk for periodontitis (OR = 1.6, 95% CI: 1.2 – 2.0, $p < 0.05$), followed by ex-smokers (OR = 1.4, 95% CI: 1.1 – 1.7, $p < 0.05$) and ever-smokers (OR = 1.4, 95% CI: 1.2 – 1.7, $p > 0.05$) (Table 4). Similarly, the risk of periodontitis in healthy controls also increased by smoking habits (Table 4). The fact that we were able to detect and confirm the two well-known risk factors for periodontitis, age and smoking [93, 189], supported the overall dataset and the results obtained in *Study I*.

Table 4. Odds ratios (95% CI) for the association between periodontal diagnostic codes and smoking habits among RA cases and controls

Diagnosis	Current smokers	Ex-smokers	Ever-smokers
RA cases	1.6 (1.2 - 2.0)*	1.4 (1.1 - 1.7)*	1.4 (1.2 - 1.7)
Controls	1.8 (1.5 - 2.2)	1.1 (0.9 - 1.3)*	1.3 (1.1 - 1.5)*

The results demonstrate subjects with at least one of the diagnostic codes; increased risk of periodontitis; increased risk of peri-implantitis; periodontitis or peri-implantitis. RA, rheumatoid arthritis.

The odds ratios with a 95% confidence interval (95% CI) were adjusted for age, gender and residential area. *p value < 0.05.

Validation of the periodontal diagnostic codes in the DHR

At the time when *Study I* was performed, the DHR was a new registry. To validate the periodontal diagnostic codes in the DHR, one hundred dental records from EIRA participants (50 RA and 50 controls) were screened. Overall the results from the dental records confirmed the diagnoses obtained from the DHR in 90% of all investigated participants. The positive predictive value (PPV), indicating the probability that periodontitis was present when the patient had diagnostic codes for periodontitis or peri-implantitis in the DHR, was 89% (95% CI: 78 – 95%). The sensitivity of the DHR was 77% (95% CI: 65 – 86%), which meant that 77% of the participants with periodontitis were identified when using the periodontal diagnostic codes in the DHR. The specificity, indicating the accuracy of the DHR registry to correctly identify the patients that did not have periodontitis, was 71% (95% CI: 49 – 87%). The results above were in line with previous reports of the validity of both Swedish and other health databases, showing for example that the PPV ranges between 64 – 96% and the sensitivity between 51 - 90% [191-194]. Because the dental records in *Study I* were recruited from EIRA participants that self-reported having periodontitis, we were not able to report the negative predictive value for the DHR. The negative predictive value would have assessed the probability that periodontitis is not present when no periodontitis/peri-implantitis codes have been registered in the DHR.

STUDY II

Effect of pre-existing periodontitis on experimental arthritis

In *Study II*, experimental periodontitis was induced to assess the effects of pre-existing periodontitis on the initiation, rate of progression and severity of experimental arthritis (PIA). A severe localized periodontitis was established in all rats induced with experimental periodontitis (the PA group) prior to induction of experimental arthritis (week 8). In contrast, the animals in the group with experimental arthritis without pre-existing periodontitis (the A group), and healthy rats (without periodontitis or arthritis) did not show any clinical signs of periodontitis throughout the experimental period of 15 weeks. Macroscopic signs of inflammation, such as swelling and redness in limbs, appeared 2 weeks post PIA induction both in the PA and the A group (Figure 7). A period of remission, indicated by the decrease in mean arthritis score, occurred 4 weeks after the induction of PIA, as previously described [195]. Our results revealed also that here were no differences in the development or severity of arthritis based on the presence of pre-existing periodontitis, throughout the experiment (Figure 7).

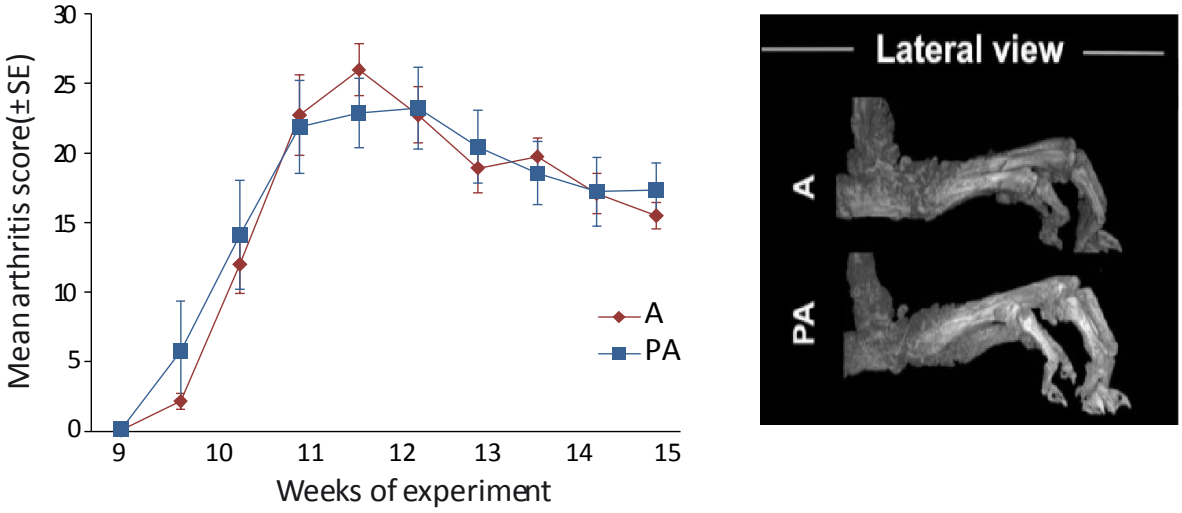

Figure 7.
The arthritis disease severity as assessed by the mean arthritis score (±SE), and micro-CT imaging of paws in arthritis rats with and without periodontitis.
PA, rats with experimental periodontitis and PIA; A, rats with PIA without periodontitis.

It has previously been suggested that pre-existing periodontitis, or infection with periodontal pathogens (such as *P. gingivalis*), may be associated with the development and severity of RA [19, 84]. In animal models, some studies have indicated that infection with periodontal pathogens (mainly *P. gingivalis*), administered subcutaneously or by oral gavage, can exacerbate the development and severity of experimental arthritis [84, 161, 163, 165]. Conversely, in agreement with our findings, others report no effect of periodontitis on the development or severity of experimental arthritis [159]. The inconsistent reports from different studies could potentially be due to different experimental models of periodontitis

and arthritis, or differences between strains of the periodontal pathogens administered. Most of the previous animal studies have been conducted in mice, where the size of the animals makes it difficult to obtain a more localized periodontal infection, and the administration of pathogens is therefore done subcutaneously or by oral inoculation/gavage [159, 161-163, 165]. In *Study II* we used the rat model due to its larger size, which enabled us to induce a more local periodontitis by tying ligatures around the teeth (that promote bacterial adhesion) and combining them with application of local swabs containing periodontal bacteria. Moreover, the lack of effect of periodontitis on arthritis reported by us and others [159] could also potentially be due to the *P. gingivalis* strains used or potentially the amount of bacteria administered. Since different *P. gingivalis* strains may vary in their ability to promote bone loss [164] they could also differ in the ability to exacerbate arthritis. Most previous animal studies reporting an effect of periodontitis or *P. gingivalis* infection on arthritis development have used the strains W50 or W83. In *Study II*, *P. gingivalis* (strain CCUG 14449) was administered in combination with *F. nucleatum* because *F. nucleatum* is known to promote the colonization of *P. gingivalis* [196]. Moreover, an infection caused by the combination of these two bacteria is known to induce a stronger inflammatory response as compared to either pathogen alone [197]. The discrepancies in the reported results could also be due to different models of experimental arthritis. In studies reporting a more severe arthritis as a result of co-induction with periodontitis, mostly collagen-induced arthritis (CIA) or collagen antibody-induced arthritis (CAIA) have been used [161-163, 165]. In *Study II*, we used the PIA model because it is a well-established experimental model of arthritis that closely mimics RA, including characteristics such as the presence of RF, symmetrical involvement of peripheral joints as well as cartilage and bone destruction [195]. In contrast to CIA and CAIA, which induce an acute arthritis, the PIA model triggers a chronic relapsing form which makes it suitable for studying different phases of the disease [195]. By using the PIA model in combination with the extended experimental period allowed us to investigate the effect of experimental periodontitis during both active and chronic forms of arthritis.

Effect of pre-existing periodontitis on arthritis associated acute phase proteins

α-1-AGP and CRP are two acute phase proteins associated with disease activity and degree of disability in patients with RA [198, 199]. The levels of CRP are part of the ACR classification criteria for RA disease [46], and α-1-AGP, described as a major acute phase protein in both humans and animals (e.g. rats and mice), has been suggested as a useful biochemical marker of RA disease activity [199, 200]. During the first 8 week of *Study II*, at the time of the periodontitis induction, the levels of plasma α-1-AGP were comparable in rats with and without periodontitis. After PIA induction there was a rapid increase in α-1-AGP, with a peak around 3 weeks post immunization. During the time of remission, indicated by the mean arthritis score, the levels of α-1-AGP decreased. There were no significant differences in plasma α-1-AGP between the groups, only a tendency (p = 0.07) towards higher levels in the group with periodontitis. A similar pattern was seen for the plasma levels of CRP. After PIA induction the levels of CRP increased in both the PA and the A group, only to decrease again following the clinical signs of remission. There were no significant

differences in CRP concentrations at baseline, after PIA induction or at endpoint based on the pre-existence of periodontitis.

Plasma levels of both α-1-AGP and CRP are typically elevated as a result of infection, inflammation or injury [200, 201]. Although the levels of both α-1-AGP and CRP have previously been investigated in rats with arthritis [202, 203], *Study II* was to our knowledge the first study reporting the plasma levels of α-1-AGP in rats induced with both periodontitis and arthritis. In a previous study the levels of α-1-AGP have been shown to correlate with the development of arthritis in rats with PIA [202]. In our study, the levels of both α-1-AGP and CRP reflected the clinical manifestations of the disease, but did not significantly differ based on the existence or absence of periodontitis.

Effect of pre-existing periodontitis on levels of inflammatory mediators in experimental arthritis

In the pathogenesis of RA and periodontitis, both cytokines and chemokines act as important signal mediators [6, 35]. By activating immune cells, fibroblasts and osteoclast as well as releasing proteolytic enzymes, these signal mediators collectively contribute to the characteristic bone and tissue destruction seen in RA and periodontitis [6, 35]. Interestingly, in *Study II* the mean levels of the investigated cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12, IL-13, IL-17, IL-18, TNF-α, RANTES, GM-CSF, M-CSF, MCP-1, GRO/KC, MIP-3α, EPO and VEGF) were increased in rats with both periodontitis and PIA, as compared to arthritis affected animals without pre-existing periodontitis. However, none of the differences reached statistical significance.

Several of the cytokines measured in *Study II* have previously been reported in periodontitis and RA, acting as either anti-inflammatory (such as IL-4 and IL-10) or pro-inflammatory (such as IL-1, IL-12, IL-17, IL-18 and TNF-α) mediators during inflammatory conditions [6, 204]. Chemokines such as MCP-1 and RANTES can induce chemotaxis and attract immune cells to the affected inflamed tissues [6]. Together these cytokines and chemokines play a crucial role in bone and tissue destruction [6]. The constantly higher plasma levels of the investigated cytokines in the PA group could potentially suggest that there might be an increased activation of the immune system as a result of the additional inflammatory stimuli due to periodontitis.

Effect of pre-existing periodontitis on antibody levels against RgpB and citrullinated PPAD peptides

Rats with pre-existing periodontitis showed elevated plasma levels of antibodies against arginine-gingipain B (RgpB) protease, an important virulence factor for periodontitis. In the PA group, 89% of the animals had antibodies against RgpB, whereas both group A (without pre-existing periodontitis) and healthy control animals tested negative for RgpB antibodies. Furthermore, the rats in the PA group also showed significantly ($p < 0.05$) increased plasma levels of antibodies against CPP3, a PPAD citrullinated peptide as compared to the periodontitis free animals. In contrast, there were no differences between the groups in

antibody levels against the uncitrullinated PPAD peptide, the RPP3, suggesting that the increased antibody response against CPP3 was citrulline-specific and not directed against other parts of the peptide.

The RgpB is one of *P. gingivalis* major virulence factors and is also important for *P. gingivalis* ability to citrullinate proteins [174]. These enzymes cleave protein at arginine residues before a citrullination by PPAD can occur [19, 174]. In patients with RA, the antibody levels of RgpB have been shown to be elevated as compared to non-RA controls [205]. To our knowledge, *Study II* is the first report showing the levels of RgpB in animals with experimental arthritis and pre-existing periodontitis. Our results indicate that local oral infection with *P. gingivalis* was able to stimulate an immune response in the periodontitis challenged animals with PIA (PA group), as compared to animals with PIA without pre-existing periodontitis (A group) and healthy controls.

The association between RA and periodontitis has been suggested to, at least in part, to be due to *P. gingivalis´* ability to secrete a citrullinating enzyme (PPAD) [87]. In support of this hypothesis, in arthritic mice inoculation with *P. gingivalis* has been demonstrated to cause significantly more cartilage and bone destruction compared to animals without *P. gingivalis* infection or even in animals infected with a PPAD deficient strain of *P. gingivalis* [162, 165]. Furthermore, non-significantly increased levels of ACPAs in serum have been detected in mice with both periodontitis and arthritis [162]. Significantly elevated levels of ACPAs have been reported following a subcutaneous infection with *P. gingivalis* in mice [163]. *Study II* was the first study reporting higher antibody levels against also a PPAD citrullinated peptide in arthritic animals with pre-existing periodontitis. It is however possible that the antibodies detected in previous studies might not be citrulline-specific, as these antibodies might be directed against some other part of the peptide rather than the citrulline-specific part [206]. Therefore, in *Study II* we also investigated the levels of antibodies against the non-citrullinated controls peptides (RPP3), showing no differences in these antibodies between rats with and without periodontitis. Taken together these results indicate that pre-existing periodontitis can induce a systemic antibody response against citrullinated peptides derived from PPAD, in rats with experimental arthritis. In patients who are positive for *P .gingivalis*, PPAD has been suggested to be responsible for citrullinating proteins in the gingival tissue, breaking the immune tolerance and thus contributing to the production of ACPAs [24, 87]. The host immune response, directed against PPAD citrullinated peptides, may then target host-citrullinated proteins present in the joint, and thereby contribute to the initiation and the autoantibody response in RA [24, 87, 207].

STUDY III

To further explore the possibility of citrullination in periodontitis-affected gingival tissue, the presence of citrullinated proteins and expression of citrullinating PAD enzymes (PAD2 and PAD4) were investigated in gingival tissue biopsies from patients with periodontitis and healthy controls.

Presence of citrullinated proteins in gingival tissue from patients with and without periodontitis

In *Study III* a monoclonal antibody (F95) was used for detection of citrullinated proteins [101]. The advantages when using monoclonal antibodies is that they produce less background staining and are less likely to cross-react with other proteins that are not of interest, as they only target one epitope per antigen [208]. In patients with periodontitis, increased staining of citrullinated proteins was observed in gingival connective tissue (80%, 12 out of 15 samples), as compared to the gingival connective tissue of healthy controls (27%, 4 out of 15 samples) ($p < 0.01$). With regard to gingival epithelium, there were no differences in the expression of citrullinated proteins in patients with and without periodontitis. The citrullinated proteins were detected mainly in association with inflammatory cells, fibroblast-like cells and the extracellular matrix of the gingival connective tissue, and were not correlated with the presence of *P. gingivalis*. In line with our results, higher levels of citrullinated proteins have been reported in inflamed gingival tissue collected from 29 patients undergoing periodontal surgery, when compared to 11 subjects without gingival inflammation [98]. The expression of these citrullinated proteins was mainly localized to the endothelial cells, fibroblasts and infiltrating inflammatory cells which are predominantly expressed in the connective gingival tissue, in agreement with our results [98, 209]. Likewise, in stroma from patients with periodontitis citrullinated proteins were detected in 80% (n = 12) of the periodontitis affected samples as compared to 33% (n = 2) in non-inflamed control tissues, when stained with polyclonal antibodies (AB5612) [101]. Taken together the results from these studies indicate that periodontitis may lead to the production of certain types of citrullinated proteins that are expressed in the gingival connective tissue.

Presence of PAD2 and PAD4 in gingival tissue from patients with and without periodontitis

Similar to the staining of citrullinated proteins, the expression of PAD2 and PAD4 enzymes were also significantly ($p < 0.01$) higher in the connective tissue from periodontitis affected individuals, compared to controls. In the gingival epithelium, no differences were reported in PAD2 or PAD4 enzymes. No correlation was found between the presence of *P. gingivalis* and the expression of the PAD2 or PAD4 endogenous enzymes.

The PAD2 and PAD4 enzymes are present in synovial tissue from patients with RA, and citrullination of proteins in the synovium has been attributed to these enzymes [210, 211]. In gingival tissue, in agreement with our findings, significantly higher expression of PAD2 and PAD4 enzymes have been detected in patients with periodontitis as compared to non-periodontitis controls [98]. The presence of these enzymes indicates the potential for citrullination in gingival tissue during periodontitis [98]. However, since the periodontitis associated pathogen *P. gingivalis* can invade gingival tissue [212], the increase of citrullinated proteins in periodontal tissue, detected in the present study and others, might have been produced as a result of PPAD rather that PAD enzymes [98]. In our study, there was no correlation between the citrullinated proteins and the presences of *P. gingivalis* in gingival tissue. Nevertheless, we did not investigate the expression of PPAD in the biopsies.

Unfortunately, there are no commercially available antibodies against PPAD and the presence of this bacterial enzyme in gingival tissue has therefore not yet been investigated. Even so, the results of increased expression of the important PAD enzymes and presence of citrullinated proteins in gingival tissue from patients with periodontitis suggest that citrullination may indeed present in the gingiva during periodontitis.

STUDY IV

Severity of periodontitis in patients with RA, in relation to ACPA and RF status

To evaluate the severity of periodontitis in patients with RA, 40 subjects with established RA underwent a full-mouth dental examination performed by a single examiner. The clinical and radiographic examinations revealed that the majority of the participants (75%, n = 30) had moderate to severe periodontitis. These RA patients had significantly higher PPD, CAL, number of furcation involved, mobile and missing teeth as compared to RA patients with no or mild periodontal disease (n = 10). APCA positivity was significantly (p = 0.032) more frequent in patients with moderate/severe periodontitis (86%) compared to the group with no/mild periodontitis (50%). Although RF positivity also seemed more frequent in patients with moderate/severe periodontitis (73% versus 50%), these differences were not statistically significant. Moreover, there were no between-group differences with regard to plaque or bleeding index based on periodontitis severity or ACPA status. Patients with moderate/severe and no/mild periodontitis were comparable regarding gender distribution, RA disease duration, type of RA medication used, comorbidities (such as diabetes, cardiovascular disease etc), body mass index (BMI), alcohol consumption, education and place of birth. In contrast, based on periodontitis severity, only age and smoking habits differed between the groups, in line with previous reports [93, 213]. Patients with no/mild periodontitis were significantly younger (mean age 50 versus 64 years, p = 0.010) and the majority of patients in this group were never smokers (60% versus 17%, p = 0.014) compared to subjects with moderate/severe periodontitis.

More severe forms of periodontitis, including increased PPD and CAL, have previously been reported in German, Iraqi, Indian, Australian and American populations with RA when compared to osteoarthritis or healthy controls [108, 117, 120-123, 128]. In *Study I*, we reported that Swedish patients with established RA showed no differences in the prevalence of periodontal diseases, when compared to healthy controls without RA. However, in that study we were not able to investigate the severity of periodontitis based on clinical measurements such as PPD and CAL. Therefore, in *Study IV* a clinical assessment of periodontitis severity was performed in patients with established RA. Our results indicate that more severe forms of periodontitis are frequent in patients with RA, which is in line with previous published data [111, 116]. Moreover, ACPA-positive RA was also more common in patients with moderate/severe periodontitis compared to no/mild periodontal disease. Similar associations between periodontitis severity and ACPA status or increased ACPA levels have been observed in American subjects with RA [116, 128]. Nevertheless, some studies do not

report significant association between periodontitis severity and ACPA-positive RA [115, 188]. For example, in a recent study including 264 patients with RA only bleeding on probing, but not PPD or CAL, was associated with ACPA status [115]. Also, when it comes to the association between RF status and periodontitis severity the results from previous studies are inconsistent. Some studies report an association between the prevalence and severity of periodontitis and increased RF antibody titers [113, 116], while other studies report a lack of association [115, 118]. The inconsistent results regarding periodontitis and seropositive RA may be due to differences between study populations such as ethnicity, or differences in classification criteria used to diagnose periodontitis. Moreover, it is also possible that an association between periodontitis and RA, or seropositive RA, exists only in susceptible individuals such as carriers of specific genes (e.g. HLA-DRB1 SE), or in subpopulations exposed to factors not investigated in these studies (e.g. other pathogens or environmental factors).

RA disease activity in relation to periodontitis severity

Several indicators of RA disease activity were investigated in patient with no/mild and moderate/severe periodontitis. There were no significant differences in the DAS28 score reported by the rheumatologists, the patients self-assessed HAQ score or the levels of the acute phase protein CRP in serum, saliva or GCF, based on periodontitis severity. Importantly, the two groups of patients were comparable for several potential confounding factors that could have affected the results such as RA disease duration, type of RA medication used and comorbidities.

In agreement with our results the lack of association between periodontitis severity and RA disease activity has been reported in other studies [118, 121, 123]. In both American (n = 50) and Indian populations with RA (n = 100), no association was found between DAS28 score and periodontitis severity [121, 123]. Moreover, serum levels of the acute phase proteins CRP and ESR have been reported to be comparable between RA patients with gingivitis and RA subjects with periodontitis [118]. In contrast to our results, several studies do report an association between loss of alveolar bone and RA disease activity and severity [114, 116, 119, 129]. In one cohort consisting of 287 American subjects with RA, tree studies have reported a significant ($p < 0.05$) correlation between periodontitis diagnosis/alveolar bone loss/self-reported "loose teeth" and increased DAS28 score, HAQ score or tender/swollen joint count [114, 116, 129]. In addition, higher DAS28 score was also reported in a Dutch population of RA subjects with severe periodontitis when compared to patients with moderate or no periodontal disease [119]. However, in this study the use of RA medication was not evaluated [119]. RA medications have previously been shown to improve periodontal parameters such as CAL and PPD [148, 154, 214]. It is therefore important to include the use of RA medication when investigating the associations between RA and periodontitis severity. In *Study IV*, we did account for RA medication, showing no differences in the prevalence of different types of medications based on the severity of periodontitis, which is in line with previous reports [114, 116]. However, it is possible that patients in the two groups with no/mild and moderate/severe periodontitis respond differently to RA medications. Moreover,

different subtypes of RA medications, such as TNF-α blockers and anti-IL-6 receptor antibodies (subtypes of biological DMARDs), could potentially differ in their ability to affect periodontitis severity. Therefore, accounting for subtypes of RA medications may provide additional information about potential differences in periodontitis severity between RA patients, not identified here. However, to be able to study such subdivisions of RA medications, larger cohorts are needed in order to have a sufficient number of patients to compare in different subgroups.

Prevalence of *P. gingivalis* in patients with RA, in relation to periodontitis severity and ACPA status

The reigning hypothesis of the link between periodontitis and RA proposes that an association may be due to *P. gingivalis* ability to secrete a citrullinating enzyme, resulting in citrullination of proteins potentially contributing to the production of ACPAs. Therefore, in *Study IV*, we also investigated the prevalence of *P. gingivalis* in patients with RA, in relation to ACPA status. The majority (60%) of the investigated RA subjects tested positive for *P. gingivalis* in subgingival plaque samples. There were, however, no significant differences in the prevalence of *P. gingivalis* based on ACPA status, or based on the presence/severity of periodontitis. The prevalence of *P. gingivalis* obtained in our study corresponded to the prevalence of *P. gingivalis* previously reported in patients with RA (47% - 67%) [114, 119]. Similar to our results, *P. gingivalis* was not associated with autoantibody status (ACPA or RF) in Southern European subjects at risk of RA, or with the presence of periodontitis in Dutch patients with established RA [180, 187]. On the other hand, an association between the prevalence of subgingival *P. gingivalis* and increased ACPA levels in serum has been reported in an American cohort of RA subjects [114].

Inflammatory mediators in serum, saliva and GCF of patients with RA, in relation to periodontitis severity

In RA subjects with moderate/severe periodontitis the levels of several inflammatory mediators were increased either in serum, saliva or in GCF, as compared to RA with no/mild periodontal disease. In serum the increased inflammatory mediators were APRIL, sCD30 and gp130, in GCF the levels of INF-α2, IL-19, IL-26, MMP-1 and sTNF-R1 were elevated, whereas in saliva only APRIL was increased. Interestingly, the levels of a proliferation-inducing ligand (APRIL) were up-regulated in both serum and saliva samples of RA subjects with moderate/severe periodontitis. APRIL is a cytokine that can stimulate the proliferation and maturation of B-cells [215, 216]. B-cells are involved in autoimmune diseases through different mechanisms including the secretion of antibodies (such as ACPAs) and presentation of autoantigens [217, 218]. APRIL could therefore potentially be involved also in the RA-periodontitis association, although our observation needs to be confirmed in other studies. Even though, in serum, increased levels of APRIL have previously been reported in patients with RA [216] and in patients with periodontitis [219] this is the first study reporting increased levels of APRIL based on periodontitis severity in subjects with RA.

STRENGTHS AND LIMITATIONS (*STUDIES I - IV*)

When interpreting the results of a study it is important to consider the studies design as it may affect the interpretation [220]. For example, when investigating the association between an exposure (e.g. a disease) and its effect on a population, factors such as bias (a systematic error of measurements due to faults in the studies design or conduct) can affects the estimated statistic, giving an incorrect assessment of the association [221, 222]. Flaws in the design are an issue in virtually all studies [222]. The designs of the studies included in this thesis have several strengths, but also some limitations, which are discussed below.

Study I

Study I was an observational case-control study where the association between periodontitis and RA was evaluated retrospectively by linking the information from two registries. The advantages of observational studies include relatively fast execution of the study and the opportunity to address a broad range of questions [222]. Moreover, using a registry study design allowed us to investigate the association between periodontitis and RA in a large population, thereby increasing the power of the analysis and the chance to detect an association if there was one. In fact, *Study I* is to our knowledge the largest study investigating the association between ACPA status and periodontitis in patients with RA. In addition, the well-defined population of the EIRA registry is another strength of this study. All RA cases in the EIRA registry were diagnosed by a rheumatologist following international consensus criteria for RA. Moreover, all controls were matched to RA cases for gender, age and residential area and were recruited at the same time as the RA cases. Furthermore, information was also available regarding important factors such as education level and smoking habits that could have affected the results. Because both low education level and smoking are associated with the prevalence and risk of periodontitis [93, 223], taking these factors into account is a strength in *Study I*. However, it is important to note that in this study we did not investigate whether periodontitis is a risk factor for RA or ACPA-positive RA before the onset of disease, since the RA patients included in this study had established RA at the time when DHR was initiated. Instead, *Study I* investigated whether there was an association between the presence of periodontitis and established RA disease.

A limitation of *Study I* was the lack of information about some potential confounding factors that could have affected the results. For example we did not have information about comorbidities, including diabetes mellitus that has been associated with increased risk of both periodontitis and RA [23, 38]. We also lacked information about RA disease activity and type of RA medication used at the time of periodontal diagnosis in the DHR. Anti-inflammatory medication used in treatment of RA has been proposed to decrease the progression of periodontitis and may have masked an association [148, 224]. Furthermore, when investigating the association between the prevalence of periodontitis and RA in relation to age, the number of participants in the younger age groups was low. Still, despite these limitations, two well-known risk factors/indicators for periodontitis (smoking and age) were

confirmed to increase the risk and prevalence of periodontitis in our cohort, supporting the dataset and the overall results of the study.

Study II

The animal study was an experimental study designed to assess the effect of an intervention, in this case the pre-existence of periodontitis on the development and severity of experimental arthritis. In this study the animals were randomly assigned to the different experimental groups and followed for 15 weeks. The long experimental period allowed us to assess the effects of pre-existing periodontitis not only in active arthritis but also during a period of remission. Additionally, when investigating the level of antibodies against PPAD citrullinated peptides we included a non-citrullinated control (RPP3) to ensure the specificity of the result. Because it is possible that antibodies may bind to other parts of the protein and not necessarily to the citrulline-specific part, the investigation of antibodies against RPP3, the uncitrullinated PPAD peptide, is an important strength of *Study II*.

The low number of animals, especially in the healthy control group is, however, a limitation of this study. We decided to assign the majority of the animals to the two experimental groups (the periodontitis-arthritis and arthritis without periodontitis) in order to increase the sample size and the potential to detect significant difference between these groups, and to still minimize the number of animals in the study for ethical and practical reasons. Additionally, because the objective of the study was to investigate the effect of periodontitis on arthritis, and not the effect of arthritis on periodontitis, we did not include an experimental group with periodontitis only. Another possible limitation of *Study II* could be the choice of the arthritis model, as the experimental arthritis used in this study (PIA) may simply have been too strong to demonstrate any additional effects that periodontitis may have had. However, other arthritis models such as CIA, widely used in studies investigating the association between periodontal pathogens and arthritis are comparable or more severe than the PIA model in rats [225]. Although it would have been possible to inject a lower dose of pristane, we decided to induce PIA using a standardize protocol thereby decreasing the risk of variability and increasing the chance to detect significant differences [226].

Study III

In *Study III*, we used a case-control study design were the gingival biopsies were collected from patients with the outcome of interest (here periodontitis) and compared to the gingival tissue from healthy controls. The groups with and without periodontitis were comparable with respect to gender and age distribution, eliminating these factors as potential confounders. Another strength of *Study III* was the blinded investigators evaluating the immunohistological stainings of the gingival tissues. None of the tree independent examiners had information about the periodontal status of the patients from whom the biopsies were collected, thus minimizing the risk of bias due to preconceived expectations [222].

A potential limitation of *Study III* was that we were not able to investigate the presence of the PPAD citrullinating enzyme in the biopsies, as there are no commercially available antibodies

for the staining against PPAD. This makes it difficult to speculate about the specific origin of the citrullinated proteins detected in the biopsies in this study, as they could potentially have been produced as a result of citrullination by PPAD rather than PAD enzymes. Moreover, it would have been interesting to investigate the presence of citrullinated proteins and PAD enzymes also in gingival biopsies from RA subjects with and without periodontitis, as well as patients at risk of RA. However, we did not have accesses to gingival tissues from these patients preventing us from investigating the relationship between periodontitis and the presence of citrullinated proteins/citrullinating enzymes in the gingiva of subjects with RA.

Study IV

Study IV was a pilot study with a cross-sectional study design. Cross-sectional studies are used when the purpose is to report the prevalence of the outcome of interest (e.g. periodontitis severity) or to describe a population at a specific time point [227]. The cross-sectional studies are a type of observational studies where the risk factors are measured at the same time as the outcome [222]. For example, in *Study IV* smoking habits were recorded at the same time as the severity of periodontitis in RA. The well-characterized data set is a strength of *Study IV*, containing information about several potential confounding factors for periodontitis and RA including RA disease duration, type of RA medication, comorbidities, BMI, smoking and alcohol habits, education level and place of birth. Moreover, the gender distribution among patients with RA reflected that of the general population indicating a representative data set.

There are however some limitations in *Study IV*. Because the main focus of this study was to investigate the severity of periodontitis in patients with RA no healthy controls were included. Moreover, the low sample size, especially in the group with no/mild periodontitis, makes it difficult to draw definite conclusions. In addition, due to the lack of information about the durations of different RA medications, we cannot exclude that this could have had an impact on for example the levels of inflammatory mediators analyzed in this study [228].

CONCLUDING REMARKS

This section summarizes the main finding of the thesis, including a brief discussion of potential future perspectives on the relationship between periodontitis and RA.

MAIN FINDINGS

- The prevalence of periodontitis in Swedish patients with established RA, included in the EIRA study, was not increased compared to matched healthy controls. Among subjects with RA, no differences in prevalence of periodontitis were detected based on ACPA or RF antibody status.

- Clinical investigations of oral health conditions revealed that moderate/severe periodontitis was common in patients with established RA. In addition, ACPA positivity was significantly more frequent in RA subjects with moderate/severe periodontitis compared to no/mild periodontal disease. There were no differences in RA disease duration or disease activity or type of RA medication based on periodontal diagnosis.

- The levels of several inflammatory mediators were significantly different in serum, saliva and GCF of RA patients with moderate/severe periodontitis compared to those with no/mild disease. The levels of the proliferation factor APRIL, involved in B-cell survival and maturation, were significantly increased in both serum and saliva of RA patients with moderate/severe periodontitis.

- Increased presence of citrullinated proteins and expression of PAD2 and PAD4 enzymes was detected in gingival connective tissue from patients with periodontitis as compared to periodontally healthy controls. The presence of *P. gingivalis* did not correlate with PAD2 or PAD4 expression or with the presence of citrullinated proteins.

- In animals with pristane-induced arthritis, the pre-existence of periodontitis resulted in increased levels of antibodies against a citrullinated peptide derived from PPAD. Rats with arthritis and pre-existing periodontitis also generated higher systemic immune response and antibody levels against RgpB, an important virulence factor of *P. gingivalis*. The development and severity of pristane-induced arthritis was not affected by the pre-existence of periodontitis.

FUTURE PERSPECTIVES

The relationship between periodontitis and RA has received increased attention the last two decades due to the hypothesis that oral infections by periodontal bacteria may play an important role in the pathogenesis of RA, as well as studies indicating that patients with RA may have an increased risk of periodontitis. However, many aspects of this association still remain unclear and warrant further investigation into the temporal relationship, the predisposing risk factors, the mechanisms involved, as well as the effects of both periodontal treatment and RA medications in the association between these two diseases.

The results from our and other studies indicate that periodontitis prevalence may not be increased in all patients with RA, since some studies including those investigating large population do not detect an association between these two diseases. Although a recent systematic review concluded that patients with RA have a slightly increased risk of periodontitis when estimated by markers of periodontitis severity (PPD and CAL) [140], it is possible that this association may be true mainly for specific subtypes of RA and/or for susceptible individuals. For example, in patients with RA primarily susceptible individuals carrying the HLA-DRB1 SE alleles appear to generate an immune response against citrullinated peptides [24]. Therefore, the inconsistent results between different studies could potentially be due to differences in study population such as genetic background, environmental factors or the composition of the oral microbiota. Thus, the relationship between periodontitis and RA warrants further investigation into the predisposing environmental and genetic risk factors, as well as the immune-inflammatory mechanisms linking periodontitis with RA.

RA medications have been suggested to be potential confounding factors masking an association between periodontitis and RA by reducing periodontal inflammation [229]. In light of the results from our pilot study however, only the type of RA medication alone may not entirely explain a potential lack of association, as our results showed no differences with regard to type of RA medications taken by RA patients with moderate/severe and no/mild periodontitis. Another explanation could be that patients respond differently to RA medications not only with respect to RA [230] but also regarding periodontitis, or that different subtypes of DMARDs/biological DMARDs may affect periodontitis severity differently. In our study, we were not able to investigate the association between different subtypes of DMARDs/biological DMARDs and the severity of periodontitis. Thus, future large prospective epidemiological studies, accounting for subtypes of different RA medications as well as the duration of treatment, may give further insight into a possible relationship between RA medication and the prevalence/severity of periodontitis.

The periodontal bacteria *P. gingivalis* may potentially be involved in the underlying mechanisms linking periodontitis to RA, as supported also by the results from our animal experiments. By inducing a systemic immune response to citrullinated peptides, *P. gingivalis*/PPAD enzyme could be involved in generating an RA specific immune response resulting in ACPA production. However, some studies also report a lack of association

between *P. gingivalis* and ACPA production [180, 187]. Moreover, recently the periodontitis associated pathogen *A. actinomycetemcomitans* has also been implicated in RA specific autoimmunity, due to its ability to trigger a dysregulated activation of citrullinating enzymes in neutrophils [104]. In this context, future research could benefit from studies investigating the microbiota profile in RA patients with and without periodontitis, conducted in large cohorts.

Given that ACPA status may be associated with periodontitis severity, future research on the association between periodontitis and RA should also focus on investigating the potential beneficial effects of periodontal treatment in patients with RA. Previous studies have indicated that non-surgical periodontal treatment in RA patients with periodontitis may reduce RA disease activity [142, 143, 145, 146, 148, 149]. However, all of these studies have a short follow-up time (6 weeks to 6 months) and the results should therefore be confirmed by additional studies conducted over a longer period of time. Moreover, longitudinal studies following patients at risk of RA as well as patients with newly diagnosed RA are also warranted to explore the role of periodontitis/periodontal infection in the development of RA.

To sum up, further research is needed including studies on the mechanisms linking periodontitis and RA before a causative relationship between periodontitis and RA can be established [107, 126, 140, 231]. Future research on the relationship between these two diseases should also focus on investigating the predisposing genetic and environmental risk factors for this association, the oral microbiota profile, subtypes of different RA medications and duration of these therapies, as well as the effects of periodontal treatment in patients with RA. Moreover, additional studies with focus on patients at risk of RA as well as newly diagnosed RA cases may also provide more insight into this relationship.

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