Expression of rheumatoid arthritis markers in normal and inflamed gingival tissues

A report submitted to the University of Adelaide in partial fulfilment of the requirements of the Degree of Doctor of Clinical Dentistry (Periodontology)

Geoffrey Harvey
# Table of Contents

Declaration .................................................................................................................................................. i

Acknowledgements ..................................................................................................................................... ii

Chapter 1. Literature Review .................................................................................................................. 1

1.1 Introduction ........................................................................................................................................ 1
1.1.1 Periodontitis .................................................................................................................................. 1
1.1.2 Rheumatoid arthritis .................................................................................................................... 2
1.2 Rheumatoid Arthritis markers ......................................................................................................... 3
1.2.1 Rheumatoid Factor ...................................................................................................................... 3
1.2.2 Antibodies against citrullinated proteins ..................................................................................... 3
1.2.3 Peptidyl arginine deiminase ....................................................................................................... 5
1.2.3.1 Peptidyl arginine deiminase type 2 ...................................................................................... 5
1.2.3.2 Peptidyl arginine deiminase type 4 ...................................................................................... 6
1.3 Rheumatoid arthritis and periodontitis ............................................................................................. 7
1.3.1 Studies examining prevalence/severity of periodontitis in RA patients ....................................... 7
1.3.2 Studies examining prevalence/severity of RA in periodontitis patients ...................................... 10
1.3.3 Effect of periodontal treatment on RA ....................................................................................... 10
1.3.4 Other links between periodontitis and RA .................................................................................. 12
1.4 Periodontal pathogens and RA ......................................................................................................... 13
1.4.1 Porphyromonas gingivalis ......................................................................................................... 13
1.4.2 Aggregatibacter actinomycetemcomitans .................................................................................... 15
1.4.3 Other oral bacteria ...................................................................................................................... 16
1.5 Drug treatment of RA and effects on periodontitis .......................................................................... 16
1.5.1 Non-steroidal anti-inflammatory drugs ....................................................................................... 16
1.5.2 Disease-modifying anti-rheumatic drugs .................................................................................... 19
1.5.3 Antibiotics .................................................................................................................................... 20
1.5.4 Cytokine blocking agents .......................................................................................................... 21
1.6 Rheumatoid Arthritis markers in periodontitis patients .................................................................. 22
1.6.1 Rheumatoid Factor in serum of periodontitis patients ............................................................... 22
1.6.2 Local production of RF in periodontal tissues ........................................................................... 23
1.6.3 Inflammatory markers in periodontitis and RA patients ............................................................. 23
1.6.4 Antibodies to citrullinated proteins in serum of periodontitis patients ...................................... 24
1.6.5 Local production of anti-CCP in the periodontium .................................................................... 25
1.7 Conclusion ......................................................................................................................................... 25
1.8 Hypothesis ......................................................................................................................................... 26
1.9 Aims of the study ............................................................................................................................... 26
1.10 References ......................................................................................................................................... 27

Chapter 2. Expression of rheumatoid arthritis markers in normal and inflamed gingival tissues ................................................................................................................................. 40

2.1 Introduction ......................................................................................................................................... 40
2.2 Materials and methods ....................................................................................................................... 41
2.2.1 Patient selection ........................................................................................................................... 41
2.2.2 Gingival tissue collection and processing .................................................................................... 42
2.2.3 Routine histological staining ........................................................................................................ 42
2.2.4 Immunohistochemistry for RF, CCP, PAD2 and PAD4 ............................................................... 43
2.2.4.1 Staining procedures ............................................................................................................. 43
2.2.4.1.1 Anti-Citrulline ............................................................................................................. 43
2.2.4.1.2 Peptidylarginine deiminase 2 ......................................................................................... 44
2.2.4.1.3 Peptidylarginine deiminase 4 ......................................................................................... 44
2.2.4.1.4 Rheumatoid Factor ...................................................................................................... 44
Appendix 1. Figures and Tables ................................................................. 67
Declaration

I, Geoffrey Harvey, declare that this work to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text. It contains no material which has been accepted for the award of any other degree of diploma in any university or tertiary institution.

I give consent to this copy of my thesis to be made available to the University Library, for loan or photocopying, subject to the provisions of the Copyright Act 1968, as well for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Declared by: ___________________________  Witnessed by: ___________________________
Geoffrey Harvey
Date: ______  Date: ______
Acknowledgements

I would like to take this opportunity to thank a number of people, whose support and input was crucial in the completion of my research:

First and foremost, I would like to express my utmost gratitude to Professor Mark Bartold for devising the original concept for this research, and for being a constant source of wisdom, guidance and expert advice throughout the duration of my studies.

I would like to extend special thanks to Dr Kencana Dharmapatni and Dr Tracy Fitzsimmons, whose expertise, unwavering patience, and support in the laboratory was of vital importance to the success of this project. Both Kencana and Tracy also provided me with constructive feedback which was highly valuable in the compilation of this report. I would also like to thank Ms Ceilidh Marchant for her technical assistance with the Periotron 8000, and advice on immunohistochemistry.

I am grateful to Dr Ghafar Sarvestani, who provided me exceptional instruction in the use of the NanoZoomer for digitising the immunohistochemistry slides, and also to Mr Yen-Liang Liu, who assisted with statistical analysis.

Many thanks go to my colleagues, Mrs Kerry Page, Ms Kylie Jones, Dr Kere Kobayashi, Dr Emma Megson, Dr Tina Choo, Dr Alexandre Hermann du Bois, Dr Siobhan Gannon, Dr Chris Bates and Dr Raymond Chan. The assistance with sample collection was much appreciated, but the friendship and memories will last for many years to come.

Thanks must also go to Ms Catherine Offler for her assistance in the final editing and preparation of this manuscript.

This project was funded by a grant from the Australian Dental Research Foundation, and I am sincerely thankful for this financial assistance. I would also like to acknowledge the Royal Australian Army Dental Corps, for providing excellent support throughout my studies and my career to date.

Finally, I’d like to convey my greatest appreciation and thanks to my family, and to my beautiful partner, Brittny Roberts. The support, kindness and understanding shown in the last three years has been fantastic, and has been an ongoing source of inspiration for which I am extremely grateful.
Chapter 1. Literature Review

1.1 Introduction

Chronic periodontitis and rheumatoid arthritis (RA) are common inflammatory diseases of complex aetiology. Various research groups have investigated a possible relationship between the two diseases, and whilst evidence of a link is emerging, the number of studies investigating this link is still relatively small. Several antibodies have been identified as markers for rheumatoid arthritis, and diagnostic tests are available to test for these markers in blood serum. Whilst studies have investigated a possible link between these diseases by correlating clinical parameters, relatively few studies have measured RA markers in periodontitis patients. Even fewer have investigated the presence/expression of RA markers in the periodontal tissues, and in these studies, thus far only rheumatoid factor (RF) has been studied.

1.1.1 Periodontitis

Periodontitis is a destructive inflammatory disease of the supporting tissues of the teeth and is a significant cause of tooth loss. The aetiology is very complex, multifactorial, and not completely understood. Hard- and soft-tissue destruction in periodontitis arises from a host-mediated immunoinflammatory response to bacteria in the plaque biofilm (Flemmig 1999). Whilst a large number of Gram-positive and Gram-negative bacterial species have been identified in plaque biofilm, only a few have been strongly implicated in the aetiology of periodontitis, including *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Tannerella forsythia* (World Workshop in Periodontology Consensus report 1996). Disease progression is determined by the host response, and this is influenced by a complicated interplay between a number of systemic, environmental and genetic factors (Kornman et al 1997, Page et al 1997). A number of inflammatory cytokines have been associated with hard and soft tissue destruction in the periodontium, including interleukin-1 (IL-1), interleukin-6 (IL-6), prostaglandin E2 (PGE2), and tumour necrosis factor-alpha (TNF-α); however, the mechanisms are still not completely understood (Listgarten 1987, Page 1991, Gemmell et al 1997, Reynolds and Meikle 1997).

Whilst it was previously thought that all individuals were equally susceptible to periodontitis, Hirschfeld and Wasserman (1978) described three distinct subpopulations with respect to progression of periodontitis: those who experience little or no disease progression (well-maintained periodontitis), those who experience moderate disease progression, which can generally be controlled by a combination of simple and advanced treatment, along with long-term maintenance (downhill periodontitis), and those who exhibit more rapid disease progression which tends not to respond to treatment, and often results in loss of teeth (extreme downhill periodontitis). In the following decades, numerous researchers reported similar findings, identifying three groups with comparable distribution to those reported by Hirschfeld and Wasserman (McFall 1982, Goldman et al 1986, Pearlman 1993, Matthews et al 2001). It is estimated that severe periodontitis affects 5-15% of the adult population (Papapanou 1999, Albandar 2005, Borrell et al 2005, Burt 2005, Albandar 2011).

The concept of varying susceptibility to periodontitis led to investigations into the possible contribution of genetic factors. Cutress et al (1982) reported distinct periodontal disease experience in two South Pacific Island populations who have relatively separate gene pools and yet had similar diets, lifestyles...
and oral hygiene practices. The prevalence of plaque, calculus and gingival inflammation was very high in all subjects at all in both populations, and yet the periodontitis experience was significantly worse in the Tongan population than the Western Samoan population, strongly suggesting a role for genetic factors in susceptibility. Studies on groups of twins have reported that approximately half of the disease variation in periodontitis is due to genetic influences (Michalowicz 1994, Michalowicz et al 2000). The exact role of genetics in periodontitis is still not well understood. Whilst a number of researchers attempted to investigate links between periodontitis and polymorphisms in genes which regulate particular cytokines, for example the IL-1 gene cluster (Kornman et al 1997), it has become evident that a complex interaction between many genes and environmental factors underlies disease susceptibility. As such, a ‘candidate gene’ approach is unlikely to identify a particular genetic polymorphism responsible for susceptibility to periodontitis.

1.1.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterised by persistent inflammatory infiltrate in the synovial membrane of joints, leading to joint damage, bone destruction, and (often severe) disability. Rheumatoid arthritis is relatively common, affecting approximately 1% of the population, and most commonly affects the elderly (Symmons et al 1994). The disease is more common in females than males, possibly suggesting a hormonal component in disease susceptibility (Arnett et al 1988, Mercado et al 2003, Alamanos and Drosos 2005). Disease progression may occur over long periods, with most patients showing radiographic evidence of joint damage within the first few years of diagnosis (Fuchs et al 1989). It is also common for RA patients to experience a progressive reduction in joint function (Scott et al 1987) and RA is associated with increased mortality (Mitchell et al 1986, Gabriel et al 2003).

Similar to periodontitis, three distinct subpopulations of RA patients have been reported: self-limiting RA, whereby disease commences but does not significantly damage the joint(s); easily-controlled RA, where disease is initiated but may be controlled by simple treatment, such as “first-line” medications; and progressive RA, where disease continually progresses and is often resistant to “second-line” medications (O'Sullivan and Cathcart 1972, Pincus et al 1984, Scott et al 1987, Mercado et al 2003).

Genetic influences are thought to be responsible for 50-60% of the risk of developing RA (MacGregor et al 2000a, MacGregor et al 2000b). An association between RA and alleles of the HLA-DRB1 locus has long been established (Gregersen et al 1987), and along with a single nucleotide polymorphism on another gene which has been reproducibly associated with RA (the protein tyrosine phosphatase, non-receptor type 22 gene), specific alleles in this locus confer a higher genetic risk of RA and account for approximately 50% of known familial aggregation of RA (Begovich et al 2004, Hinks et al 2007). A number of other potential genetic risk factors (including non-HLA genes) have been linked to RA, although there is significant racial variation, and few genome-wide associations have been carried out (reviewed in Tobon et al 2009). Recent studies have identified numerous potential genetic risk factors for RA, with many of these showing a possible association with genetic regulation of immunological pathways (reviewed in Bax et al 2011).

Just as the host response has become increasingly recognised in periodontitis, the inflammatory response in a susceptible host are key in the development and progression of RA. A number of potentially arthritogenic agents have been investigated, including exogenous infectious agents (Ebringer and Wilson 2000, Carty et al 2004), endogenous substances, such as connective tissue proteins (e.g. collagens and proteoglycans), and altered immunoglobulins.
The periodontal pathogens share several features with some species which appear to be arthritogenic in a susceptible host (in animal models) (Bahr et al 1988). This has lead to the theory that bacteria may be a common aetiological factor linking the two diseases. Although RA is still not largely recognised as a disease resulting solely from bacterial infection, a number of the pathogens implicated in periodontitis have been investigated for their possible role in RA (discussed in Section 1.4).

1.2 Rheumatoid Arthritis markers

The formation of autoantibodies is a common manifestation of RA (Vander Cruyssen et al 2005). The serum in RA patients contains a variety of autoantibodies, and these contribute to the disturbance in immunoregulation (Corrigall and Panayi 2002). The first of these antibodies to be discovered was the rheumatoid factor (Waaler 1940).

1.2.1 Rheumatoid Factor

Rheumatoid factor (RF) autoantibodies are found in every immunoglobulin subclass (IgA, IgE, IgG, and IgM), and they target the constant region (Fc) of human IgG (van Boekel et al 2002, Lee et al 2008). Early studies showed that whilst RF can be produced by plasma cells present in both inflamed and non-inflamed tissues, localised inflammation in the synovial compartment of RA patients provides favourable conditions for RF production (Mellors et al 1959, Vaughan et al 1976). Serum testing for RF in patients has been performed for over half a century (Jacobson et al 1956, Epstein et al 1957), and for many years the RF test has been among the revised American College of Rheumatology (ACR) diagnostic criteria for RA (Arnett et al 1988, Aletaha et al 2010). The long-term use of RF as a diagnostic factor is based on its high sensitivity for RA, with up to 85% of RA patients being seropositive for RF (Samanci et al 2005). Seropositivity for RF has also been associated with a number of deleterious clinical outcomes, such as poorer prognosis, progressive radiological signs of damage, and extra-articular manifestations (Chatfield et al 2009).

Whilst RF has a high sensitivity for RA, it has relatively low specificity. It has been detected in the sera of 5-10% of apparently healthy (non-RA) individuals, and 10-30% of healthy (non-RA) elderly individuals (van Boekel et al 2002, Lee et al 2008). It has been reported that seropositivity for three RF isotypes (IgM, IgG, and particularly IgA) predate clinical onset of disease by several years in patients who went on to develop RA (Rantapaa-Dahlqvist et al 2003).

Seropositivity to RF is also found in patients with other autoimmune diseases such as Sjögren’s syndrome, systemic lupus erythematosus, and mixed connective tissue disease, and some infectious diseases such as hepatitis (van Boekel et al 2002, Renaudineau et al 2005).

1.2.2 Antibodies against citrullinated proteins

Antibodies directed against citrullinated proteins are highly specific for RA (Schellekens et al 2000, van Venrooij et al 2006). Citrullination, or deimination, is the post-translational enzymatic modification of proteins, in which a positively charged arginine residue is converted into a neutral citrulline residue by an enzyme family known as peptidylarginine deiminases (PAD). Citrulline is a non-standard amino acid, which is a deiminated form of the amino acid arginine. Metabolism of citrulline is tightly regulated, and it is involved in the citric acid and ornithine cycles (Suzuki et al 2007). Citrullination is a physiological process...
which occurs during keratinisation of epithelial cells, inflammation, and apoptosis (Vossenaar et al 2003, Chang et al 2005, Szekanecz et al 2008). Since no citrulline tRNA exists, citrulline is not incorporated into proteins during translation, and thus the presence of citrulline in proteins is always the result of post-translational modification (Rosenstein et al 2004). It has been proposed that, in susceptible patients, presentation of citrullinated proteins to the immune system results in the production of auto-antibodies directed against citrulline or proteins containing citrulline (Schellekens et al 1998).

These anti-citrulline autoantibodies, known as anti-cyclic citrullinated protein (anti-CCP) antibodies, can be detected in almost 80% of RA patients’ sera, giving them a comparable sensitivity to RF. However, the specificity for RA is much higher than that of RF, with 88-100% specificity being reported in a number of systematic reviews and meta-analyses (van Venrooij et al 2002, Riedemann et al 2005, Avouac et al 2006, Nishimura et al 2007).

Studies in RA-affected synovial joints have shown that anti-CCP antibodies are produced locally in the joint at the site of inflammation by local plasma cells (Masson-Bessiere et al 2000, Reparon-Schuijt et al 2001). Autoantibodies to a number of different citrullinated proteins have been investigated for possible roles in RA pathogenesis, and also as potential diagnostic markers for disease onset/progression. The presence of citrullinated fibrin during joint inflammation has been confirmed in chronic and acute mouse models for arthritis (Vossenaar et al 2003). Another study showed that α- and β-citrullinated chains of fibrin deposited in joints are targeted by pathogenic autoantibodies in RA (Masson-Bessiere et al 2001). Vimentin (also known as the Sa antigen) is an intermediate filament protein expressed in mesenchymal cells, and it is citrullinated in macrophages undergoing apoptosis (Vossenaar et al 2004b). Antibodies to citrullinated vimentin have been associated with RA (Vossenaar et al 2004a), and seropositivity to these antibodies has been reported by some authors to be highly specific for RA either alone (Mathsson et al 2008) or in combination with other assays (Liu et al 2009).

Anti-CCP antibodies are showing promise as markers of disease, not only due to their specificity and sensitivity, but also due to their presence very early in the disease process (Schellekens et al 2000, Forslind et al 2001, Zendman et al 2004). It has also been shown that seropositivity for anti-CCP antibodies can predict future development of RA in both asymptomatic individuals and in patients with undifferentiated arthritis (Rantapaa-Dahlqvist et al 2003, van Venrooij et al 2006). Seropositivity for the anti-CCP antibody has also been associated with disease activity and disease severity (Bongi et al 2004, van der Helm-van Mil et al 2005). Whilst the presence of the anti-CCP antibody in serum has been linked to more severe disease, some studies have reported that serum levels of this antibody do not correlate well with disease activity or severity (Papadopoulos et al 2008).

It has been proposed that the anti-CCP antibodies may not only be markers for RA, but may be actively involved in the pathogenesis of the disease (Holers 2006). Indeed, an earlier study by Vossenaar et al (2004c) showed that the presence of citrullinated proteins in inflamed synovial tissue was not exclusive to rheumatoid arthritis. They suggested that the presence of citrullinated proteins in the synovium may be common to a number of inflammatory conditions, and that the high specificity of antibodies to these proteins in RA probably reflects an altered or abnormal humoral response to the citrullinated proteins in RA patients.

While anti-CCP antibodies are highly specific markers for rheumatoid arthritis, they do not exclude the diagnosis of other autoimmune diseases including psoriatic arthritis (Bogliolo et al 2005), systemic lupus (Hoffman et al 2005) and Sjögren’s syndrome (Gottenberg et al 2005, Atzeni et al 2008).
1.2.3 Peptidyl arginine deiminase

The peptidyl arginine deiminase (PAD, also known as protein arginine deiminase) family of enzymes was first reported over three decades ago (Rogers et al 1977) and was given its current name a few years later (Fujisaki and Sugawara 1981). In these seminal studies, the PAD enzyme was extracted from guinea pig hair follicles and the epidermis of newborn rats, and it was found to catalyse the conversion of arginine residues to citrulline residues within proteins. The reaction was found to be dependent on the presence of calcium ions, and only acts upon peptidylarginine; PAD are unable to convert free L-arginine to L-citrulline (Sugawara et al 1982).

So far, five isotypes of PAD have been cloned from mouse, rat, human, and other mammals: PAD1 to PAD4, and PAD6. As discussed in Section 1.4.1, one bacterial enzyme capable of citrullinating proteins has been reported in the literature (McGraw et al 1999). Produced by *P. gingivalis*, this bacterial PAD is evolutionarily and structurally unrelated to eukaryotic PAD, and whereas human PAD is only able to citrullinate peptidylarginine, *P. gingivalis* PAD is also able to citrullinate free L-arginine.

Whilst the different PADs share a number of primary structural features, tissue expression varies between classes, and certain proteins are citrullinated more rapidly than others by individual PADs (Senshu et al 1999). Of the PADs that have been described to date, the expression of PAD2 and/or PAD4 has been detected in the synovial tissues (Chang et al 2005, De Rycke et al 2005, Nakayama-Hamada et al 2005, Foulquier et al 2007) and synovial fluid of RA patients (Vossenaar et al 2004b, Kinloch et al 2008), suggesting that either or both may play a role in the pathogenesis of RA.

1.2.3.1 Peptidyl arginine deiminase type 2

Peptidyl arginine deiminase type 2 (PAD2) is the most widely expressed type of PAD, and has been detected in a number of different cell types, including macrophages, across a broad range of tissue types such as brain, exocrine glands, skeletal muscle, and bone marrow (Vossenaar et al 2004b). Until recently, PAD2 expression and function was thought to be limited to the cytoplasm, but a study by Cherrington et al (2010) reported detection of PAD2 in the nucleus of canine mammary epithelial cells. Cherrington et al (2010) suggested that it is possible that PAD2 localisation and function is at least partially dependent on the type of tissue in which it is expressed. Another recent study also detected PAD2 in the nuclei of hippocampal astrocytes and neurons in mice, and found that expression was similar to the levels detected in cytosol (Jang et al 2011).

De Rycke et al (2005) attempted to further investigate the RA specificity of citrullinated proteins, and in particular focused on PAD2 expression. They reported that PAD2 was expressed more frequently and at higher levels in the synovium of RA patients compared to control synovium. They also detected citrullinated proteins co-localised with PAD2, and suggested that it was likely that PAD2 is at least partially responsible for the presence of these proteins in RA synovium. The previously referenced study by Foulquier et al (2007) aimed to identify which PAD isotypes are expressed in the synovium of RA patients, and whether or not any are involved in the citrullination of fibrin. It was shown that PAD2 and PAD4 are the only PADs present in RA synovium, and also that synovial expression of PAD was not exclusive to RA. No correlation was found between the synovial expression of PAD2 or -4 and anti-CCP antibodies, although a significant correlation was found between PAD expression and the level inflammation. PAD4 was found at higher levels than PAD2, and whilst simultaneous detection in the same area was not detected frequently, both were observed
directly or in the close vicinity of fibrin deposits in the synovium. The results suggest that both PAD2 and PAD4 are involved in the citrullination of fibrin in the synovium of RA patients, and the authors concluded that both are likely involved in generation of autoantibodies which is thought to play a role in the pathogenesis of RA.

1.2.3.2 Peptidyl Arginine Deiminase Type 4

Peptidyl arginine deiminase type 4 (PAD4) is largely expressed in bone marrow and white blood cells, and is thus able to be detected in a number of tissue types (Ishigami et al 1998, Yamakoshi et al 1998). Unlike most of the other PADs, PAD4 has been detected in cell nuclei (Nakashima et al 2002) as well as cytoplasm. It has been implicated in a number of physiologic processes, such as apoptosis, histone deimination and regulating gene expression (Cuthbert et al 2004, Wang et al 2004), although most of these are still not completely understood.

In addition to these physiological functions, the role of PAD4 in a number of pathological conditions (including RA) has also been studied (Jones et al 2009). Chang et al (2005) found that PAD4 is expressed in a number of haemopoietic cells at high levels in RA synovium, and only at very low levels in controls. Citrullinated proteins and apoptotic cells were co-located with the PAD4, suggesting a role in citrullination and also in apoptosis in RA patients. Foulquier (2007) also reported the presence of PAD4 in the synovium of RA patients, co-located with PAD2.

There has also been some research suggesting that PAD4 may be involved in the genetic component of disease susceptibility to RA. Using single nucleotide polymorphism analysis, Suzuki et al (2003) showed that a functional haplotype of the gene which codes for PAD4 is associated with susceptibility to RA and is also related to the production of anti-CCP in RA patients. From this finding, they suggested that this gene (known as the PADI4 gene) may play an important role in the complex polygenetic pathogenesis of RA.

In a subsequent study, the same research group developed an ELISA system using recombinant human PAD4 to identify antibodies to PAD4 in serum (Takizawa et al 2005). They found that the prevalence and the titres of antibodies to PAD4 are significantly higher in RA patients than in healthy controls and patients with other rheumatic diseases. In conjunction with the results of their previous study, the authors concluded that antibodies to PAD4 are over-expressed in RA, and they suggested that PAD4 plays a role in the pathogenesis of RA.

A more recent study examined both of these factors, and reported a possible link between over-expression of PAD4 and RA pathogenesis (Harris et al 2008). Sera from RA patients and healthy controls were analysed for the presence of antibodies to PAD4, and genotyping was also conducted to examine expression of the PADI4 gene. The authors reported that the previously reported ‘susceptible haplotype’ of PADI4 gene was significantly associated with the expression of antibodies to PAD4. Interestingly, the same association was not found for anti-CCP antibodies. The authors also reported that expression of antibodies to PAD4 was associated with more severe disease. As such, the authors concluded that polymorphisms of the PADI4 gene influence the immune response to PAD4, and that this in turn may contribute to disease severity and progression.

A number of European studies have shown no association between polymorphisms in the PADI4 gene and susceptibility to RA (Martinez et al 2005, Burr et al 2010), suggesting that this gene may not play a role in disease susceptibility in all racial groups.
Another study by Auger et al (2009) examined the possible role of PAD4 as an autoantibody in RA, and also suggested the possibility that it not only citrullinates other proteins, but also citrullinates itself. They found that anti-PAD4 antibodies were positively correlated with anti-CCP antibodies, and also detected anti-PAD4 antibodies in some RA patients who were anti-CCP negative. The authors suggested that the citrullination of PAD4 may be involved in initiating the formation of autoantibodies to citrullinated proteins in susceptible patients. A follow-up study to examine the nature of the anti-PAD4-antibodies’ influence on citrullination found that these autoantibodies inhibited the citrullination of fibrin by PAD4 (Auger et al 2010). Another study by Kolfenbach (2010) retrospectively analysed stored pre-diagnosis serum samples from RA patients, and found that anti-PAD4 antibodies were present years before clinical onset of disease in a significant proportion of patients. Seropositivity for anti-PAD4 antibodies was also significantly related to the presence of anti-CCP antibodies. Whilst further research is required to investigate the temporal relationship, the results of this study appear to add weight to the theory that PAD4 is related to the presence of anti-CCP antibodies, and that both play a role in the development of RA prior to clinical onset.

1.3 Rheumatoid arthritis and periodontitis

Whilst evidence of a link between these diseases is emerging, the number of studies investigating this link is still relatively small. Recent reviews of the literature reported that meaningful conclusions from past studies are often difficult to draw. There have been conflicting results reported, which appears to be largely due to lack of uniformity in classification and indicators being measured (Bartold et al 2005).

1.3.1 Studies examining prevalence/severity of periodontitis in RA patients

An early study of periodontal conditions among of RA patients compared with those of an age and sex-matched random sample of non-RA subjects actually reported better periodontal health in RA patients (Sjostrom et al 1989). Severe periodontitis was observed less frequently in RA patients (12%) than in the controls (16%). The authors suggested that the difference in periodontal status between the two groups could reflect an effect of the long-term administration of non-steroid anti-inflammatory drugs (NSAID) in the RA patients. This suggestion is supported by a large volume of literature in which it has been demonstrated that NSAID can suppress the development of gingival inflammation and periodontal breakdown, originating with Nyman’s beagle study (1979) and progressing to a number of further animal and clinical trials (see Section 1.5.1). The RA patients in Sjostrom’s study (1989) were also found to have less plaque and calculus than the control group, which the authors suggested could indicate a difference in periodontal care.

Yavuzilma et al (1992) compared a number of clinical and immunological parameters in RA patients and periodontitis patients. The periodontitis group had significantly greater pocket depths and gingival inflammation than the RA group, although there was no difference in the number of missing teeth between the groups. The authors concluded from their findings that RA patients are not a risk group for advanced periodontal problems in comparison with age- and sex-matched systemically healthy controls.

In spite of the seemingly negative findings returned in these early studies, a number of studies have reported worse periodontal health in RA patients than systemically healthy controls.

Tolo and Jorkjend, (1990), examined 37 RA patients and age- and sex-matched controls for number of teeth, alveolar bone loss, serum immunoglobulins, and serum antibodies to three bacterial species (Porphyromonas gingivalis, Capnocytophaga ochracea and Eubacterium saburreum). On average, patients
in the RA group had significantly fewer teeth and increased alveolar bone loss compared to the control group. The RA patients also had a significantly increased level of serum IgG and IgA, but not IgM. In the RA group, a significant association was found between alveolar bone loss and age, IgG antibody against *P. gingivalis* and the level of total serum IgG.

Kasser *et al* (1997) attempted to quantify periodontitis in patients with long-standing active RA and controls, and to examine any relationship between the tissue destruction resulting from each disease. A number of clinical parameters, including gingival bleeding index, probing depths, and clinical attachment level, were recorded to determine the patients’ periodontal condition. Tissue destruction in RA was determined radiographically using a scoring system first described by Larsen *et al* (1977). Rheumatoid arthritis activity was assessed by clinical and nephelometric examinations, and functional capacity was also tested so its potential effect on oral hygiene (and, possibly, periodontitis) could be determined. Potential confounders such as smoking, sex, age, and oral hygiene efficiency were controlled. The results showed significantly worse periodontal condition in the RA group, with a 50% greater rate of gingival bleeding, 26% greater probing depths, 173% greater attachment loss, and 29% more missing teeth than non-RA controls. Even in RA patients with severe functional impairment in the hands, oral hygiene was apparently not affected, with the authors finding no correlation between joint functionality measures and plaque formation. No correlation was found between the radiographic joint destruction (Larsen score) and periodontal attachment loss. The authors concluded that patients with longstanding active RA have a substantially increased frequency of periodontal disease and increased tooth loss compared with controls. Rheumatoid arthritis patients taking anti-inflammatory drugs had significantly less periodontitis, and the authors suggested that this drug treatment might have masked a possible correlation between clinical incidences of the two diseases. A slightly expanded follow up study on the same population was published by the same group a year later (Gleissner *et al* 1998). The authors found no correlation between the duration of drug treatment and periodontal parameters, and deduced that there is no connection between long-term use of NSAIDs, corticosteroids and disease-modifying anti-rheumatic drugs (DMARD) and severe periodontitis observed in RA patients.

Mercado *et al* (2001) studied RA patients from a hospital rheumatology clinic to determine the extent of their periodontitis and investigated possible correlations with clinical indicators of RA. As well as testing for a relationship between periodontal bone loss and rheumatological parameters, the authors compared bone loss in RA patients with healthy controls. Whilst no differences were found between the control and rheumatoid arthritis groups in plaque and gingival bleeding indices, tooth loss was greater in the RA group, and a higher proportion of RA patients had moderate to severe periodontitis. A number of clinical and laboratory indicators for RA showed a positive association with periodontal bone loss: swollen joints, health assessment questionnaire scores, levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). The authors postulated that the apparent relationship between clinical and laboratory parameters of the two diseases may reflect an underlying dysregulation of the inflammatory response. They also noted that there was no evidence that limited manual dexterity in RA patients is associated with greater plaque accumulation, and that this does not significantly contribute to the periodontitis experience in these patients.

A study by Zhang *et al* (2005) also concluded that individuals with rheumatoid arthritis are more likely to experience periodontal disease compared to healthy subjects, and that there is an increased likelihood of these patients’ periodontitis being moderate to severe.
Another study investigating periodontitis in RA patients and comparing to healthy controls was performed by Ishi Ede et al (2008). The authors reported that on average RA patients had fewer teeth, more plaque, and more sites with advanced attachment loss than non-RA controls. Despite the increased plaque levels found in RA patients, there was no difference in gingival bleeding on probing between the groups.

A recent study of US veterans found that patients with RA were almost twice as likely to have moderate to severe periodontitis compared to control patients who did not have RA (Dissick et al 2010). Interestingly, it was also found that seropositivity to either RF or anti-CCP antibodies was associated with more severe periodontitis in RA patients compared to seronegative RA patients and non-RA controls.

It has been postulated that the loss of joint function in RA patients may result in significant impairment of oral hygiene procedures, which in turn may affect periodontitis experience in susceptible patients (Feldman et al 1983). A number of studies have mentioned this concept as a minor factor, rather than investigating it as a primary study outcome, and numerous studies have reported no significant effect on periodontitis experience (Sjostrom et al 1989, Kasser et al 1997, Mercado et al 2001). A recent study by Pischon et al (2008), considered the effect of oral hygiene and risk factors for periodontitis when examining the association between RA and periodontitis in 57 subjects with RA and 52 healthy controls. Subjects were matched for age and gender, and potential risk factors for periodontitis, such as smoking, as well as chronic diseases associated with RA and periodontitis, were assessed through questionnaires. The results showed that RA was significantly associated with higher odds of periodontitis compared to controls (odds ratio 6.21, 95% CI), and this association remained when potentially confounding factors were controlled. In examining the effect of oral hygiene on this association, the authors reported that plaque index accounted for 12.4% of this association, gingival bleeding accounted for 11.1%, and the two parameters combined accounted for 13.4%. As such, the authors concluded that patients with RA have significantly increased periodontal attachment loss compared to controls, and that oral hygiene may only partially account for this association.

While most of these studies have investigated possible links between RA and chronic periodontitis, one group studied young (20-35 year old) patients with aggressive forms of the disease (Havemose-Poulsen et al 2006). This is particularly interesting because this form of periodontitis has previously been associated with genetic and/or familial aetiology more so than other forms of periodontitis, and it has previously been estimated that genetic factors may be responsible for up to 50-60% of the aetiology of RA (MacGregor et al 2000a). Blood samples were taken from patients in five different groups: localised aggressive periodontitis, generalised aggressive periodontitis, juvenile idiopathic arthritis, RA, and healthy controls. The samples were analysed for ESR, CRP, and a number of antibodies associated with RA. The patients’ periodontal condition was also assessed, and numerous clinical parameters were recorded. The authors hypothesised that patients with these periodontal or arthritic conditions would share similar haematological and periodontal characteristics, indicating a shared systemic immune response in these patients (as distinct from healthy controls). The results showed that RA patients had greater periodontal attachment loss and alveolar bone loss than healthy controls, which is in accordance with most of the previously mentioned studies on older RA patients. It was noted that none of the RA or juvenile idiopathic arthritis (JIA) patients in this study had severe periodontal pocketing of ≥6 mm, and indeed none of the arthritis patients had periodontal destruction as severe as that seen in the generalised aggressive periodontitis group. The number of interproximal sites with pocketing of ≥4 mm was comparable in the RA and localised aggressive periodontitis groups. The results showed an association between two types of rheumatoid factor (IgM-RF and IgA-RF) and antibodies to citrullinated proteins (anti-CCP, as discussed below), but did not find any significant association between
the anti-CCP antibodies and any of the periodontal parameters measured. Due to a number of confounding factors, such as treatment with anti-rheumatoid drugs, the authors did not make any definite conclusions from their results. However, they did suggest that there were sufficient similarities in haematological and periodontal measures in the periodontitis and arthritis groups, and sufficient distinction between these groups and the healthy controls, to warrant further studies including more markers of inflammation and tissue destruction.

1.3.2 Studies examining prevalence/severity of RA in periodontitis patients

Mercado et al (2000) investigated the relationship between the disease experience of periodontitis and RA patients, assessing 1412 dental patients in a university clinic. They hypothesised that periodontitis patients would have a higher prevalence of RA than patients without periodontitis. As a secondary objective, they also sought to investigate the notion that RA patients would have a higher prevalence of severe periodontitis than patients with periodontitis who did not have RA. Self-reported health questionnaires were used to evaluate patients’ RA status, with the authors acknowledging the limitation of doing so, but asserting the need for a large sample of baseline data. Patients referred for treatment of periodontitis had a higher prevalence (3.95%) of self-reported RA than patients attending for other dental treatment (0.66%), and this figure is significantly higher than the figures previously reported for the general population (1%) (Symmons et al 1994). More than 60% of the RA patients identified in the study had severe periodontitis, whilst 43% of the periodontitis patients without RA were classified as having moderate or severe disease. Whilst the number of RA patients (n=32) was relatively small compared to the number of non-RA periodontitis patients (n=777), the difference in prevalence of moderate or severe disease was statistically significant between the two groups. Whilst the study had a number of limitations, it was nonetheless important research, and provided promising evidence that not only are patients with moderate or severe periodontitis at a higher risk of developing RA, but that RA patients who develop periodontitis are more likely to suffer from severe disease than patients without RA.

A recent cross-sectional study attempted to assess the associations between periodontitis and a number of systemic conditions, including RA, cardiovascular disease and diabetes (Nesse et al 2010). In particular the authors wanted to examine the possible contribution of confounding factors to any observed associations. Whilst the low prevalence of RA made it difficult to make a meaningful evaluation of the data, the authors nonetheless found an increased prevalence of RA among periodontitis patients. They reported that this increased prevalence could not be attributed to confounding factors such as smoking, whereas the increased prevalence of a number of the other diseases was at least partially due to such factors.

1.3.3 Effect of periodontal treatment on RA

Whilst a large volume of literature exists on the efficacy of non-surgical and surgical periodontal treatment, little has been published on the possible effect of such treatment on the severity or progression of RA. Although one case report from Japan was published, describing the remission of RA following periodontal treatment (Iida and Yamaguchi 1985), it was a further 20 years before larger studies were conducted to investigate this further.

Ribeiro et al (2005) evaluated the clinical effects of periodontal treatment on a number clinical and laboratory indicators of disease severity in RA patients with periodontitis. In a sample of 42 patients, all
patients received (supragingival) “professional tooth cleaning” and oral hygiene, while half of the patients also received full-mouth subgingival scaling and root debridement over multiple appointments (up to eight visits). Patients were followed up monthly, and further treatment (as per the original protocols in each group respectively) was provided as required. Clinical periodontal charting was performed at baseline and three months after treatment. Biochemical markers measured included changes in RF levels and erythrocyte sedimentation rate (ESR), and each patient’s medication regime was assessed and recorded. Periodontal parameters improved in both groups, although clinical attachment and reduction in deep pockets was only observed in the subjects who received subgingival scaling and root debridement. There were minor reductions in the RA parameters observed, although only ESR was significantly reduced for the second group after SRP. Serum levels of RF did not show statistical reductions and there was no significant improvement in joint function in either group. The authors found no correlation between RA severity and periodontal clinical parameters, and suggested that this may be due to the small sample size and/or the relatively low severity of periodontitis in the patients studied. They concluded that the significant reduction in ESR might be due to the periodontal treatment, and that there was a tendency towards improvement in RA parameters (although statistical significance was not achieved).

Al-Katma et al (2007) studied the effect of non-surgical periodontal therapy on the severity of active RA in patients with mild-to-moderate generalised chronic periodontitis. Over 300 RA patients were identified from university rheumatology clinics, although only 38 patients commenced the trial, and a number of these patients dropped out or were excluded due to poor compliance. All patients included in the study had a confirmed diagnosis of RA and mild-to-moderate chronic periodontitis of at least three years' duration. The activity of RA was assessed using the disease activity score test (DAS28), a numeric index that was introduced and validated by Prevo et al (1993, 1995), and ESR. All patients continued their RA treatment, including use of disease-modifying anti-rheumatic drugs (DMARD), as directed by treating rheumatologists, and the groups were matched for common risk factors for both periodontitis and RA. The treatment group (n=17) received periodontal treatment consisting of scaling/root planing and oral hygiene instruction whereas the 12 patients in the control group received no periodontal treatment. The treatment group also received weekly periodontal examination, prophylaxis, and reinforcement of oral hygiene instruction for the entire study period. Baseline measurements of RA and periodontal parameters were recorded, and there were no differences between the groups. Measurements of RA parameters and periodontal indices were recorded at baseline and at eight weeks for each participant. Improvement in RA scores was noted in 58.8% of the treated group and 16.7% of the untreated group, with a statistically significant difference in DAS28 and ESR between the treatment and the control groups. The authors deduced that this improvement was not due to DMARD or other systemic factors, as the groups had been matched for these factors and no alteration to RA treatment was implemented. The authors concluded that control of periodontal infection and gingival inflammation by scaling/root planing and plaque control in subjects with moderate periodontitis may reduce the severity of signs and symptoms of RA. They also noted that patients reported subjective improvement following periodontal treatment.

Ortiz et al (2009) examined the effect of non-surgical periodontal treatment on the signs and symptoms of RA in patients treated with or without tumour necrosis factor-alpha (TNF-α) blocking agents. Subjects who received periodontal treatment showed improvement in a number of RA indicators, including DAS28, ESR, number of swollen joints and tender joints, and self-reported symptom severity as measured on a visual analogue scale. This improvement was not observed in patients who received no periodontal
treatment. Periodontal treatment also resulted in a reduction in serum TNF-α levels, which was not observed in untreated patients. It is worth noting that all patients in this study were also taking disease-modifying anti-rheumatic drugs, which may or may not have affected the results.

A recent Brazilian study examining the effect of periodontal treatment on clinical and serological indicators of RA failed to show any significant differences after six months (Pinho Mde et al 2009).

1.3.4 Other links between periodontitis and RA

A review by Mercado et al (2003) examined a number of similar features in periodontal disease and RA, acknowledging that whilst it is likely that the aetiology of these two diseases is quite different and that a causal relationship is unlikely, the underlying pathogenic mechanisms appear to be similar. From a microbiological perspective, it was postulated that chronic exposure to virulence factors associated with the periodontal pathogens, namely, lipopolysaccharides (LPS), may trigger the inflammatory cascade seen in RA. This review also discussed possible roles for acute phase proteins and inflammatory mediators in the pathogenesis of both RA and periodontitis.

Scardina and Messina (2007) investigated the differences in periodontal microcirculation between 30 healthy subjects and 30 RA patients. In light of previously published studies reporting vascular lesions in RA, Scardina and Messina examined microcirculation in the periodontal tissues to determine whether the vasculature in the periodontium was affected, with the inference being that impaired circulation in these tissues may increase the risk of periodontitis. Capillaroscopy was used to evaluate a number of characteristics of the microvasculature of the periodontium. Visibility, course, tortuosity, average calibre of the capillary loops and the number of visible capillary loops per square millimetre were evaluated for each patient, and the presence of any microhaemorrhages was noted. In patients suffering from RA, a greater number of capillaries were noted, and it was also reported that the capillaries were elongated and of smaller calibre than the average reported in healthy controls. No significant differences relating to oral capillaroscopic pattern were detected between RA patients that were seropositive for RF, antinuclear antibodies (ANA), rheumatoid arthritis nuclear antigens (RANA) and RA patients that were seronegative. The authors concluded that capillary alterations in RA patients are manifested in the periodontal microcirculation, and suggested that these alterations may contribute significantly to the complex aetiology of periodontitis in RA patients.

A study by de Pablo et al (2008) used data from the third National Health and Nutrition Examination Survey (NHANES III) to test for an association of periodontitis and tooth loss with RA. Of the 4461 participants, 103 had RA. Compared to the non-RA subjects, the RA patients had more missing teeth, but a lower caries rate. After adjusting for possible confounding factors such as age, sex, race/ethnicity, and smoking, the authors reported that RA patients were more likely to be edentulous and have periodontitis compared with non-RA subjects. Whilst rheumatoid factor (RF) seropositivity in RA patients seemed to be associated with edentulism, there was no association found between RF seropositivity and periodontitis. The authors concluded that RA may be associated with tooth loss and periodontitis. Interestingly in this study, only 30% of RA patients were seropositive for RF, which is significantly lower than the prevalence reported in the literature. It has been suggested by another author (Dissick et al 2010) that this represents misclassification, and this may have affected the results.
1.4 Periodontal pathogens and RA

Following reports suggesting that bacteria such as mycobacteria (Bahr et al 1988) and Escherichia coli (Albani et al 1992, 1995) may be associated with the induction of RA, and in light of the burgeoning research linking periodontitis to RA, researchers in the 1990s began to investigate possible relations between the putative periodontal pathogens and RA.

1.4.1 Porphyromonas gingivalis

Porphyromonas gingivalis was identified as a periodontal pathogen at the World Workshop in Periodontology (AAP Consensus Report 1996), and is a member of the so-called “red complex” of periodontal pathogens (Socransky et al 1998). P. gingivalis is a Gram-negative, non-motile, asaccharolytic anaerobe, and utilises numerous virulence factors in order to evade host defence mechanisms. For many years, its presence has been linked to periodontitis, although no causal role has yet been proven. It has also been identified as the only prokaryotic species capable of producing a variant of PAD (McGraw et al 1999). This family of enzymes catalyses enzymatic modification of the arginine into citrulline (Fujisaki and Sugawara 1981) which, as discussed in Sections 1.2.2 and 1.7, is thought to be important in the development and progression of RA. Whilst PAD produced by P. gingivalis (sometimes referred to as P. gingivalis PAD [PPAD] or bacterial PAD) does not display similar amino acid sequencing to the human PAD enzymes, and seems to target a different part of the arginine residues, its ability to citrullinate proteins led some researchers to postulate that it may play a role in the development of RA. In 2004, one group proposed that infection with P. gingivalis in patients susceptible to periodontitis could lead to systemic exposure to citrullinated antigens, predisposing patients to development of anti-cyclic citrullinated peptide antibodies (Rosenstein et al 2004). They hypothesised that citrullinated peptides may break immune tolerance to endogenous citrullinated antigens, resulting in the generation of an immune response to citrullinated self-antigens, and they went on to suggest that systemic circulation of these antibodies could result in infiltration of the synovial joints. As discussed in Section 1.2.2, the anti-cyclic citrullinated peptide antibodies are highly specific markers of RA, even in early onset disease, and a number of reports have implicated them as being important in the pathogenesis of the disease. An article recapitulating Rosenstein’s hypothesis was recently published by another group (Liao et al 2009), and other groups have since tested this hypothesis.

A study by Wegner et al (2010) tested Rosenstein’s hypothesis, examining citrullination by P. gingivalis, and comparing it to other oral bacteria. They also created modified P. gingivalis strains, which had either the PAD gene removed or the gingipains inactivated. Of the bacteria tested, only P. gingivalis displayed the ability to citrullinate endogenous proteins. Deletion of the PAD gene completely removed this ability, and it was also reported that the P. gingivalis strains with inactivated arginine gingipains showed decreased citrullination. They concluded that the production of citrullinated proteins by P. gingivalis results firstly from proteolytic cleavage at arginine-X peptide bonds by arginine-gingipains, and then by citrullination of the short peptides which contain carboxy-terminal arginine residues. They also showed that P. gingivalis PAD not only citrullinates bacterial protein fragments, but also human fibrinogen and human α-enolase.

Prior to the discovery that P. gingivalis produces PADs, and the subsequent investigations into the possibility that it leads to citrullination in the periodontium, a number of studies investigated a possible role of P. gingivalis infection in the development and progression of RA.
Yusof et al (1995) used Enzyme-Linked Immunosorbent Assay (ELISA) to measure the levels of IgG antibodies against *P. gingivalis* in serum from four groups of patients: RA, chronic periodontitis, aggressive periodontitis, and healthy controls. The results showed no significant differences between the controls and RA patients, and similarly no differences between the two periodontitis groups. However, the levels in both periodontitis groups were significantly higher than both the controls and RA patients. Thus, whilst repeating previous findings that antibodies to *P. gingivalis* are found at elevated levels in the serum of patients with periodontitis, this study did not find evidence of a role for *P. gingivalis* in RA.

Moen et al (2003) expanded on the ideas investigated by Yusof’s group, analysing serum and synovial fluid from RA patients, and comparing them to samples from patients with various forms of arthritis and also healthy blood donors used as controls (no synovial fluid samples were obtained from non-arthritis patients, so only serum was available for the control group). Using ELISA, samples were screened for antibody levels to a number of oral bacteria. Specifically, IgG and IgA antibody levels to *P. gingivalis, P. intermedia, B. forsythus*, and *Candida albicans* were measured in the samples from all of the abovementioned groups. Whilst the levels of antibodies to *P. gingivalis* found in the RA patients’ sera were generally higher than those in the control group, the difference was not statistically significant. This was in agreement with the findings of Yusof’s 1995 study, but in contrast to Ogrendik’s 2005 study which found a significant increase in antibodies to *P. gingivalis* compared to controls. Furthermore, Moen’s group did not find a statistically significant correlation between the levels of IgG antibodies against *P. gingivalis* and the total IgG level in RA sera. Antibodies to the other bacteria examined in the study were found at higher levels, and will be discussed below. A subsequent study by Moen’s group looked for DNA from the same oral bacteria in serum and synovial fluid samples (Moen et al 2006). The mean DNA counts were higher in synovial fluid than in serum for both RA and for psoriatic arthritis patients, and there was DNA from a greater number of oral bacterial species in the synovial fluid than the serum of these patients. From these findings, the authors suggested that synovial inflammation in RA and other forms of arthritis may favour the ‘trapping’ of oral bacterial DNA once it reaches circulation, and that this might play a role in the initiation and progression of arthritic disease.

Martinez-Martinez et al (2009) studied 19 patients with periodontitis and refractory RA, taking subgingival plaque, serum and synovial fluid samples and using PCR to identify bacterial DNA from periodontal pathogens. Bacterial DNA from at least one of the periodontal pathogens was identified in all of the plaque and synovial fluid samples, and in 16 of 19 serum samples. *P. gingivalis* was detected in 78.9% of plaque samples, 57.8% of synovial fluid samples, and 42.1% of serum samples, but the differences between the prevalence in each sample type were not statistically significant. Similar to Moen et al (2006), the authors of this study also concluded that the presence of *P. gingivalis* DNA, as well as the DNA of other periodontal pathogens (see Section 1.4.3), in synovial fluid of RA patients might suggest a role for the DNA of periodontal bacteria in the development of rheumatoid arthritis. As they found no evidence of viable bacterial cells in the synovium, they suggested that the movement of the oral bacterial DNA from the periodontium to the synovium occurs in ‘free DNA’ form.

Mikuls et al (2009) published the results of a study comparing prevalence and serum concentration serum of antibodies to *P. gingivalis* in RA patients, periodontitis patients, and healthy controls, and examining a possible association between these antibodies and those traditionally associated with RA (i.e. RF and anti-CCP). Significant differences were found between antibody titres to *P. gingivalis* among the three groups, being lowest in healthy controls, higher in RA patients, and highest in periodontitis patients. In RA
patients, the *P. gingivalis* titre was significantly associated with C-reactive protein (an acute phase protein, associated with tissue damage and inflammation) and also with a number of anti-CCP antibody isotypes (anti-CCP-IgM, anti-CCP-IgG-2, and anti-CCP-IgG-4). Weak associations were also found between *P. gingivalis* titre and total anti-CCP antibody, but this was not statistically significant, and there was no association of immunity to *P. gingivalis* with RF concentrations. As the authors analysed samples stored from other studies, this study was inherently retrospective and cross-sectional in nature. As such, the authors were careful not to make any conclusions as to a causal role of *P. gingivalis* infection in the onset of RA, but the results lent some support to the possibility that it may have some affect on risk of disease onset and progression.

Hitchon et al (2010) investigated a possible association between immune responses to *P. gingivalis* and the presence of RF and/or anti-CCP antibodies in Native American patients with RA and relatives who did not have the disease. Sera from these subjects were analysed for anti-CCP antibodies, RF, and specific IgG antibodies to LPS from *P. gingivalis*. The serum levels of the anti-*P. gingivalis* antibodies were higher in patients with RA compared to their relatives and compared to controls. The results also showed an association between anti-*P. gingivalis* antibodies and anti-CCP antibodies, but there was no association between anti-*P. gingivalis* antibodies and RF. The authors interpreted this to mean that the immune response to *P. gingivalis* may be involved in breaking immune tolerance to citrullinated proteins, thus playing a role in the pathogenesis of RA.

A recent study examined serum levels of immunoglobulin G (IgG) antibodies to *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Eikenella corrodens*, and *Prevotella intermedia*, and also serum levels of anti-CCP antibodies and rheumatoid factor in RA patients and non-RA controls who were matched for age, smoking status, gender and periodontal status (Okada et al 2011). They reported that the sera of RA patients showed significantly higher levels of anti-*P. gingivalis* antibodies, and significantly lower IgG responses to the other species. The levels of anti-*P. gingivalis* antibodies correlated significantly with serum RF, probing depth and attachment levels. They suggested that these results may reflect a role of serum antibody responses to *P. gingivalis* in the pathogenesis of RA. However, all RA patients in the study were medicated, which may have affected serum responses and also periodontal inflammation, and they also reported a much higher prevalence of periodontitis among RA patients (~98%) than is reported in other studies, so the results must be interpreted with caution.

### 1.4.2 *Aggregatibacter actinomycetemcomitans*

Yoshida et al (2001) examined antibodies in sera from RA patients for reactivity to a particular protein from *A. actinomycetemcomitans* (known as DnaJ) using ELISA. The results showed a significant increase in IgG levels in the sera of RA patients compared to the levels found in healthy controls. The authors also compared the RA group to sera taken from age- and sex-matched non-RA patients with infectious diseases (namely, enteronitis, pneumonitis, maxillary sinusitis, and cellulitis of the leg). They reported a significant increase in mean titres of an antibody to one particular fragment of the DnaJ protein (anti-DnaJ1 IgG) in RA patients compared to the “disease controls”. The authors concluded that the N-terminal region of DnaJ from *A. actinomycetemcomitans* may be immunodominant in RA patients, and that this may be an important factor in determining the aetiology of the disease in future.

One study analysed serum samples from RA patients for pathogen-specific IgG antibodies to a number of oral anaerobic bacteria often associated with periodontitis (Ogrendik et al 2005). Whilst the
authors reported higher levels of antibodies to a number of these species (see Section 1.4.3), no differences were found between RA patients and controls for serum levels of IgG to *A. actinomycetemcomitans*.

### 1.4.3 Other oral bacteria

While Moen’s 2003 study found no significant increase in antibodies to *P. gingivalis* in the serum of RA patients compared to controls, antibodies against *B. forsythus* and *P. intermedia* were found in significantly higher levels in RA patients compared to other arthritis patients (serum and synovial fluid) and controls (serum only). The authors concluded that these findings are indicative of the presence of an active antibody response in synovial tissue and suggest “a potential connection between periodontal and joint diseases”.

Another ELISA analysis of blood samples from RA patients and controls by Ogrendik *et al* (2005), found that the levels of IgG antibodies to *P. intermedia*, *P. melaninogenica*, and *B. forsythus* were significantly higher in the serum of RA patients than in controls. They concluded that the antibodies formed to these bacterial species may be important in the aetiopathogenesis of RA.

The Martinez-Martinez study discussed above also analysed plaque, synovial fluid and serum samples from refractory RA patients for DNA from *Tanerella forsythensis*, *Prevotella intermedia*, *A. actinomycetemcomitans*, *Prevotella nigrescens*, and *Treponema denticola*. In particular, *P. intermedia* (100%) and *T. denticola* (84.2%) were found in a high proportion of plaque samples, with *P. intermedia* also being found in a majority of synovial fluid and serum samples (89.4% and 73.6%, respectively). There was a significant difference in the prevalence of *P. intermedia*, *T. forsythensis*, *P. nigrescens* and *T. denticola* among the three sample types taken. All subjects who were positive to any bacteria in serum and/or synovial were positive to the same species in subgingival plaque, lending some support to the notion that the periodontium may be the source of the systemic circulation of these bacteria.

### 1.5 Drug treatment of RA and effects on periodontitis

Pharmacological therapy plays an important part in the management of RA in many patients. Numerous drugs have been used in RA treatment, and currently there are three classes in widespread use: NSAID, corticosteroids, and disease-modifying anti-rheumatic drugs (DMARD) such as methotrexate, hydroxychloroquine, and gold salts. Often RA patients will take one or more of these medications for long periods. As such, the potential effects of these drugs on periodontitis must be considered when examining possible links between the two diseases.

#### 1.5.1 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAID) such as aspirin, ibuprofen, and diclofenac, are used to reduce tissue inflammation and pain, by inhibiting the formation of prostaglandins, and are indicated in a variety of chronic inflammatory diseases. They are considered a first-line drug treatment for RA, due to their analgesic and anti-inflammatory properties, which are often effective in managing symptoms. However, it has been reported that these drugs are not effective in reducing the severity of tissue destruction in RA or controlling disease progression in the long-term (Jain and Lipsky 1997). In the 1970s, it was discovered that NSAID interfered with the arachidonic acid metabolite pathway by blocking cyclooxygenase, which in turn
blocked prostaglandin production (Vane 1971). This discovery followed reports showing that prostaglandins, and in particular PGE₂, were capable of inducing bone resorption (Klein and Raisz 1970, Goldhaber 1971). Following Vane’s discovery, researchers began to investigate whether this action might have an effect on the bone resorption in periodontitis, with studies in animals initially, and later in clinical trials (reviewed in Williams 2008).

Nyman et al (1979) tested the efficacy of systemic doses of an NSAID (indomethacin) on the suppression of alveolar bone resorption and gingival inflammation in a ligature-induced periodontitis model in beagle dogs. The results showed that indomethacin delayed the onset and suppressed the magnitude of the acute inflammatory response and decreased the amount of alveolar bone resorption.

In the same year, an experiment on a cat model also showed that systemic indomethacin suppressed alveolar bone destruction and periodontal ligament destruction (Torbinejad et al 1979). In this study, immune complexes were injected via the root canal into the periapical tissues of cat teeth, causing bone resorption which could be detected radiographically and histologically within seven days. In animals that received systemic administration of indomethacin (10 mg/kg daily) prior to and during the 28-day experimental period, inhibition of periodontal soft and hard tissues was observed without having an effect on the accumulation of inflammatory cells.

Williams et al (1984) were the first to report an in vivo effect of NSAID in naturally occurring periodontitis in animal studies. The effects of non-surgical and surgical periodontal therapy were compared over a 12-month period, and the animals were dosed daily either with 0.02 mg/kg flurbiprofen or with a placebo. Observation at 3, 6, 9 and 12 months showed significantly decreased rates of alveolar bone loss in both surgically and non-surgically treated animal groups when compared to baseline levels. The control (placebo-treated) groups did not show significantly decreased levels of bone loss. The animals were examined following withdrawal of the drug regimen, and the data published in a follow-up study (Jeffcoat et al 1986). In the NSAID-treated groups the decrease of alveolar bone loss was sustained up to 3 months of the post-treatment period but was lost 6 months after termination of flurbiprofen therapy.

Following these reports, a large number of animal experiments investigating the effects of NSAIDs on periodontal disease progression emerged (reviewed in Salvi and Lang 2005). Studies were published reporting the effects of systemic and topical NSAID administration on gingival prostaglandin levels, gingival inflammation, and periodontal hard- and soft-tissue destruction. A variety of NSAID drugs were trialled, over time periods varying from 14 days to 13 months, and the results generally showed that rate and amount of bone resorption was reduced following administration of topical or systemic NSAID. Some of the longer studies also showed that this effect was diminished upon withdrawal of the drug.

Human clinical studies followed, examining the effects of NSAID on gingival inflammation, alveolar bone loss, and as an adjunct to both non-surgical and surgical periodontal therapy. Again, a range of different NSAID, including flurbiprofen, meclofenamate, ibuprofen, ketorolac, naproxen and aspirin, were systemically or locally administered at a variety of dosages, over periods ranging from 10 days to 24 months. A number of the studies showed a significant benefit with respect to alveolar bone preservation when NSAIDs were associated with conventional therapy (Salvi and Lang 2005, Kirkwood et al 2007).

Studies examining the effect of NSAID on the levels of inflammatory markers in the gingival crevicular fluid (GCF), namely prostaglandin E₂ (PGE₂) and thromboxane B₂, found significant reduction in these markers compared to the placebo group. However, one week after drug withdrawal, the levels had returned to baseline (Abramson et al 1992, Heasman et al 1993).
Most of the clinical studies examining NSAID use in treatment of periodontitis involve the use of the drugs as an adjunct to surgical and/or non-surgical treatment. However, before the first such study was published, a paper was published by a group examining the periodontal condition of patients taking NSAID for other conditions, namely arthritis or ankylosing spondylitis (Waite et al 1981). Twenty-two subjects taking NSAID for either disease and 22 age-matched controls were examined and periodontal parameters compared. The NSAID patients were found to have lower gingival index and shallower pocket depths, and there was a trend towards less clinical attachment loss compared to the controls.

A similar study on patients taking either aspirin or a combination of aspirin and indomethacin for arthritis for long periods (>5 years) retrospectively showed that the patients taking the NSAIDS long term had significantly fewer sites with interproximal bone loss than did healthy controls (Feldman et al 1983).

In the first clinical study using NSAID specifically as an adjunct to periodontal treatment, the effects of short-term systemic flurbiprofen administration (50 mg twice daily) following periodontal treatment in 15 patients with "refractory periodontitis" were compared to placebo controls (Jeffcoat et al 1988). Standardised radiographs were used to measure bone loss over a period of two months, and the results showed significantly less alveolar bone loss during the treatment period in the flurbiprofen group compared with the placebo group.

In a 2-year longitudinal study, 44 patients with advanced chronic periodontitis were followed to investigate the long-term and post-treatment effects of flurbiprofen on the progression of alveolar bone loss (Williams et al 1989). Results were determined by interpreting standardised radiographs. Thirty-three compliant patients available for follow-up were also monitored for six months after withdrawal of the drug. The results indicated significantly lower rates of bone loss at 12 and 18 months with flurbiprofen treatment, but a return to baseline rates by 24 months. Indeed, there were no significant differences between the rates of bone loss at 24 months between the test and control groups.

The same group published further studies in patients with “rapidly progressing periodontitis”. The first paper reported a significant decrease in bone loss in patients taking 500mg naproxen twice daily for three months when compared with the placebo group, as measured by digital subtraction radiography (Jeffcoat et al 1991). A further study from this group reported a 6-month controlled clinical trial examining the efficacy of systemic meclofenamate sodium at two different doses as an adjunct to scaling and root planing. Subtraction radiography was again used, and it was shown that patients taking low (50 mg twice daily) and high (100 mg twice daily) doses of meclofenamate sodium showed significant bone gain when compared to the placebo group (Reddy et al 1993). A subsequent study compared the effect of a topical (0.1% ketorolac mouth rinse twice daily) and systemic (50mg flurbiprofen) NSAID taken twice daily on inhibition of alveolar bone loss, with standardised radiographs taken at baseline, 3- and 6-months. Clinical parameters were not significantly affected in any of the groups. However, the topical and systemic NSAID groups showed significantly reduced alveolar bone loss rate and GCF- PGE₂ levels when compared to the placebo group (Jeffcoat et al 1995).

Another study examined the effects of systemic administration of flurbiprofen (50 mg t.i.d.) on tissue healing one month after periodontal surgery in patients with moderate to severe chronic periodontitis (Bragger et al 1997). Radiographic and clinical examination revealed improvement in surgically treated sites, irrespective of whether flurbiprofen or placebo had been administered. As such, the authors deduced that the use of flurbiprofen conferred no additional benefit and was not indicated.
Despite some apparently promising results, long-term use of NSAID has a number of serious adverse systemic effects, and for this reason they are not currently in wide use as an adjunctive treatment for periodontal disease. The studies have shown that daily administration for extended periods is necessary for a beneficial effect on the periodontium, and NSAID are associated with significant unwanted effects, including gastrointestinal problems, internal haemorrhage (as a result of decreased platelet aggregation), and renal and hepatic impairment. It is thought that the inhibition of both COX-1 and COX-2 is responsible for the gastrointestinal side effects of non-selective NSAID (Wallace et al 2000, Warner and Mitchell 2004). Selective NSAID such as COX-2 inhibitors are not associated with the same adverse effects as non-selective NSAID, and this prompted research interest into these drugs as a possible treatment alternative.

Animal studies showed some promise for the role of selective COX-2 inhibitors in treating periodontitis, with a study in a rat model showing that celecoxib altered the progression of alveolar bone loss (Holzhausen et al 2002). A recent animal study found that both COX-1 and COX-2 participate in the early stages of experimental periodontitis, and that inhibition of these pathways reduced the clinical signs of disease in a rat model (Queiroz-Junior et al 2009). The authors also reported that local administration of selective COX inhibitors was just as effective as systemic dosage. Both COX-1 and COX-2 inhibitors were effective in reducing attachment loss and leukocyte numbers in gingival tissues, but only the COX-2 inhibitors were effective in preventing alveolar bone loss.

A human study investigating the effectiveness of selective NSAID as an adjunct to root surface instrumentation in patients with chronic periodontitis found little clinical benefit of COX-2 inhibitors in the management of such patients, but significant reductions in gingival tissue levels of PGE$_2$ and PGF$_2$ (Vardar et al 2003). More recently COX-2 inhibitors have been linked with severe cardiovascular adverse effects (e.g. heart attack and stroke), and as such, they are unlikely to be used in periodontal treatment (Couzin 2004, Wardle 2004).

Overall, results of the currently available studies are mixed, and whilst numerous authors have reported promising results, cessation of the NSAID regimen saw a return to the rate of bone loss seen prior to drug therapy in a number of these studies. Furthermore, long-term therapy appears to be required for significant improvement, and a number of adverse effects have been associated with long-term use of NSAID. As such, NSAID are not widely used as an adjunctive treatment for periodontitis. However, given their common use in RA patients, it is possible that the periodontitis experience in these patients is underestimated or masked by the anti-inflammatory effect of these drugs.

1.5.2 Disease-modifying anti-rheumatic drugs

These drugs are used as a second-line treatment in patients whose RA cannot be managed by first-line drugs (such as NSAID), and have been shown to have significant effects on disease progression in the medium to long term (reviewed in Jain and Lipsky 1997). A number of these drugs have been associated with significant side effects, although generally it is considered that the risk of side effects is outweighed by the potential for disease progression if treatment is delayed (O'Dell 2004). There have been very few studies examining the effects of disease-modifying anti-rheumatic drugs on periodontitis. An early animal study showed that intramuscular administration of gold salts was effective in reducing inflammation in a ligature-induced experimental periodontitis model (Novak et al 1984). Another animal study investigated the possible effects of methotrexate on experimental periodontitis in a rat model (Verzeletti et al 2007). The authors
reported that there was no difference in alveolar bone levels between the test and control groups, and that dosage had no effect.

Whilst no clinical studies have been conducted to specifically examine the effects of DMARD treatment on periodontitis, the use of DMARD by RA patients with periodontitis has been noted in several studies. In their cross-sectional study quantifying periodontitis in RA patients, Gleissner et al (1998) found that, despite strong correlation between duration of DMARD and corticosteroid therapy and the radiographic signs of RA, there was no association between the duration of drug treatment and clinical periodontal parameters. They concluded that the long-term use of these drugs had no effect on the increased incidence of severe periodontitis in these patients. Dissick et al (2010) examined a US veteran population for possible associations between periodontitis and RA. They noted that 91% of the RA patients were taking at least one DMARD, and whilst there was no detailed comment about the possible effects of the medication, they reported no association between severity of periodontitis and medication use in these patients.

**1.5.3 Antibiotics**

As discussed earlier, a number of bacteria strongly implicated in the pathogenesis of periodontitis are found commensally throughout the body, and a possible role in RA has been investigated. Antibiotics have been trialled as potential treatments for periodontitis and RA, initially due to their antibacterial action. Some studies have shown beneficial effects of antibiotics as part of periodontal treatment, but the current consensus is that while they may provide a useful adjunct to conventional periodontal treatment in some circumstances, antibiotic ‘monotherapy’ is not a valid treatment option for periodontitis (Herrera et al 2002, Haffajee et al 2003, Heitz-Mayfield 2009). As such, even though bacteria are known to be the primary aetiological factor in periodontitis, the antimicrobial effects of these drugs is of limited effectiveness in periodontal treatment. However, Golub et al (1983) reported that the tetracycline antibiotics (and their semi-synthetic analogues) inhibited matrix metalloproteinase (MMP) activity, thus imparting an anti-inflammatory effect as well as an antimicrobial effect. Further research revealed that this inhibition of MMPs still occurred at subantimicrobial doses, and as such, was independent of the antibacterial action of the drugs (Golub et al 1990). MMPs have been identified in the synovial tissues and fluid of RA patients (Brinckerhoff 1991). Tetracycline analogues such as minocycline have also been shown to have an inhibitory effect on the production of nitric oxide synthases (Amin et al 1996).

Several studies investigating the use of tetracyclines as anti-inflammatory treatment in RA have shown them to be more effective than placebos (Kloppenburg et al 1994, Tilley et al 1995, O'Dell et al 1997, O'Dell et al 1999, O'Dell et al 2001). Most trials have been of relatively short duration (<1 year), although O’Dell et al (1999) reported continued efficacy after four years. A number of side effects, including hyperpigmentation, have been documented in patients taking these drugs for the treatment of RA (Wasel et al 1998, O'Dell et al 1999, Mehrany et al 2003).

Another class of antibiotics which has been trialled in the treatment of RA is the macrolide class. As well as their antibacterial activity, these drugs have been shown to interfere with several inflammatory processes (Ianaro et al 2000, Zalewska-Kaszubska and Gorska 2001, Kamemoto et al 2009). A recent animal study showed that a modified formulation of azithromycin was effective in reducing the severity of experimentally-induced RA. Interestingly, the drug had been modified to remove the antimicrobial activity, and the researchers reported that the therapeutic effect arose from inhibitory effects in regulation of NF-κB, a transcription factor which mediates the expression of genes for inflammatory signals (Mencarelli et al 2011).
A recent human study reported improvement in signs and symptoms in RA patients with no history of DMARD treatment after a relatively short-term (3 month) course of a macrolide (roxithromycin) compared to a placebo (Ogrendik 2009).

A recent randomised double-blind placebo-controlled trial investigated the effectiveness of a combined antibiotic therapy in RA patients and compared it to a placebo (Smith et al 2011). According to the authors of that study, the combination of oral tetracycline with intravenous administration of the lincosamide antibiotic, clindamycin, is commonly used in clinical practice in the absence of strong evidence. A pilot study by the same group had previously showed limited positive results within six months of treatment, and clinical improvements were not generally maintained following cessation of the treatment (Gompels et al 2006). In the recent study, it was noted midway through the trial that there were minimal differences between the test and control groups, and the trial was ceased. The authors concluded that the antibiotic combination being tested did not appear to be effective, despite being widely used in clinical practice.

1.5.4 Cytokine blocking agents

Drugs which block proinflammatory cytokines such as TNF-α have been studied as possible treatments for RA (Toussirot and Wendling 2007, Ranganathan 2008). Results from animal studies have shown that these drugs may reduce the severity of periodontitis (Assuma et al 1998, Di Paola et al 2007). A number of human studies have been conducted examining the effects of these drugs on periodontitis and RA in patients with both conditions. Generally, the sample sizes have been quite small, and the results have been conflicting – certainly no consensus has been reached to date.

Pers et al (2008) evaluated the periodontal condition of two groups of RA patients, half of whom had taken 6-weekly injections of infliximab for at least 22 months, and half of whom had not yet commenced the drug treatment (although half of these 'controls' were receiving another DMARD - methotrexate) at the commencement of the study. The authors reported that while blocking the activity of TNF-α tended to aggravate the gingival tissues and increase inflammation, bone resorption was inhibited by this treatment. The authors proposed that destruction and inflammation could indeed be distinct components of the disease, thus explaining the decreased bone loss in spite of increased inflammation.

A study by Mayer et al (2009) examined the clinical and immunological effects of anti-TNF-α drugs on the periodontal tissues in 10 RA patients, and compared them to 10 RA patients not taking the drugs, and also to 10 healthy controls. Subjects in the test group were injected with 200 mg of infliximab every eight weeks for up to six months, with GCF samples taken from all three groups and TNF-α levels were measured using ELISA. The RA patients on anti-TNF-α therapy recorded lower TNF-α levels, and had significantly improved periodontal parameters (including lower bleeding on probing, less clinical attachment levels, and lower mean probing depths) compared with the other two groups. Whilst acknowledging the limited power of such a small sample size, the authors suggested that anti-TNF-α agents may be effective in controlling periodontal inflammation and bone loss, and as such may be promising as future treatment for periodontitis.

Nilsson and Kopp (2008) conducted a study to examine the relationship between a number of inflammatory mediators and periodontitis in 19 RA patients. In this study, high circulating (plasma) levels of TNF-α were associated with gingival inflammation (gauged by bleeding on probing), greater clinical attachment loss and deeper periodontal probing depths compared to patients with low plasma levels. The authors concluded that high systemic levels of TNF-α are related to periodontal inflammation and tissue destruction in RA patients.
Ortiz et al (2009) assessed the effect of anti-TNF-α therapy on periodontitis in a human study. This study also examined the effect of non-surgical periodontal treatment on the signs and symptoms of RA in patients with moderate-to-severe RA and severe chronic periodontitis, treated with or without anti-TNF-α medications. Whilst patients receiving periodontal treatment and anti-TNF-α therapy showed a significant improvement in all clinical periodontal parameters measured, anti-TNF-α therapy without periodontal treatment had no significant effect on the periodontal condition.

1.6 Rheumatoid Arthritis markers in periodontitis patients

1.6.1 Rheumatoid Factor in serum of periodontitis patients

Thé and Ebersole (1991) investigated the occurrence of the autoantibody, IgM rheumatoid factor (IgM-RF), in the sera of 260 subjects. The study population included periodontitis patients, controls with gingivitis but no periodontitis, and RA patients. Sixteen of the periodontitis patients (9.4%) were seropositive for IgM-RF, and most of these patients had severe chronic or aggressive periodontitis. For comparison, a random population of seronegative periodontitis patients was constructed that was matched for sex and approximate age to the seropositive group. The total immunoglobulin levels of the two groups were not significantly different and the means of both were slightly lower than the RA group. Comparison of the antibody profiles in the periodontitis populations revealed that the RF-positive group showed significantly elevated titres of IgM to Capnocytophaga species, E. corrodens and F. nucleatum, and IgG antibody that was significantly elevated to F. nucleatum.

Thé and Ebersole (1991) hypothesised that RF production could result from chronic antigenic stimulation in periodontitis subjects. The results supported this hypothesis, with the authors concluding that the chronic inflammation associated with periodontitis appears to significantly increase the formation of IgM-RF. They also noted that there appeared to be a relationship between IgM-RF and elevated titres of antibodies to the oral bacterial species examined.

The same authors further investigated the antigenic specificity of RF in the serum of patients with periodontitis (Thé and Ebersole 1996). Serum samples were taken from five RF-seropositive RA patients and 14 RF-seropositive periodontitis patients and IgM-RF was isolated and examined for specificity to human IgG and selected oral bacteria: namely Capnocytophaga gingivalis, Fusobacterium nucleatum, and A. actinomycetemcomitans. The results showed that the IgM-RF reacted with polyclonal human IgG non-specifically bound to microtitre plates. The reactivity of the IgM-RF was increased when incubated with IgG that bound as antibody to C. gingivalis, F. nucleatum or A. actinomycetemcomitans. They reported that RF from the seropositive patients cross-reacts with epitopes of the selected oral bacteria, and suggested that the RF detected in the serum of these patients may have been produced by immune reactions to subgingival plaque bacteria.

A recent study by Dissick et al (2010) examined patients at a rheumatology outpatient clinic, with the authors reporting a correlation between RF-positivity and severity of periodontitis. Of the RA patients who were seropositive for RF (56/67 – 89%), 59% had moderate to severe periodontitis, 25% had mild periodontitis, and 16% had no periodontitis. In contrast, among patients who were RF negative, only 15% had moderate to severe periodontitis, while 54% had mild periodontitis, and 31% were periodontally healthy.
1.6.2 Local production of RF in periodontal tissues

Thus far, few studies have examined local production of these antibodies in the periodontal tissues. One early study of patients with moderate chronic periodontitis used latex slide agglutination to test for RF in subgingival plaque, inflamed gingival tissues, stimulated pooled saliva, and serum (Gargiulo et al 1982). Following sample processing, electron microscopy revealed the presence of RF in each of these substances/tissues in periodontitis patients. The same authors conducted another study testing inflamed and non-inflamed gingival tissues for the presence of RF, using similar measurement methods but also utilising ELISA to analyse samples (Gargiulo et al 1985). It showed good agreement between the ELISA and the latex slide agglutination, and positive and negative serum controls for RF confirmed the accuracy of the tests. RF was detected in 66% of inflamed tissue samples. The authors concluded that this system is a “proper and suitable system” for detection of “RF and other autoantibodies”, and suggested that this may be used clinically. They did not speculate as to the reason for the presence of RF in the periodontitis samples, nor on any possible significance of these findings. Hirsch et al (1989), used ELISA to determine the presence and concentration of IgG, IgA, and IgM immunoglobulin-secreting cells and IgA- and IgM-RF-producing cells in the gingival tissues and peripheral blood of adult periodontitis patients. Tissue samples were obtained from 29 patients undergoing either oral surgery or periodontal surgical procedures, and cells were extracted from these samples, and then incubated for quantitative and qualitative analysis. The results showed a high frequency of IgA-RF- and IgM-RF-secreting cells in the gingival tissues of patients with chronic periodontitis. The authors found that in these patients, local production of RF occurred even in the absence of RF production by peripheral blood mononuclear cells, which they suggested might be evidence that local autoimmune reactions occur in periodontitis.

A recent study by Bostanci (2010) used mass spectrometry to quantify 154 proteins in the GCF of five patients with generalised aggressive periodontitis and five healthy controls. In this study, rheumatoid factor was detected in the GCF of periodontitis patients, and was not detected in the GCF of non-periodontitis controls. Whilst the level of RF detected in the periodontitis GCF was very low ([2.7 ± 0.3] x 10^{-15} mol), and the amount of GCF collected from these patients was statistically significantly higher than the amount collected from healthy controls (mean 0.58 ± 0.30 μL vs 0.16 ± 0.09 μL), the results nonetheless give some indication that production of rheumatoid factor may be upregulated in association with periodontitis.

1.6.3 Inflammatory markers in periodontitis and RA patients

Bozkurt et al (2000), measured and compared interleukin-6 (IL-6) levels in GCF clinical periodontal parameters in patients with RA and chronic periodontitis. Three groups of 15 patients were studied: patients with RA and chronic periodontitis, patients with periodontitis but no RA, and healthy controls. Clinical parameters of periodontal health were measured, and GCF samples were taken and analysed with ELISA for IL-6 levels. Of the clinical parameters measured, only plaque index was found to be significantly different between the RA and chronic periodontitis groups. Whilst the RA group recorded the highest mean IL-6 levels in GCF samples, there was no statistically significant difference between any of the groups. The authors hypothesised that RA patients would be at risk for increased local tissue destruction due to autoimmune processes, and also due to higher plaque index recordings, but no statistical evidence supported this. The authors suggested that anti-RA medications such as DMARD and NSAID may have been responsible for the lack of differences observed.
With increasing information about the roles of numerous cytokines in the pathogenesis of RA and periodontitis, the same group carried out another study examining the GCF levels of two cytokines known to inhibit inflammation (IL-4 and IL-10) and not measured in the original study (Bozkurt et al 2006). ELISA analysis revealed that healthy controls had statistically significantly higher levels of IL-4 and IL-10 than both the RA and the periodontitis groups. RA patients had higher levels than periodontitis patients. Whilst the authors acknowledged that the small samples sizes limited the statistical power of the results, and that further research is required in this area, they suggested that the initiation and progression of periodontal inflammation may be due to a lack or inappropriate response of the anti-inflammatory cytokines in both chronic periodontitis and RA.

One study has reported on the effect of polymorphisms in the IL-1 gene cluster on cytokine profiles in young patients with various forms of periodontitis or RA, and healthy controls (Havemose-Poulsen et al 2007). There did not seem to be any correlation between the different polymorphisms and the various disease types, and the frequency of polymorphisms did not differ between disease and health. The authors reported some association between certain IL-1 genotypes and cytokine levels in aggressive periodontitis patients and RA patients. They suggested that rather than specific cytokine profiles being associated with each disease type, there may be a shared genetic background for high- and low-cytokine responders. If this is shown to be true, the authors postulated that genetic testing may be useful in determining which patients are more likely to respond to drug treatments which inhibit pro-inflammatory cytokines.

1.6.4 Antibodies to citrullinated proteins in serum of periodontitis patients

In a recent review article, Lundberg et al (2010) reported results of a preliminary immunohistochemical study which showed the presence of citrullinated proteins in the gingival tissues of patients with periodontitis. If substantiated by further research, these findings provide support to the hypothesis that citrullination occurs in the periodontium, and anti-CCP antibodies may be produced against these citrullinated proteins in susceptible patients.

Prior to Lundberg (2010) reporting the presence of citrullinated proteins in the gingival tissues, a small number of authors investigated the presence of anti-CCP antibodies in the serum of periodontitis patients. The previously mentioned study by Havemose-Poulsen et al (2006) was the first to examine anti-CCP levels in blood samples from periodontitis patients. Whilst only a small proportion of the non-RA patients were positive for these antibodies (2 of 27 generalised aggressive periodontitis patients, and no localised aggressive periodontitis patients or healthy controls), given the high specificity for RA of these antibodies, this is an interesting finding, and is worthy of follow-up.

In a study investigating the possible involvement of autoimmune reactions in the pathogenesis of periodontitis, Hendler et al (2010) tested serum samples from gingivitis and periodontitis patients for the presence of autoantibodies to a number of proteins, including CCP. They theorised that citrullination may play a role in susceptibility to periodontitis, “due to similar inflammatory processes in RA and periodontitis and also due to the fact that P. gingivalis is the only bacteria that is known to express PAD”. They found that a small number of samples from aggressive periodontitis patients showed high autoactivity against CCP (2 of 25 samples), while a few more showed borderline titres (4 of 25 samples). None of the samples from the chronic periodontitis or gingivitis groups displayed any autoactivity to CCP. Whilst no strong conclusions could be made from these results, the authors suggested that autoimmunity may be a significant factor in the pathogenesis of aggressive periodontitis.
Few studies have examined chronic periodontitis patients for the presence of anti-CCP antibodies. A study by Ballini et al (2010) investigated the presence of anti-CCP antibodies in the serum of periodontitis patients, in order to investigate the possible utility of this marker as diagnostic tool. In a population of 22 patients (20 with chronic periodontitis, two with RA), only one of the periodontitis patients was found to be anti-CCP seropositive. The authors concluded from their results that a role for anti-CCP antibodies in periodontitis is unlikely. Neither of the RA patients in that study were found to be seropositive, and the sample size was quite small, so these results should probably be interpreted with some caution.

One study has reported a correlation between serum levels of anti-CCP antibodies and chronic periodontitis (Dissick et al 2010). Patients with RA who were seropositive for anti-CCP antibodies (87%) were more likely to have moderate to severe periodontitis than seronegative RA patients. Of the 59 RA patients who were seropositive for the anti-CCP antibody, 56% had moderate to severe periodontitis, 31% had mild periodontitis, and 14% had no periodontitis. Among patients who were anti-CCP negative (including RA and non-RA patients), 22% had moderate to severe periodontitis, 22% had mild periodontitis, and 56% were classified as periodontally healthy.

1.6.5 Local production of anti-CCP in the periodontium

To our knowledge, no studies have been published reporting the local production of antibodies to CCP in the periodontium. A summary of a recent (as yet unpublished) study was presented at a conference in 2009, and an abstract has been printed (Nesse et al 2009). The abstract reports that citrullinated proteins and HC pg-39 were present in the gingival tissue of periodontitis patients. The authors also attempted to detect PAD2 in the tissues, but no results were reported due to ‘technical matters’. Gingival crevicular fluid samples were collected at sites exhibiting gingival inflammation from eight healthy control patients and analysed by Western Blot analysis. Citrullinated proteins were detected in four of the eight GCF samples, and PAD2 was present six out of eight samples. The authors reported that their study was the first to show that citrullination occurs in vivo in the periodontium, and suggested that their findings lend support to the theory that periodontitis may be involved with the formation of anti-CCP antibodies. An abstract of another unpublished study with a very small study population reported the presence of PAD2 in ‘periodontal tissues’ (Bingham et al 2010).

Due to the very small sample sizes and the paucity of detail in these abstracts, no conclusions can be drawn from these publications. Other than these two abstracts, we found no other publications investigating the local production of citrullinated proteins, or production of autoantibodies against these proteins, in the periodontium.

1.7 Conclusion

There is increasing evidence of a relationship between periodontitis and rheumatoid arthritis, with a number of potential mechanisms being investigated. There has been no definitive evidence of a causal relationship between the two diseases, and indeed this seems unlikely. Rather, it seems likely that there may be common susceptibility factors. It also seems likely that, whilst neither disease directly causes the onset of the other, the diseases can impact on the severity and progression of each other in individuals affected by both conditions. There is no evidence that impaired joint function in RA patients is associated with ineffective oral hygiene or increased plaque accumulation in these patients. Periodontal treatment has been
shown to have a positive effect on clinical signs of RA in some studies, and this has lead to the theory that periodontitis may indeed be a modifiable risk factor for RA. Numerous possible mechanisms have been proposed in support of this theory. Antibodies to oral bacteria have been detected in systemic circulation, and isolated from synovial tissue, and it is possible that the periodontium provides an extra-articular source of pathogens which may be involved in the pathogenesis of RA. It has also been suggested that chronic inflammation arising from untreated periodontitis may prime the susceptible patient, and in the presence of this persistent inflammation, introduction of arthritogenic stimuli may lead to an exaggerated host response, and the initiation of RA. Conversely, the sustained inflammation present in RA may result in an exaggerated response to plaque bacteria in the periodontium, leading to initiation of periodontitis. Production of autoantibodies has been strongly associated with RA, and it has been suggested that local production of these antibodies in the periodontium may lead to systemic distribution which, in susceptible patients, may lead to the onset of RA. Whilst a small number of studies has attempted to explore a relationship between periodontitis seropositivity for these antibodies, very little research has investigated the possibility of local production in the periodontium. Presence of these autoantibodies in the periodontal tissues may be indicative of local production, which would provide support to the theory that the periodontium may be a source of these antibodies. It has been shown that *P. gingivalis* produces an enzyme which may citrullinate proteins, but thus far, the presence of human PAD has not been examined in the periodontium. Given the importance of the PAD family in the citrullination process, their presence in the periodontium would be further evidence that citrullination occurs in these tissues. Whilst it is clear that citrullination is a physiological process that occurs in many tissue types, production of autoantibodies to citrullinated proteins has been strongly associated with disease (specifically RA). If the periodontium is found to be a source of these autoantibodies, this would lend significant support to the possibility periodontitis is involved in the onset of RA.

### 1.8 Hypothesis

The hypothesis is that local production of rheumatoid factor, citrullinated proteins, anti-cyclic citrullinated peptide autoantibodies, and peptidylarginine deiminase types 2 and 4 in periodontal tissues provides an extra-synovial source of autoantibodies, and that expression of these proteins is increased in periodontitis.

### 1.9 Aims of the study

The primary aim of the study is to determine the presence of rheumatoid factor, citrullinated proteins, and PAD2 and PAD4 in periodontal tissues by immunohistochemistry, and to determine the presence of rheumatoid factor and anti-cyclic citrullinated protein antibodies in gingival crevicular fluid by ELISA. The secondary aim of the study is to compare the expression of these proteins between periodontitis and non-periodontitis patients.
1.10 References


Havemose-Poulsen, A., Sorensen, L. K., Bendtzen, K. & Holmstrup, P. (2007) Polymorphisms within the IL-1 gene cluster: effects on cytokine profiles in peripheral blood and whole blood cell cultures of patients with aggressive periodontitis, juvenile idiopathic arthritis, and rheumatoid arthritis. *J Periodontol* 78, 475-492.


36


Chapter 2. Expression of rheumatoid arthritis markers in normal and inflamed gingival tissues

2.1 Introduction

Periodontitis is a common chronic inflammatory disease of complex aetiology, in which a host-mediated immunoinflammatory response to bacteria results in the destruction of hard and soft tissues of the periodontium in susceptible patients. In the past 20 years, a large volume of research has been undertaken investigating links between periodontitis and a number of systemic conditions, including diabetes mellitus (Löe 1993), cardiovascular disease (Beck et al. 1996), adverse birth outcomes (Offenbacher et al. 1996), and rheumatoid arthritis (Bartold et al. 2005, de Pablo et al. 2009).

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterised by persistent inflammatory infiltrate in the synovial membrane of joints, leading to joint damage, bone destruction, and (often severe) disability. Formation of autoantibodies is common in RA (Bridges 2004), and is thought to cause disturbance in immune regulation in RA patients. Of the numerous autoantibodies described in the serum of RA patients, rheumatoid factor (RF) and anti-citrullinated protein (anti-CCP) antibody are currently the only two which are routinely used clinically as diagnostic aides (Aletaha et al. 2010). It has been reported that seropositivity to these antibodies often predate clinical onset of RA, and may be useful predictors of disease progression (Rantapaa-Dahlqvist et al. 2003).

Rheumatoid factor is an autoantibody which acts against the Fc portion of IgG, forming immune complexes thought to contribute to the disease process. Antibodies to the citrullinated proteins are highly specific to RA (Vander Cruyssen et al. 2005), and their production in susceptible patients has been implicated in the onset and progression of RA (Schellekens et al. 1998). Citrullination is a post-translational modification of proteins, in which a positively charged arginine residue is converted into a neutral citrulline residue by an enzyme family known as peptidylarginine deiminases (PAD). It is a physiological process which occurs during keratinisation of epithelial cells, inflammation, and apoptosis. Of the five known human peptidylarginine deiminases which citrullinate proteins, only PAD2 and PAD4 have been shown to be associated with RA (Vossenaar et al. 2004a, Foulquier et al. 2007). In addition, in vitro studies have shown that one of the periodontal pathogens, Porphyromonas gingivalis, produces a variant of PAD (sometimes referred to as P. gingivalis PAD or PPAD) which is capable of citrullinating proteins (McGraw et al. 1999).

Whilst the aetiology of both diseases is not completely understood, there are numerous similarities between the two. Both periodontitis and RA are considered to be complex diseases (Ghosh and Collins 1996), as there are almost certainly multiple genes involved in disease susceptibility, and a number of environmental factors, such as smoking, are known to contribute to disease severity and progression in genetically susceptible patients. Soft and hard tissue destruction in both diseases arises from an exaggerated inflammatory response, mediated by host factors. The plaque biofilm is known to be the primary aetiological agent in periodontitis, and there have been numerous studies suggesting a role for bacteria in RA, with some authors suggesting a role for periodontal pathogens (Thé and Ebersole 1996, Rosenstein et al. 2004).

As well as these apparent similarities in pathogenesis, numerous clinical studies have reported a significant relationship between the two conditions. Increased incidence of tooth loss and periodontal attachment loss has been reported in RA patients compared to non-RA patients (Malmstrom and Calonius...
1975, Kasser et al 1997, Mercado et al 2001, Havemose-Poulsen et al 2006, de Pablo et al 2008, Ishi Ede et al 2008, Martinez-Martinez et al 2009). Furthermore, some authors have reported a higher incidence of RA in periodontitis patients than in the general population (Mercado et al 2000), and the possibility that periodontitis increases severity of RA has also been investigated (Ribeiro et al 2005). Whilst treatment of the two diseases differs significantly, there is some evidence that non-surgical periodontal treatment may reduce the severity of RA (Ribeiro et al 2005, Al-Katma et al 2007, Ortiz et al 2009).

The production of autoantibodies is thought to play a role in the pathogenesis of RA, and detection of these antibodies in serum is well documented in RA research. It has been theorised that these autoantibodies may play a role in periodontitis, and a small number of studies have examined serum from periodontitis patients for RF (Thé and Ebersole 1991, Thé and Ebersole 1996, Dissick et al 2010) or anti-CCP antibodies (Havemose-Poulsen et al 2006, Dissick et al 2010, Hendler et al 2010). Even fewer studies have investigated the possibility of local production of these antibodies in the periodontium. Gargiulo et al (1982, 1985) reported the presence of RF in inflamed gingival tissues. Hirsch et al (1989) analysed gingival tissue samples and blood samples of periodontitis patients, and reported that there was local production of RF in the periodontium, even in the absence of RF in the mononuclear cells in the peripheral blood. They suggested that this may be evidence that autoimmune reactions occur in the periodontium, and that this may contribute to the onset or progression of periodontitis. More recently, a mass spectrometry study identified RF as one of 154 proteins isolated from GCF in periodontitis patients, but not in the non-periodontitis controls (Bostanci et al 2010). A preliminary study by Lundberg et al (2010) reported the presence of citrullinated proteins in the gingival tissues of periodontitis patients. However, to date no studies have reported local production of anti-CCP antibodies in the periodontium. With the mounting evidence of a relationship between periodontitis and RA, and some studies suggesting a link between the presence of autoantibodies and periodontitis, determination of the presence of these antibodies in the periodontium may provide useful insight into the possible mechanisms for this association. To our knowledge there are no studies investigating the presence or expression of PAD in the periodontal tissues. Thus, the aim of this study was to determine if anti-CCP antibodies, rheumatoid factor, and PAD types -2 and -4 are present in the periodontium, and whether or not their expression is related to periodontitis. The hypothesis is that local production of rheumatoid factor, citrullinated proteins, anti-cyclic citrullinated peptide autoantibodies, and peptidylarginine deiminase types 2 and 4 in periodontal tissues provides an extra-synovial source of autoantibodies, and that expression of these proteins is increased in periodontitis.

2.2 Materials and methods

2.2.1 Patient selection

Ethics approval was sought and obtained from the University of Adelaide Human Ethics Research Committee and also the South Australian Dental Service, in accordance with the guidelines of the National Health and Medical Research Council of Australia. Informed consent was obtained prior to treatment, and patients were given the option to withdraw from the study at any time.

Patients attending the Periodontology Department at the Adelaide Dental Hospital for periodontal surgery were invited to participate in this study. Following an explanation of the project and the surgical procedure, written and verbal informed consent were obtained from each patient prior to enrolment in the study and sample collection.
Patients were excluded if there were relative or absolute contraindications to periodontal surgery, such as pregnancy, use of anticoagulants (as listed in Section 3b of MIMS 2009), risk of bacterial endocarditis, uncontrolled diabetes, haematological disorders, any form of neoplasm and/or chemotherapy or radiotherapy. Patients were also excluded if they had taken antibiotics, steroids, or anti-inflammatory drugs in the previous 6 months.

The study included gingival samples taken from 50 patients, divided into two groups: periodontitis and non-periodontitis. The periodontitis group consisted of patients receiving periodontal surgery at sites of persistent deep pocketing (probing depth [PD] ≥5 mm) having previously undergone at least one course of non-surgical periodontal treatment and re-evaluation after a three-month healing period. Patients in this group were diagnosed with chronic or aggressive periodontitis, according to the American Academy of Periodontology 1999 classification system (Armitage 1999). This system grades the severity of periodontitis by the amount of clinical attachment loss, and also categorises periodontitis as either localised (if ≤30% of the teeth are affected by periodontitis) or generalised (if >30% of sites are affected). The majority of patients in the periodontitis group in this study had moderate or severe generalised chronic periodontitis, and received open flap debridement surgery. One patient in this group had mild generalised chronic periodontitis, and for this patient, the surgery performed was crown lengthening prior to prosthetic treatment at a site with no deep pocketing (maximum PD = 2mm). The pocket depth for the remainder of the periodontitis group ranged from 4 – 10mm, with a mean of 6.55 mm (SD ± 1.72mm).

The control group consisted of patients without periodontitis. The majority of these patients had either healthy periodontal tissues, or gingival inflammation without periodontal attachment loss or alveolar bone loss, and required crown lengthening surgery to remove tissue either for aesthetic purposes, or prior to undergoing prosthetic treatment. Three of the patients in this group were undergoing surgery to remove soft tissue as treatment for localised or generalised gingival enlargement.

### 2.2.2 Gingival tissue collection and processing

Tissue biopsies were collected during periodontal surgical procedures undertaken by dentists in the University of Adelaide Postgraduate Periodontics Training Programme at the Adelaide Dental Hospital. Samples were collected from tissue removed during surgery, which otherwise would have been discarded. Where possible, the samples included both connective tissue and epithelial tissue, and often included granulation tissue associated with periodontal defects. Due to the variation in defect morphology between patients, it was impossible to control the size of tissue samples or the exact site of collection. Following collection, each sample was immediately placed into a 2mL vial of 10% normal buffered formalin solution, and stored at room temperature for 24 – 48 hours. Samples were washed three times in phosphate-buffered saline (PBS, pH 7.2) for thirty minutes per wash, and then embedded in paraffin. Using a microtome, the tissue blocks were cut into 5μm thick sections, which were then mounted on glass slides.

### 2.2.3 Routine histological staining

The first section of tissue cut from each sample was sent to the University Histology laboratory for routine staining with haematoxylin and eosin. The sections were viewed under a light microscope for standard histological assessment. Samples which were too small or which did not contain any connective tissue were excluded. Each sample was also given an inflammatory score from 1-3 based on the amount of
inflammatory cell infiltration, using a grading system based on previous publications by Tak et al (1995) and Kraan et al (2000). A score of 1 indicated nil or mild infiltration; a score of 2 indicated moderate infiltration, and a score of 3 indicated heavy infiltration.

Two observers, who were blinded to the periodontal status of the patients, allocated a score to each tissue sample according to the grading system (illustrated in Figures 7A-7F, in Appendix 1).

2.2.4 Immunohistochemical Detection of RF, CCP, PAD2 and PAD4

The expression of RF, CCP, PAD2 and PAD4 in gingival tissues was investigated by immunohistochemistry using commercially available antibodies. For each of the antibodies, preliminary experiments were conducted to optimise antigen retrieval, primary antibody concentration, and detection (as per IHC-Paraffin protocol - http://www.abcam.com/ps/pdf/protocols/ihc_p.pdf). Both heat-mediated (either sodium citrate buffer – pH 6.0, or Tris-ethylenediaminetetraacetic acid [EDTA] – pH 9.0), and enzymatic (Proteinase K) antigen retrieval methods were tested, and each of these is described below.

2.2.4.1 Staining Procedures

Prior to staining, slides were de-waxed and rehydrated. Slides were placed into a rack, and washed in two changes of xylene for 10 minutes each, then in 100% ethanol for five minutes, and finally in 95% ethanol for five minutes. The slides were then washed twice in deionised water for five minutes, and remained in deionised water until antigen retrieval was carried out.

2.2.4.1.1 Anti-Citrulline

In order to stain for citrullinated proteins, enzymatic antigen retrieval was carried out using Proteinase K (200 μg/ml, Abcam, Cambridge, MA, USA) diluted in EDTA buffer. Proteinase K was added to each section by manual pipette, and then the slides were placed in an incubator at 37°C for 30 minutes. Following antigen retrieval, endogenous peroxidase (EP) activity was inhibited by adding 0.3% v/v H₂O₂ in PBS/0.1% Sodium Azide to each section for 10 minutes. A blocking serum (normal horse serum, supplied in the Vectastain Universal Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) was placed and left for 20 minutes to block non-specific binding. The primary antibody (Anti-Citrulline - rabbit immunoaffinity purified IgG, Upstate, Lake Placid, NY, USA) was then placed on the sections at a concentration of 2.66 µg/mL in PBS. For control sections, the primary antibody was omitted and replaced with PBS. The slides were incubated overnight at room temperature in a wet chamber. The following day, the sections were incubated with biotinylated secondary antibodies in PBS for 45 minutes. Sections were then reacted with peroxidase-labelled avidin-biotin-peroxidase complex (ABC reagent, Vector Laboratories) for a further 45 minutes.

For all proteins of interest, the protocol for colour development and counterstaining was identical. The colour reaction was developed using hydrogen peroxide as substrate and 3-amino-9-ethylcarbazole (AEC Peroxidase Substrate Kit, Vector Laboratories) as the dye, resulting in red colouring of positively staining sections. The sections were washed three times in PBS, for five minutes each, between each step, except after the addition of the blocking serum, which was shaken off prior to addition of the primary antibody. Following colour development and washing in deionised water, all sections were counterstained in haematoxylin for 10 seconds. The sections were then washed in water before being immersed in saturated
lithium carbonate solution for 30 seconds. After washing in water, the slides were mounted with Aquatex (Merck, Whitehouse Station, NJ, USA) and 22x50 mm glass cover slips (HD Scientific Supplies, Wetherill Park, NSW, Australia).

2.2.4.1.2 Peptidylarginine Deiminase 2

Peptidyl arginine deiminase-2 detection required heat-mediated antigen retrieval with Tris-EDTA buffer (pH 9.0). The Tris-EDTA buffer was heated to 95°C inside a glass container in a water bath, at which point the slides were immersed for 20 minutes. After removal from the water bath, the slides were cooled on a bench at room temperature for another 20 minutes. Endogenous peroxidase activity was inhibited as per the anti-citrulline protocol. Blocking serum (normal horse serum, supplied in the R.T.U. Vectastain Universal Quick Kit (Vector Laboratories)) was added to the sections and incubated for 20 minutes. The primary antibody (PAD2 antibody - rabbit polyclonal to PAD2, Abcam, Cambridge, MA, USA) was then added to each section at a concentration of 10µg/mL in PBS. For control sections, the primary antibody was omitted and replaced with PBS. The slides were incubated overnight at room temperature in a wet chamber. The following day, sections were incubated with prediluted biotinylated pan-specific universal secondary antibody (R.T.U. Vectastain Universal Quick Kit, Vector Laboratories) for 45 minutes. Sections were then reacted with preformed streptavidin/peroxidase complex (Vector Laboratories) for a further 45 minutes. Colour development, counterstaining and mounting of slides was carried out as per the anti-citrulline protocol.

2.2.4.1.3 Peptidylarginine Deiminase 4

Peptidyl arginine deiminase-4 detection required heat-mediated antigen retrieval with sodium citrate buffer (10mM, pH 6.0). The sodium citrate buffer was heated to 95°C inside a glass container in a water bath, at which point the slides were immersed for 20 minutes. After removal from the water bath, the slides were cooled on a bench at room temperature for another 20 minutes. Endogenous peroxidase activity was inhibited and blocking serum was placed as per the anti-citrulline protocol (described above). The primary antibody (PAD4 antibody - rabbit polyclonal to PAD4, Abcam, Cambridge, MA, USA) was then added to each section at a concentration of 10µg/mL diluted in PBS. For control sections, the primary antibody was omitted and replaced with PBS. The slides were incubated overnight at room temperature in a wet chamber. The following day, the sections were incubated with biotinylated secondary antibodies in PBS for 45 minutes. Sections were then reacted with peroxidase-labelled avidin-biotin complex (ABC reagent, Vector Laboratories) for a further 45 minutes. Colour development, counterstaining and mounting of slides was carried out as per the anti-citrulline protocol.

2.2.4.1.4 Rheumatoid Factor

During the preliminary experiments, all of the above mentioned antigen retrieval methods, a number of amplification methods, and RF primary antibody (Human Rheumatoid Factor Monoclonal Antibodies - Mouse IgG2b, QED Bioscience, San Diego, CA, USA) were tested at a range of working concentrations from 4-25µg/mL. However, no consistent staining was observed in test sections, nor in positive (or negative) control sections, and thus no results were obtained.
2.2.4.1.5 HISTOLOGIC ANALYSIS

All slides were scanned for ‘virtual microscopy’ using a NanoZoomer 2.0-HT high-resolution slide scanner and NanoZoomer Digital Pathology software (Hamamatsu Photonics, Japan). Semi-quantitative analysis (SQA) was conducted to determine the proportion of cells staining positively in each section, using the scoring system previously described Kataria et al (2010), itself a modification of systems published by Tak et al (1995) and Kraan (2000) and as follows:

Scores from 0 to 4 were given depending on the proportion of cells (excluding epithelial cells) staining positive for each antibody. Scores from 0 to 4 were given depending on the proportion of cells (excluding epithelial cells) staining positive for each antibody. Score 0: 0 to 10% of the cells stain positive; Score 1: 11 to 25% of the cells stain positive; Score 2: 26 to 50% of the cells stain positive; Score 3: 51 to 75% of the cells stain positive; Score 4: 76 to 100% of the cells stain positive.

2.2.5 GCF collection and processing

GCF was collected from the site with the deepest probing depth in the area of surgery, by the method of Uematsu et al (1996), a modification of the method originally published by Offenbacher et al (1986). Briefly, any visible supragingival plaque at the collection site was removed to avoid plaque contamination of the paper strips. The teeth were gently washed with water and air-dried. Salivary contamination was minimised by isolation with cotton rolls and use of a saliva ejector. GCF was collected using paper strips (Periopaper™, Oraflow, Plainview, NY, USA), with one strip being used for each collection site. The paper strips were carefully inserted 1 mm into the gingival crevice, avoiding trauma to the gingival tissues, and allowed to remain in place for 30 seconds, after which they were transferred for volume measurement. Strips with obvious blood contamination were discarded.

Volume determination was performed using a Periotron 8000™ (Oraflow, Plainview, NY, USA). This machine was located in the clinic, to minimise the delay between sample collection and analysis, and was calibrated regularly. The paper strips were transferred to the Periotron after sample collection, and the volume of GCF determined by converting the Periotron reading to a volume using the standard curve generated during calibration. The Periopaper strips were then wrapped in foil, placed in a sterile 1mL microcentrifuge tube, and stored at -20°C.

To retrieve the GCF, the Periopaper strips were placed into individual wells of a sterile 96-well microtitre plate (Flow Laboratories, McLean, VA, USA) and eluted using a previously published method (Megson et al 2010) with minor modifications. In order to achieve a volume that would allow further analysis by Enzyme Linked Immunosorbent Assay (ELISA), an initial volume of 225 μL of sterile PBS (pH 7.2) was added to each well. The plate was then sealed and placed on a plate shaker for 30 minutes at room temperature. The eluted samples were then transferred to 1.5 mL microcentrifuge tubes containing a further 225 μL sterile PBS and stored at -20°C until analysis by ELISA.

2.2.6 Detection of anti-CCP antibodies and Rheumatoid Factor by ELISA in GCF

Commercially available ELISA kits were used to analyse the GCF samples for the presence of anti-CCP antibodies (Diastat™ Anti-CCP FCCP200, Axis-Shield Diagnostics, Dundee, UK) and RF (Rheumatoid
Factor Screen [GD06], Genesis Diagnostics, Cambridgeshire, UK). As there were no commercially available ELISA kits for PAD2 or PAD4, GCF was not tested for the presence of either of these proteins.

The ELISAs were run in accordance with the manufacturers’ instructions for the respective kits:

In testing for the presence of anti-CCP antibodies, the semi-quantitative protocol of the Diastat Anti-CCP kit was followed. Wash buffer and reagents were prepared as per the manufacturer’s instructions. Five reference standards (ranging from 0 U/mL to 100 U/mL) and positive and negative controls (human plasma, <0.1% [w/v] sodium azide) were run in the same plate as the GCF samples, and incubated for 60 minutes. Wells were then decanted and washed three times with 200 μL of wash buffer. Conjugate (100 μL alkaline phosphatase-labelled murine monoclonal antibody to human IgG, Tris buffer, protein stabiliser, <0.1% [w/v] sodium azide) was added to each well, and the plates were incubated for 30 minutes. Following emptying and washing (as described above), 100 μL of substrate (Mg2+, phenolphthalein monophosphate [PMP], buffer solution) was added and the plates incubated for 30 minutes. A stop solution was added (100 μL) to each well, and the wells were gently tapped to mix. All standards and GCF samples were assayed in duplicate. A microplate reader (BioTek Powerwave, BioTek Instruments, Winooski, VT, USA) was used to read the optical density at 550 nm. Computer software (KC4, BioTek Instruments) was used to generate a weighted 5-parameter logistic curve, and this allowed determination of antibody activity in each sample.

In testing for the presence of RF, the wash buffer and reagents were prepared as per the manufacturer’s instructions. Five reference standards (ranging from 25 U/mL to 500 U/mL) and positive and negative controls were run in the same plate as the GCF samples, and incubated for 30 minutes at room temperature. Wells were then decanted, filled with wash buffer, and then emptied by inversion. This wash procedure was carried out three times. After washing, 100 μL of conjugate was added to each well and incubated for 15 minutes. Following this step, the wash procedure was carried out four times, and then tetramethylbenzidine solution was added and incubated for 15 minutes. Stop solution was added to each well, and the optical density was read in the plate reader at 450 nm, with 620 nm as a reference wavelength. KC4 software was used to generate a weighted 5-parameter logistic curve, and this allowed determination of antibody activity in each sample.

The results returned from both kits were expressed in an arbitrary measure of antibody activity (Units/mL), and as such did not give us information on the concentration of antibody in each sample.

2.2.7 Statistical analysis

A commercial statistical software package (SPSS version 11.5, SPSS Inc, Chicago, IL, USA) was used for the statistical analysis of the SQA results. For comparison of non-parametric values between the periodontitis and non-periodontitis groups, the Mann-Whitney U test was used to detect any difference between the groups. Non-parametric data was analysed using the Fisher’s exact test. Correlation between ordinal data was assessed using Kendall’s τb test. All tests were 2-sided, and results with \( p < 0.05 \) were considered statistically significant.

2.3 Results

The periodontitis group consisted of 29 patients (18 males, 11 females, mean age = 55.6 years) diagnosed with either chronic (n = 27) or aggressive (n = 2) periodontitis. With the exception of the one patient requiring crown lengthening described above, pocket depths for the periodontitis group ranged from 4
– 10 mm, with a mean of 6.55 mm (SD ± 1.72 mm). The non-periodontitis group consisted of 21 patients (11 males, 10 females, mean age = 38.3 years) who did not have periodontitis. The probing pocket depths (PD) in the non-periodontitis group ranged from 1 – 8 mm, although the deepest two pockets measured were associated with gingival enlargement without attachment loss, and thus represented ‘pseudopockets’. Aside from these two pockets, the range was 1 – 5 mm. The mean PD in the non-periodontitis group was 3.05 mm (SD ± 1.63 mm). The study population consisted of 29 males and 21 females, aged 15-84 years (mean age = 48.3 years), as shown in Table 1.1.

<table>
<thead>
<tr>
<th>Total</th>
<th>Periodontitis</th>
<th>Non-periodontitis</th>
<th>p-value **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>50</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>48.3 ± 20.4</td>
<td>55.6 ± 13.1</td>
<td>38.3 ±24.3</td>
</tr>
<tr>
<td>Male</td>
<td>29/50 = 58%</td>
<td>18/29 = 62%</td>
<td>11/21 = 52%</td>
</tr>
<tr>
<td>Female</td>
<td>21/50 = 42%</td>
<td>11/29 = 38%</td>
<td>10/21 = 48%</td>
</tr>
<tr>
<td>PD (mm)*</td>
<td>5.08 ± 2.41</td>
<td>6.55 ± 1.72</td>
<td>3.05 ± 1.63</td>
</tr>
<tr>
<td>Recession (mm)*‡</td>
<td>0.54 ±1.34</td>
<td>1.10 ±1.32</td>
<td>-0.24 ± 0.94</td>
</tr>
<tr>
<td>BoP positive</td>
<td>27/50 = 54%</td>
<td>22 / 29 = 75.9%</td>
<td>5/21 = 23.8%</td>
</tr>
<tr>
<td>Smoking history†</td>
<td>11/50 = 22%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCF volume (μL)*</td>
<td>0.36 ± 0.44</td>
<td>0.45 ± 0.54</td>
<td>0.24 ± 0.22</td>
</tr>
</tbody>
</table>

* Scores represent mean ± SD
** p-values according to Mann-Whitney U test
‡ Negative recession was recorded where the gingival margin was coronal to the cementoenamel junction, in cases of gingival enlargement.
† Patients were categorised as ‘smokers’ if they were current smokers or ex-smokers, or ‘non-smokers’ if they had never smoked. Further categorisation by length of smoking habit, time of quitting, or pack years resulted in a number of very small groups.
NS – not statistically significant (i.e. p ≥ 0.05).

Table 1.1. Patient demographics and clinical parameters measured.

As expected, statistically significant differences in probing pocket depth, bleeding on probing, gingival recession, and age were observed between the periodontitis and non-periodontitis groups (see Table 1.1). However, there was a poor correlation between clinical diagnosis and the level of inflammation observed histologically in each sample (Kendall’s τb = 0.171, p = 0.217). As illustrated in Table 1.2, over a third of the periodontitis tissue samples displayed only mild inflammation histologically, and half of the non-periodontitis samples were either moderately or severely inflamed. Although there was a tendency towards more severe inflammation in the periodontitis samples, the difference was not statistically significant.

<table>
<thead>
<tr>
<th>Inflammation</th>
<th>Periodontitis</th>
<th>Non-periodontitis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>10 (37%)</td>
<td>10 (48%)</td>
<td>20 (42%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>11 (41%)</td>
<td>10 (48%)</td>
<td>21 (44%)</td>
</tr>
<tr>
<td>Severe</td>
<td>6 (23%)</td>
<td>1 (5%)</td>
<td>7 (15%)</td>
</tr>
<tr>
<td></td>
<td>27*</td>
<td>21</td>
<td>48*</td>
</tr>
</tbody>
</table>

* Two periodontitis samples excluded following histological assessment.

Table 1.2. Histological inflammation vs clinical diagnosis
The majority of the subjects in the study (29/50 = 58%) reported no systemic conditions and no regular medication use. A summary of the conditions and medications reported by subjects in this study can be found in Table 3.13, in Appendix 1.

Whilst GCF samples and gingival tissue biopsies were collected from each of the 50 subjects, a number of the samples could not be used in the analysis. Gingival tissue samples from two subjects in the periodontitis group were discarded after initial histologic assessment (one section was too small to be analysed, and one section contained only epithelial tissue). Following staining with the respective antibodies, some of the tissue samples were damaged during antigen retrieval and processing. This left a number of samples in each group unsuitable for analysis, and thus these samples were excluded. Therefore, of the 50 subjects participating in the study, immunohistochemistry results were obtained from 45 samples for CCP, 40 for PAD2, and 41 for PAD4.

As previously mentioned, staining tissues for rheumatoid factor was unsuccessful, and no results were obtained for this part of the study.

In the immunohistochemical study, the SQA scores for each protein were compared between the periodontitis and non-periodontitis groups. Expression of CCP staining was generally cytoplasmic, and fibroblasts and blood vessels also tended to stain positively. Most of the tissues showed some positivity to CCP although the strength of expression did not appear to be associated with the clinical diagnosis. In fact, the periodontitis samples had a lower mean SQA score than the non-periodontitis (1.72 ± 0.23 versus 2.15 ± 0.30, see Figure 1), although there was no significant difference between the two (p = 0.240). Table 1.3 shows the frequency of each SQA score in the tissues stained for CCP.

Figure 1. Mean SQA scores for CCP, PAD2 and PAD4 in periodontitis and non-periodontitis samples
Table 1.3. Distribution of CCP semiquantitative analysis scores in periodontitis and non-periodontitis samples

Staining for PAD2 tended to be expressed more strongly than the CCP, in both the periodontitis and non-periodontitis samples (see Figure 1). The mean SQA score was higher in the periodontitis group than the non-periodontitis samples ($2.86 \pm 0.24$ versus $2.33 \pm 0.27$), but the difference was not statistically significant ($p = 0.128$). Table 1.4 shows the frequency distribution of the samples staining for PAD2 in the periodontitis and non-periodontitis groups, with most samples in the periodontitis group scoring towards the upper end of the scale. Ten samples were excluded in this group, due to tissue damage during the antigen retrieval procedures. This represents 20% of the initial sample size, and it is likely that such a reduction had an impact on the statistical power of the analysis.

Table 1.4. Distribution of PAD2 semiquantitative analysis scores in periodontitis and non-periodontitis samples

Tissue expression of PAD4 was generally lower than the expression of the other proteins, according to the SQA. Expression was generally higher in the periodontitis samples than the non-periodontitis group, although there was no significant difference between the means ($1.78 \pm 0.25$ versus $1.22 \pm 0.30$, $p = 0.134$). Table 1.5 shows the distribution of the SQA scores, and the low expression is clear, with half of the samples scoring either 0 or 1.

Table 1.5. Distribution of PAD4 semiquantitative analysis scores in periodontitis and non-periodontitis samples

* 5 samples excluded

* 10 samples excluded

* 9 samples excluded
The SQA scores were also analysed for any correlation between the level of inflammation and expression of any of the proteins. Tables 1.6-1.8 and Figures 2-4 show the distribution of SQA scores for each protein, categorised by the level of inflammation, with an apparent trend towards increasing expression of each of the proteins with higher levels of inflammation. A significant correlation was observed between PAD4 SQA score and inflammation (Kendall’s $\tau_b = 0.284$, $p = 0.040$). Correlation between inflammation and expression of each of the other two proteins, CCP (Kendall’s $\tau_b = 0.230$, $p = 0.080$) and PAD2 (Kendall’s $\tau_b = 0.259$, $p = 0.063$), approached statistical significance.

### Table 1.6. CCP semi-quantitative analysis scores vs inflammation

<table>
<thead>
<tr>
<th>Inflammation</th>
<th>CCP SQA scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>3</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>Severe</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
</tr>
</tbody>
</table>

* 5 samples excluded

### Table 1.7. PAD2 semi-quantitative analysis scores vs inflammation

<table>
<thead>
<tr>
<th>Inflammation</th>
<th>PAD2 SQA scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>0</td>
</tr>
<tr>
<td>Moderate</td>
<td>1</td>
</tr>
<tr>
<td>Severe</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
</tr>
</tbody>
</table>

* 5 samples excluded

### Table 1.8. PAD4 semi-quantitative analysis scores vs inflammation

<table>
<thead>
<tr>
<th>Inflammation</th>
<th>PAD4 SQA scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>5</td>
</tr>
<tr>
<td>Moderate</td>
<td>5</td>
</tr>
<tr>
<td>Severe</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
</tr>
</tbody>
</table>

* 5 samples excluded
**Figure 2.** Distribution of CCP SQA scores vs level of inflammation

**Figure 3.** Distribution of PAD2 SQA scores vs level of inflammation
Figure 4. Distribution of PAD4 SQA scores vs level of inflammation

The statistical analysis of the SQA scores did not reveal any significant correlation between expression of CCP with either PAD2 or PAD4. There was a weak correlation between expression of PAD2 and PAD4, although this did not quite reach statistical significance (Kendall’s $\tau_b = 0.271, p = 0.055$).

An unexpected finding was the statistically significant negative correlation between probing pocket depth and SQA scores for CCP expression (Kendall’s $\tau_b = -0.248, p = 0.036$). There was no relationship between pocket depths and expression of either PAD2 or PAD4. There was also a statistically significant correlation between the SQA score for PAD2 expression and bleeding on probing (Kendall’s $\tau_b = 0.299, p = 0.041$).

2.3.1 Analysis of GCF for presence of RF and anti-CCP

Procedural errors prevented the analysis of GCF samples from seven subjects (three periodontitis, four non-periodontitis). These strips were either omitted or discarded due to obvious blood contamination or improper collection, storage or transportation to the laboratory. Of the 43 useable GCF samples obtained, 26 were from periodontitis patients and 17 were from non-periodontitis patients. Both commercial ELISA kits used were designed for analysis of serum, but our preliminary experiments indicated that they could also be used for GCF.

In testing for anti-CCP antibodies, we found two distinct groups of readings: those that were clearly negative, and those that scored from 1.7 – 5.3 U/mL. According to the manufacturer’s instructions, the lower limit of detection for the anti-CCP ELISA kit was 0.05 U/mL, and readings >5 U/mL are considered positive in serum samples. Correspondence with the manufacturer revealed that the CCP peptides in this ELISA kit are very specific for anti-CCP, and samples with no anti-CCP present generally return readings well below the 2 U/mL standard. As such, although this ELISA kit is designed for use with serum/plasma, positive scores are indicative of the presence of anti-CCP antibody activity, even at low levels, in our GCF samples.

Tables 1.9 and 1.10 show the proportion of GCF samples which were positive for anti-CCP, and a number of clinical parameters observed in the patients. A majority (9 out of 11, or 82%) of the samples which were positive for anti-CCP were from the periodontitis group. Three of the positive samples came
from former heavy smokers. Figure 5 illustrates the range and distribution of the ELISA readings, with a majority of the samples testing returning negative results, and most of the positive samples coming from the periodontitis group. Whilst most of the positive samples were from the periodontitis group, there was no statistically significant correlation between GCF positivity for anti-CCP antibody and periodontitis (Kendall’s $\tau_b = 0.256, p= 0.097$).

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Periodontitis</th>
<th>Non-periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CCP positive</td>
<td>11/43 = 26%</td>
<td>9/26 = 35%</td>
<td>2/17 = 12%</td>
</tr>
<tr>
<td>BoP positive</td>
<td>7/11 = 64%</td>
<td>6/9 = 67%</td>
<td>1/2 = 50%</td>
</tr>
<tr>
<td>Smoker/ex-smoker</td>
<td>3/11 = 28%</td>
<td>3/9 = 33%</td>
<td>0/2 = 0%</td>
</tr>
</tbody>
</table>

Inflammation

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CCP positive</td>
<td>11/43 = 26%</td>
<td>4/14 = 29%</td>
<td>5/20 = 25%</td>
<td>2/9 = 22%</td>
</tr>
<tr>
<td>BoP positive</td>
<td>7/11 = 64%</td>
<td>3/4 = 75%</td>
<td>3/5 = 60%</td>
<td>1/2 = 50%</td>
</tr>
<tr>
<td>Smoker/ex-smoker</td>
<td>3/11 = 28%</td>
<td>1/4 = 25%</td>
<td>2/5 = 40%</td>
<td>0/2 = 0%</td>
</tr>
</tbody>
</table>

* 7 samples excluded

**Table 1.9.** GCF samples positive for anti-CCP antibodies, categorised by diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CCP positive</td>
<td>11/43 = 26%</td>
<td>3/9 = 33%</td>
<td>1/2 = 50%</td>
<td></td>
</tr>
<tr>
<td>BoP positive</td>
<td>7/11 = 64%</td>
<td>4/9 = 44%</td>
<td>1/2 = 50%</td>
<td></td>
</tr>
<tr>
<td>Smoker/ex-smoker</td>
<td>3/11 = 28%</td>
<td>2/9 = 22%</td>
<td>0/2 = 0%</td>
<td></td>
</tr>
</tbody>
</table>

* 7 samples excluded

**Table 1.10.** GCF samples positive for anti-CCP antibodies, categorised by severity of inflammation

The ELISA results for RF showed a different distribution to the anti-CCP ELISA, with a broader range of readings and, overall, fewer positives. According to the manufacturer’s guidelines, readings of >40 U/mL can be considered positive. The range of readings returned from our samples was 0 – 83 U/mL, with five samples clearly positive (see Figure 6). All of the positive readings were from periodontitis samples, which were either moderately or severely inflamed (see Tables 1.11 and 1.12).

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Periodontitis</th>
<th>Non-periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF positive</td>
<td>5/43 = 12%</td>
<td>5/26 = 19%</td>
<td>0/17 = 0%</td>
</tr>
<tr>
<td>BoP positive</td>
<td>4/5 = 80%</td>
<td>4/5 = 80%</td>
<td>N/A</td>
</tr>
<tr>
<td>Smoker/ex-smoker</td>
<td>2/5 = 40%</td>
<td>2/5 = 40%</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Inflammation

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Periodontitis</th>
<th>Non-periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>0/5 = 0%</td>
<td>0/5 = 0%</td>
<td>N/A</td>
</tr>
<tr>
<td>Moderate</td>
<td>3/5 = 60%</td>
<td>3/5 = 60%</td>
<td>N/A</td>
</tr>
<tr>
<td>Severe</td>
<td>2/5 = 40%</td>
<td>2/5 = 40%</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* 7 samples excluded
Table 1.11. GCF samples positive for RF, categorised by diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Total</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF positive</td>
<td>5/43 = 12%</td>
<td>0/14 = 0%</td>
<td>3/20 = 15%</td>
<td>2/9 = 22%</td>
</tr>
<tr>
<td>BoP positive</td>
<td>4/5 = 80%</td>
<td>3/4 = 75%</td>
<td>3/5 = 60%</td>
<td>1/2 = 50%</td>
</tr>
<tr>
<td>Smoker/ex-smoker</td>
<td>2/5 = 40%</td>
<td>1/4 = 25%</td>
<td>2/5 = 40%</td>
<td>0/2 = 0%</td>
</tr>
</tbody>
</table>

Diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodontitis</td>
<td>5/5 = 100%</td>
<td>N/A</td>
<td>3/3 = 100%</td>
<td>2/2 = 100%</td>
</tr>
<tr>
<td>Non-periodontitis</td>
<td>0/11 = 0%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* 7 samples excluded

Table 1.12. GCF samples positive for RF, categorised by severity of inflammation

Statistical analysis revealed that GCF positivity for RF was strongly and positively correlated with positivity for anti-CCP, and this was highly statistically significant (Kendall’s $\tau_b = 0.452, p = 0.003$). Positivity for RF was also significantly associated with increasing probing pocket depth (Kendall’s $\tau_b = 0.274, p = 0.041$) and gingival recession (Kendall’s $\tau_b = 0.284, p = 0.048$). A weak association approaching statistical significance was also found between RF positivity and periodontitis (Kendall’s $\tau_b = 0.293, p = 0.057$), and also between RF positivity and the level of inflammation (Kendall’s $\tau_b = 0.291, p = 0.053$). No correlation was found between GCF positivity for either antibody and expression of CCP, PAD2 or PAD4 in the gingival tissues (data not shown).

**aCCP ELISA**

![aCCP ELISA](image)

Figure 5. Anti-CCP antibody activity in GCF
Figure 6. RF activity in GCF. Dotted line indicates threshold for positivity according to the manufacturer of the ELISA kit.

2.4 Discussion

To our knowledge, this is the first study investigating the presence of RF, CCP, anti-CCP antibodies, PAD2 and PAD4 in the periodontium. The immunohistochemical study showed the presence of citrullinated proteins, PAD2 and PAD4 in the gingival tissues, both in periodontitis and non-periodontitis patients. The mean SQA scores for PAD2 and PAD4 were slightly higher in the periodontitis samples than the non-periodontitis samples, while the mean CCP SQA scores were slightly higher in the non-periodontitis group. None of these differences were statistically significant.

Citrullination has been associated with a number of pathological conditions, but it also occurs in a range of normal physiological functions, including apoptosis and terminal differentiation of the epidermis (for review, see Gyorgy et al 2006).

Whilst anti-CCP antibodies have been detected in serum prior to clinical onset of RA (Rantapaa-Dahlqvist et al 2003, van Venrooij et al 2006), it has been shown in an animal study that citrullinated proteins and PAD4 were not present in synovial tissues prior to onset (Lundberg et al 2005). Following disease onset, it was shown that as inflammation proceeded, expression of both CCP and PAD4 increased. The authors of that study also reported cytoplasmic staining of PAD4 and not nuclear staining, which contrasts to previous reports in the literature (Nakashima et al 2002). This suggests that citrullination in the joint may be a consequence of RA rather than a cause, but also suggests that anti-CCP antibodies produced outside the joints may be involved in triggering the onset of RA. In the present study, citrullinated proteins were detected in the gingival tissues of both periodontitis and non-periodontitis patients, and there was a trend towards higher expression with increasing inflammation, in agreement with Lundberg’s (2005) study.
This may suggest that citrullination could be involved in periodontal inflammation, but also raises the possibility that the periodontium is an extra-articular site of citrullinated proteins to which autoantibodies are formed, eventually contributing to the RA disease process.

Staining for CCP showed a cytoplasmic and perinuclear pattern, with some extracellular staining apparent. Blood vessels and fibroblasts stained positively in a number of sections (see Figure 8 in Appendix 1). While the pattern of staining did not seem to depend on the presence or absence of periodontitis, the strength of expression tended to increase with the level of inflammation. Similar findings were reported in an immunohistochemical study of synovial tissue, which reported that increased citrullination was associated with inflammation, rather than being associated with a specific disease process (Makrygiannakis et al 2006). The expression of intracellular citrullinated proteins has been examined previously. De Rycke et al (2005) reported a much higher expression of intracellular citrullinated proteins in RA synovium than in non-RA controls, suggesting that a number of these proteins are specific to RA. They also reported staining of a number of extracellular citrullinated proteins which were not specific to RA. A cytoplasmic pattern of staining was reported in another study which showed that the presence of citrullinated proteins in synovium is not exclusive to RA (Vossenaar et al 2004b). The present study did not distinguish between the individual types of citrullinated proteins, but it appears that the observed pattern of staining for citrullinated proteins in the gingival tissues is consistent with those reported in other tissues.

Vossenaar et al (2004b) also investigated a possible correlation between the expression of staining for citrullinated proteins and anti-CCP antibodies in serum and synovial fluid. In the RA patients, there was no correlation between titres for anti-CCP antibodies and citrullinated protein expression. There was, however, a clear correlation between anti-CCP levels in serum and synovial fluid. The anti-CCP antibodies represented a higher proportion of IgG in the synovial fluid than serum, suggesting that local production in the synovium was likely. In our study, we found no significant correlation between GCF-positivity for anti-CCP antibodies and expression of citrullinated proteins in the tissues. The ELISA kit used in the present study did not allow for measurement of the concentration of anti-CCP antibodies, but rather indicated whether or not there was antibody activity.

In the present study, eleven of the forty-three GCF samples (26%) tested showed positivity to anti-CCP antibodies, including 9/26 periodontitis samples (35%), and 2/17 non-periodontitis (12%) samples. It has been shown that anti-CCP antibodies are very specific for RA, and the prevalence of seropositivity in healthy subjects is very low (0-2%) (Schellekens et al 2000). According to the self-reported medical history data we collected, none of the subjects in our study had RA. The presence of anti-CCP antibodies in the GCF of patients in our study may be indicative of early or impending RA, although it seems unlikely that this would explain such a high percentage of positive samples, considering that prevalence of RA is widely reported as being around 1% (Symmons et al 1994). Alternatively, it may support the suggestion that anti-CCP antibodies are involved in the periodontitis disease process. Presence of anti-CCP antibodies has been reported in a percentage of patients with other (non-RA) inflammatory diseases, such as psoriatic arthritis (Bogliolo et al 2005), systemic lupus erythematosus (Hoffman et al 2005) and Sjögren’s syndrome (Gottenberg et al 2005, Atzeni et al 2008). It has been suggested that a small proportion of these findings may be related to false positives, and also that a number of these patients may be in the early/subclinical stages of RA (Vander Cruyssen et al 2005).

To date, no studies analysing GCF for the presence of anti-CCP antibodies have been published, but several studies have reported its presence in the serum of periodontitis patients. Havemose-Poulsen et al
(2006) and Hendler et al (2010) reported seropositivity for anti-CCP antibodies in a small proportion (<10%) of non-RA patients with generalised aggressive periodontitis, whilst healthy controls and patients with chronic periodontitis or gingivitis were all seronegative. Ballini et al (2010) examined the sera of a small group of chronic periodontitis patients for the presence of anti-CCP antibodies found only one patient out of 20 to be seropositive. A study of RA patients with chronic periodontitis found an association between seropositivity for anti-CCP antibodies and increased severity of periodontitis (Dissick et al 2010).

Gingival crevicular fluid consists of a complex mixture of substances derived from systemic (serum) and local sources, and the systemic expression of numerous inflammatory and immune markers has been associated with localised (periodontal) expression in the GCF (Ebersole 2003). Given the results of the present study in the context of the few serological studies available, it would be valuable to analyse serum samples and compare them to our GCF samples. Unfortunately, serum samples were not obtained, but this appears to be a promising avenue for future research. Vossenaar et al (2004b) reported a correlation between anti-CCP levels in synovial fluid and serum, and observed higher levels in the synovium, suggesting local production in the joint. Quantitative analysis of the GCF for the levels of anti-CCP and comparison to serum levels could shed some light on the possibility that anti-CCP antibodies are produced locally in the periodontium.

A close association between seropositivity for anti-CCP antibodies and seropositivity for RF has been reported (Turesson et al 2007, Papadopoulos et al 2008, Dissick et al 2010). Analysis of the ELISA results from the current study revealed a strong correlation between GCF positivity for anti-CCP antibodies and RF. Again, serological data would provide a useful insight into the significance of this finding. Whilst RF positivity was only detected in a small number of GCF samples (5/43 = 12%), all were periodontitis patients, and all were from sites which returned moderately or severely inflamed tissue biopsies. The only previous report of RF positivity in GCF was an incidental finding in a mass spectrometry study which reported RF at very low levels in a small number of samples (Bostanci et al 2010).

Unfortunately, we were unable to stain the gingival tissues for the presence of rheumatoid factor, so no correlation between tissue and GCF expression could be investigated. Whilst the presence of RF in gingival tissues has been reported previously in a study which used latex slide agglutination to analyse homogenised tissue samples (Gargiulo et al 1982, Gargiulo et al 1985), there are no studies reporting immunohistochemical staining for RF. Staining and semiquantitative analysis of gingival tissues for the expression of RF may have given some further insight into the possibility that the gingival tissues are an extra-articular site of RF production.

Both PAD2 and PAD4 have been previously isolated in inflammatory cells, and it is likely that this finding underpins their proposed roles in the development of RA (Vossenaar et al 2004a, Foulquier et al 2007). In the present immunohistochemical study, both PAD2 and PAD4 were detected in a number of the periodontal tissue samples. Whilst no significant association was found between expression of PAD2 or PAD4 and periodontitis, there was a notable trend towards increased expression of each protein in more inflamed tissue. This association was statistically significant in PAD4 (p = 0.040) and approached significance in PAD2 (P = 0.063). These results support the findings of a previous study examining the expression of PAD in synovial tissue biopsies from RA patients and non-RA controls (Foulquier et al 2007). In that study, the expression of each protein was evaluated by semiquantitative analysis and compared to the level of histological inflammation present in each sample. Similar to the findings of the present study, they found that whilst the expression PAD was not exclusive to RA tissues, the expression of both PAD2 (p
and PAD4 ($p < 0.03$) showed significant correlation with the level of inflammation. Studies in animal models have also reported increased expression of PAD4 in more severe inflammation (Lundberg et al 2005).

The PAD enzymes have all been located in the cell cytoplasm, although PAD4 is often located in cell nuclei (Nakashima et al 2002). While several animal studies have recently reported nuclear staining of PAD2 under certain conditions (Cherrington et al 2010, Jang et al 2011), this has not yet been confirmed in human studies. Staining patterns observed in the current study showed cytoplasmic localisation of PAD2, generally in mononuclear cells, with some blood vessels and fibroblasts also appearing to stain positively (see Figure 9 in Appendix 1). These patterns resemble the staining reported in studies examining staining in synovial tissues (Nakayama-Hamada et al 2005, Foulquier et al 2007).

Staining for PAD4 in our study consisted of a mixture of nuclear and cytoplasmic staining, mostly occurring in mononuclear cells, but blood vessels and fibroblasts also seemed to stain positively in numerous sections (see Figures 10A-10G in Appendix 1). This is consistent with previous reports of PAD4 staining in other tissues, including RA synovium (Chang et al 2005, Foulquier et al 2007) and various tumour types (Chang and Han 2006, Chang et al 2009). As well as the cellular staining pattern, PAD4 staining showed a peculiar distribution in a number of our samples. As can be seen in Figure 10 (in Appendix 1), whilst there is a heavy generalised inflammatory infiltrate in this section, the PAD4 staining is not taken up by all inflammatory cells, and instead is expressed only in certain clusters of cells throughout the tissue.

The expression of PAD by *P. gingivalis* (PPAD) was not examined in this study. Citrullinated proteins were located in the gingival tissues, and whilst PAD2 and PAD4 were also detected, it cannot be assumed that no other PADs were involved in the citrullination process. As such, it may have been useful to investigate whether or not PPAD was present. Whilst *P. gingivalis* is strongly associated with periodontitis, it has been detected in the supra- and subgingival biofilm of a percentage of healthy controls (Riviere et al 1996). Thus, PPAD may be present in periodontitis or non-periodontitis patients, just as the presence of citrullinated proteins was detected in both groups in our study. Further research is required to determine if PPAD is present in the periodontium, and whether or not it is involved in citrullination of proteins in the gingival tissues.

Given the limited sample size and varied smoking history of the patients, statistical analysis of smoking status had to be limited to smokers versus non-smokers. Regardless of whether former smokers were categorised as smokers or non-smokers, our analyses revealed no significant relationship between smoking status and expression of any of the proteins. A previous study reported increased PAD2 expression in the lung tissue of smokers, but no difference between smokers and non-smokers in PAD4 (Makrygiannakis et al 2008).

The poor correlation between the clinical diagnosis and the degree of inflammation observed histologically in each sample is not a unique finding. It has long been established that development of gingival inflammation in response to plaque accumulation varies between individuals (Löe et al 1965), and in populations with ubiquitous plaque/calculus deposits and no oral hygiene, only a particular subset of the population developed severe periodontitis (Löe et al 1986). To this end, it is feasible that moderate or severe chronic inflammation may be present in patients who do not experience any loss of periodontal attachment or alveolar bone and thus fall into the ‘non-periodontitis’ category.
In the periodontitis tissue samples, the variation in severity of inflammation may have been influenced by the fact that the patients in this group all underwent a course of non-surgical periodontal treatment and a healing period prior to surgery.

An incidental finding of our study was that a significant proportion of periodontitis patients whose GCF samples were positive for anti-CCP (5/9) or RF (3/5) reported hypertension on their medical histories. This is in contradiction to a previous study in RA patients, which showed no association between hypertension and seropositivity for anti-CCP antibodies (Lopez-Longo et al. 2009). Given the low numbers, and the demographics of the periodontitis patients, it is unlikely that this finding represents a significant association. Furthermore, due to the heterogeneity of the medical conditions reported and the small sample size, we were unable to analyse the possible effects of general health or specific medical conditions on our results.

As stated above, there were significant differences in the clinical and demographic parameters between the periodontitis and non-periodontitis groups. For this reason, the groups were not age- or sex-matched, and thus these factors could possibly confound results to an unknown degree.

The immunohistochemical and ELISA analyses in this study present findings which have not previously been reported in the literature. Although the sample size limits the power of the statistical analysis of the results, we made a number of interesting observations. The presence of CCP, PAD2 and PAD4 in the gingival tissue samples did not appear to be related to the presence or absence of periodontitis. However, a trend towards increased expression of each of these proteins with increased tissue inflammation was observed, which is consistent with reports of their expression in other tissues. As far as the expression of autoantibodies in the GCF, a majority of the samples positive for either RF or anti-CCP antibodies came from the periodontitis group. Whilst the statistical analysis did not reveal a significant correlation between diagnosis and GCF positivity, the fact that the positive samples were almost exclusively from the periodontitis group suggests that there may be some connection. The GCF expression of these antibodies did not appear to be related to the severity of localised tissue inflammation. One theory which could possibly explain these findings is that the PADs and citrullinated proteins are involved in tissue inflammation in the gingival tissues, regardless of periodontitis status, whereas the autoantibodies are only produced in a certain susceptible subset of patients. It has previously been suggested that chronic periodontitis is actually a ‘constellation’ of several different polygenic and polymicrobial disease variants with similar clinical presentation (Armitage 2002). If true, the fact that not all of the periodontitis patients were positive for either of these antibodies could be indicative of a role for these antibodies in a particular subset of periodontitis patients. A strong correlation was also found between positivity for the autoantibodies with each other (Kendall’s $\tau_b = 0.452, p = 0.003$). As such, it is possible that the production of autoantibodies may be involved in susceptibility and/or disease progression.

### 2.5 Future directions

In this study, we detected the presence of RF and anti-CCP antibodies in the GCF. Both of these antibodies have been repeatedly detected in the serum of RA patients, and their potential role in that disease has been extensively reported. Future studies examining both GCF and serum for the presence of these antibodies in periodontitis and non-periodontitis subjects may provide more insight into the relevance of their presence in the periodontium. A larger sample size would provide more statistical power with which to
analyse any differences between the groups. Also, further investigation into the expression patterns of PAD and CCP in the immunohistochemical studies is required. Whilst the semiquantitative analysis revealed positive staining in an increased proportion of cells in more inflamed tissues, in some sections only a particular subset of cells stained positively. As such, it may be useful to conduct more advanced assays, such as dual immunohistochemistry studies, in order to identify the cell types involved in citrullination in these tissues. A possible role for bacterial PAD (PPAD) in citrullination of proteins in the periodontium has been theorised based on in vitro studies, and further studies are required to examine the presence and activity of these enzymes in animal or human periodontal tissues.

2.6 Conclusion

We have shown that citrullinated proteins and peptidylarginine deiminase types 2 and 4 are present in inflamed periodontal tissues, and that anti-CCP antibodies and rheumatoid factor can be detected in the GCF of some patients. Tissue expression of CCP and PAD appeared to increase with the severity of localised inflammation, and did not appear to be related to the patient’s clinical periodontitis status. In contrast, the presence of autoantibodies in the GCF was almost exclusive to a subset of periodontitis patients, and did not appear to be related to the localised inflammation. The anti-CCP antibodies in particular are highly specific for RA, even in early/pre-clinical disease. As such, if their detection in GCF mirrors serum findings, this may provide a minimally invasive method of screening for RA. If the proteins are detected in GCF before they can be detected in serum, this may suggest local production in the periodontium, and could add weight to the theories that periodontal infection contributes to the onset of RA in susceptible patients. Further studies are needed to relate the GCF findings to serum analysis, and to further investigate the specific cellular expression of PAD and CCP. The presence of these proteins in the periodontium, combined with their known importance in the development and progression of RA, suggests that this is a promising area of future research in investigating links between the two diseases.
2.7 References


Appendix 1. Figures and Tables

Figure 7. Haematoxylin and eosin staining of gingival tissues, depicting various grades of inflammation. A, B. Mild inflammation at 10x and 20x magnification. C, D. Moderate inflammation at 10x and 20x magnification. E, F. Severe inflammation at 10x and 20x magnification. Bar = 200µm
Figure 8. Immunohistochemical staining for CCP in gingival tissue. Red = positive staining for CCP. Blue = haematoxylin counterstaining. **A.** 20x magnification. **B.** 40x magnification. Bar = 200µm.

Figure 9. Immunohistochemical staining for PAD2 in gingival tissue. Red = positive staining for PAD2. Blue = haematoxylin counterstaining. **A.** 20x magnification. **B.** 40x magnification. Bar = 200µm.
Figure 10. A. Routine H&E of periodontitis tissue at 4x, showing heavy generalised inflammatory infiltrate. B-G. Immunohistochemical staining for PAD4 in tissue from same sample as 10A. Red = positive staining for PAD4. Blue = haematoxylin counterstaining. B. 4x magnification. C. 10x magnification. D. 20x magnification. E-G. 40x magnification. Bar = 200µm.
<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Periodontitis</th>
<th>Non-periodontitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No systemic conditions</td>
<td>29/50 = 58%</td>
<td>15/29 = 52%</td>
<td>14/21 = 67%</td>
</tr>
<tr>
<td>Systemic conditions</td>
<td>21/50 = 42%</td>
<td>14/29 = 48%</td>
<td>7/21 = 33%</td>
</tr>
<tr>
<td>Hypertension</td>
<td>13/50 = 26%</td>
<td>9/29 = 31%</td>
<td>4/21 = 19%</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>8/50 = 16%</td>
<td>6/29 = 21%</td>
<td>2/21 = 10%</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>3/50 = 6%</td>
<td>2/29 = 7%</td>
<td>1/21 = 5%</td>
</tr>
<tr>
<td>Diabetes Type 2</td>
<td>4/50 = 8%</td>
<td>3/29 = 10%</td>
<td>1/21 = 5%</td>
</tr>
<tr>
<td>Gout</td>
<td>3/50 = 6%</td>
<td>2/29 = 7%</td>
<td>1/21 = 5%</td>
</tr>
<tr>
<td>Thyroidectomy</td>
<td>2/50 = 4%</td>
<td>1/29 = 3%</td>
<td>1/21 = 5%</td>
</tr>
<tr>
<td>Asthma</td>
<td>1/50 = 2%</td>
<td>1/29 = 3%</td>
<td>0/21 = 0%</td>
</tr>
<tr>
<td>Depression</td>
<td>1/50 = 2%</td>
<td>1/29 = 3%</td>
<td>0/21 = 0%</td>
</tr>
<tr>
<td>Fibromyalgia</td>
<td>1/50 = 2%</td>
<td>1/29 = 3%</td>
<td>0/21 = 0%</td>
</tr>
<tr>
<td>Osteoporosis</td>
<td>1/50 = 2%</td>
<td>1/29 = 3%</td>
<td>0/21 = 0%</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antihypertensives</td>
<td>11/50 = 20%</td>
<td>8/29 = 28%</td>
<td>3/21 = 14%</td>
</tr>
<tr>
<td>Cholesterol lowering drugs</td>
<td>9/50 = 18%</td>
<td>6/29 = 21%</td>
<td>3/21 = 14%</td>
</tr>
<tr>
<td>Oral antidiabetic drugs</td>
<td>4/50 = 8%</td>
<td>3/29 = 10%</td>
<td>1/21 = 5%</td>
</tr>
<tr>
<td>Antihyperuricemics</td>
<td>3/50 = 6%</td>
<td>2/29 = 7%</td>
<td>1/21 = 5%</td>
</tr>
<tr>
<td>Analgesics</td>
<td>2/50 = 4%</td>
<td>1/29 = 3%</td>
<td>1/21 = 5%</td>
</tr>
</tbody>
</table>

**Table 3.13.** Systemic medical conditions and medications reported by subjects