CHANGES IN THE MICROFLORA AND HUMORAL IMMUNE RESPONSE FOLLOWING PERIODONTAL THERAPY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Aa	*Actinobacillus actinomycetemcomitans*
A. actino	*Actinobacillus actinomycetemcomitans*
ADCC	antibody-dependent cell-mediated cytotoxicity
AL	attachment loss
ANOVA	analysis of variance
ANUG	acute necrotising ulcerative gingivitis
AP	adult periodontitis
AP-PCR	arbitrarily-primed polymerase chain reaction
BANA	N-benzoyl-DL-arginine-2, naphthylamide
BCFIA	bacterial concentration fluorescence immunoassay
Bf	*Bacteroides forsythus*
B. fors	*Bacteroides forsythus*
BOP	bleeding on probing
BSA	bovine serum albumin
CB	coating buffer
CEJ	cemento-enamel junction
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl) phenyl phosphate
DIG	digoxigenin
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbant assay
EOP	early-onset periodontitis
EU	ELISA units
GCF	gingival crevicular fluid
GEOP	generalised early-onset periodontitis
GJP	generalised juvenile periodontitis
GTR	guided tissue regeneration
HCMV	human cytomegalovirus
HIV	human immunodeficiency virus
HPV	human papilloma virus
HSV	Herpes simplex virus
IB	incubation buffer
IBD	Ivan Darby
IFA	immunofluorescence assay
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
LA	latex agglutination
LEOP	localised early-onset periodontitis
LJP	localised juvenile periodontitis
MGI	modified gingival index
MHC	major histocompatibility complex
na	not applicable

PCR	polymerase chain reaction
PD	pocket depth
Pg	*Porphyromonas gingivalis*
P. ging	*Porphyromonas gingivalis*
Pi	*Prevotella intermedia*
P. inter	*Prevotella intermedia*
PJH	Dr. P. J. Hodge
PLI	plaque index
PMN's	polymorphonuclear leukocytes
PPP	prepubertal periodontitis
PROS	pathogen-related oral spirochaete
RNA	ribonucleic acid
RRP	rapidly progressive periodontitis
rRNA	ribosomal ribonucleic acid
SD	standard deviation
SRP	scaling and root planing
Supp	suppuration
Td	*Treponema denticola*
T. dent	*Treponema denticola*
XB	Checkerboard technique

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Deep in the human unconsciousness is a pervasive need for a logical universe that makes sense. But the real universe is always one step beyond logic.

Frank Herbert, Dune, 1965

Se sufre pero se aprende.

DECLARATION

This thesis is the original work of the author.

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SUMMARY

This thesis investigated the changes in the microflora and humoral immune response with periodontal therapy. Scaling and root planing (SRP) has been shown to reduce the microbial load and produce clinical improvement. Generally, culture techniques have been used to analyse the microflora in periodontitis, but this technique is now recognised as having many errors and limitations. Polymerase chain reaction is a very sensitive and accurate technique for detecting bacteria and was used in these studies to investigate the flora in adult periodontitis (AP) and generalised early-onset periodontitis (GEOP) subjects before and after SRP. In addition, the effect of SRP on the systemic and local humoral immune response in AP patients was assessed. The antibody response is thought to be protective and the response of the humoral immune system to SRP may reflect this. The relationship between the microflora and humoral immune response was also determined. Antibody serostatus has been shown to have a relationship with baseline clinical parameters and affect the magnitude of both the clinical and humoral immune response to SRP. Serostatus was assessed and its effect on other parameters investigated in AP patients. Checkerboard DNA-DNA hybridisation is a relatively new technique with which to determine the content of periodontal plaque samples, but, although it has been shown to be more sensitive than culture, it has not been compared to other microbial assays such as PCR. Another aspect of this thesis was to compare PCR and the Checkerboard technique for microbial analysis. Smoking has been shown to be a risk factor for periodontal disease, and smoker patients tend to have greater periodontal destruction levels and respond less well to periodontal therapy. The

effect of smoking on the AP and GEOP patients was also investigated, and in the AP patients, the humoral immune response between smokers and non-smokers was compared.

Fifty seven untreated patients, 33 AP and 24 GEOP, were recruited for this study. Clinical parameters were recorded and GCF and plaque samples taken before and after SRP. There were 10 AP smokers and 12 GEOP smokers. In addition, venous blood was collected from AP subjects before and after therapy. Plaque samples were analysed for the presence of *P. gingivalis*, *P. intermedia*, *B. forsythus*, *A. actinomycetemcomitans* and *T. denticola* using both PCR and Checkerboard. GCF and serum samples were analysed by ELISA for antibody titres and serum antibody avidity to these organisms.

Comparison of AP and GEOP subjects at baseline showed that GEOP subjects have lower BOP and GCF volume, which may have resulted from the greater proportion of smokers in this group. GEOP subjects also had deeper pocketing and higher prevalences of *B. forsythus* and *A. actinomycetemcomitans*. The higher prevalence of these bacteria may be due to the deeper pocketing but also suggests a role for these organisms in GEOP. SRP produced significant clinical improvement and the reductions in PD and AL were in keeping with previously published data. However SRP produced few significant changes in the microflora in AP subjects and this probably reflects the sensitivity of PCR. GEOP subjects had significant reductions in the flora in response to SRP which mirrored the greater reduction in pocket depth (PD) seen in these patients compared to AP subjects. Bleeding on probing (BOP),

suppuration (Supp) and bacterial prevalences were related to pocket depth in AP subjects and the magnitude of the reduction of PD related to initial PD in both AP and GEOP subjects. Post-SRP, *B. forsythus* and *T. denticola* were associated with deep pockets in AP and *P. intermedia* in GEOP patients. In AP subjects the presence of *P. gingivalis*, *P. intermedia*, *B. forsythus*, and *T. denticola* were inter-related and, in GEOP, *T. denticola* was always detected with *B. forsythus*.

The comparison of PCR and Checkerboard showed roughly 60% agreement and the higher bacterial prevalences using PCR reflected the lower detection limit of this technique. However, a number of technical problems prevented optimal analysis by Checkerboard. Using Checkerboard to analyse microbial prevalence before and after SRP produced a higher number of significant reductions in the flora compared to PCR suggesting that the sensitivity of PCR masked the reductions in bacterial load.

SRP produced little change in systemic and local antibody titres and also antibody avidity, in contrast to previous reports. The results suggest that the poor immune response may have been a factor in the onset of disease. Serostatus had little effect on clinical parameters, and high responder and low responder patients responded similarly to therapy. The poor response of high responder patients paralleled the poor host response generally. High responder patients displayed generally higher systemic and local antibody titres to all organisms but similar clinical parameters to low responder subjects, suggesting that other underlying mechanisms are also involved in the disease process. The correlation of anti *P. gingivalis* serum and GCF

titres with PD and the presence of the organism may indicate its pathogenicity in AP subjects, but also the greater numbers of the organism in deeper pockets. *P. gingivalis*, *A. actinomycetemcomitans*, and *T. denticola* systemic and local antibody titres correlated suggesting greater systemic input, whereas *P. intermedia* and *B. forsythus* may have greater local production or destruction. The failure of the local response to *B. forsythus* may allow it to be pathogenic.

The Modified Gingival Index (MGI) and BOP were found to be lower in smokers than non-smokers at baseline, which is in keeping with the vasoconstrictive effects of nicotine. However, PD, plaque levels, and the microflora were not significantly different. Smokers responded less well to SRP, with lower reductions in PD and microbial prevalences. The poorer response may reflect the effects of smoking on the host, and the lower reduction in the microflora may be due to the more anaerobic pockets in smokers or impaired host clearance from the effects of smoking. Reduced antibody titres and avidity were noted in smokers compared to non-smokers, again reflecting the effect of smoking on the host immune response. However, in both smokers and non-smokers, SRP produced little change in antibody titre and avidity. The reduced titres and avidity in smokers compared to non-smokers, suggests that differences in the host response rather than the microflora may be responsible for the greater destruction seen in some smokers.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Periodontal disease is a widespread infectious disease. Because of an increase in the average life span, a decrease in carious tooth loss and an increasing elderly population, its incidence is increasing (Papapanou, 1996). There is thus a need for better understanding and management of the disease. Although the last thirty years have seen a great deal of effort directed towards these goals, leading to improved diagnosis, altered treatment modalities and an enhanced range of treatments available, much is still unknown.

In particular, the aetiology and pathogenesis associated with the microbial origins of the disease need further investigation. This thesis reports laboratory studies on the nature of the periodontal microflora and the associated humoral immune response with emphasis on changes induced by treatment. It begins with a survey of published reports of the basic concepts of the periodontal diseases and their treatment, making special reference to the microbial aetiology, and then extends to cover the humoral immune responses. Clinical and microbial methods of diagnosis and treatment are reviewed, followed by an explanation of the methods actually applied in this study, enabling the results to be interpreted in the context of other published investigations.

1.1.1 The clinically healthy periodontium

The periodontium is defined as "the tissues investing and supporting the teeth" (Hassell, 1993). This consists of the alveolar bone, root cementum, periodontal

ligament, and the gingivae. Together these form a functional unit (Lindhe & Karring, 1997), and of these, only the gingiva is clinically visible.

The gingiva is normally pink in colour, has a scalloped outline, a firm texture, stipling, and is demarcated apically from the oral mucosa by the mucogingival line. Attached keratinsed gingiva extends coronally from the mucogingival line and is firmly bound to the underlying periosteum by collagen fibres. The corono-apical width of the attached gingiva can vary significantly from tooth to tooth. The free gingival margin, which is normally about 1.5mm in the corono-apical dimension, surrounds but is not attached to each tooth. In health the gingiva completely fills the embrasure space between the teeth, and this part is termed the papilla (Wennström, 1988).

The gingival sulcus consists of the tooth surface on one side and sulcular epithelium of the free gingiva on the other. The sulcular epithelium is continuous coronally with the oral epithelium and apically with the junctional epithelium. The junctional epithelium extends from the base of the sulcus to the amelo-cemental junction, about 2.5mm, and is attached to the tooth surface by its basement lamina and hemidesmosomes.

Healthy gingiva does not bleed on gentle probing, and the depth of the gingival sulcus is minimal. The alveolar bone is located 1mm apically to the cemento-enamel junction, but healthy periodontium may show recession and reduced bone height.

Histologically, no inflammatory infiltrate is present, and classically only a few polymorphonuclear leukocytes (PMN's) can be seen within the connective tissue and junctional epithelium. Recently, a number of investigations have shown that an inflammatory infiltrate, comprising both PMN's and small round mononuclear cells is always present in gingival biopsies from clinically healthy gingivae (Seymour et al., 1983; Brecx et al., 1987; Kinane & Lindhe, 1997), and indeed Wennström (1988) suggested that the term "clinically healthy" is preferable to "normal" periodontium.

"Clinically healthy" periodontium produces the complex exudate, gingival crevicular fluid (GCF). It is normally produced in small amounts in health and reaches the sulcus via the junctional epithelium. It has the effect of flushing out the sulcus and enters the oral cavity to form part of the saliva. Its components are mainly derived from microbial products, interstitial fluid, locally produced factors of host origin, plasma and tissue degradation/turnover products (Cimasoni, 1983).

1.1.2 The "diseased" periodontium

Classification

Periodontal disease is a general term which could be used to refer to all diseases affecting the periodontium. However for the purposes of this discussion we will consider periodontal disease to refer to the two most common conditions: gingivitis and periodontitis.

Gingivitis

Gingivitis refers to pathological inflammatory changes that are limited to the gingiva. These are seen clinically by a change in colour (redness), texture and appearance (swelling) of the gingiva, with an increased tendency to bleeding on probing. All these effects are reversible and are associated with increased vascularity. The increased vascularity also leads to an increase in local temperature, and GCF. The main aetiological factor in gingivitis is dental plaque (Löe et al., 1965). Other factors have only a secondary role by either enhancing plaque accumulation e.g. overhanging margins, or increasing host susceptibility to plaque e.g. pregnancy.

Periodontitis

If the disease process affects the deeper structures of the periodontium resulting in loss of periodontal support, it is called periodontitis. It is associated with the presence of periodontal pockets, bleeding on probing (BOP), and bone loss.

Periodontal disease has been broadly classified by Attström & van der Velden (1994) as follows:

1) Adult periodontitis (AP)
2) Early-onset periodontitis
    a) Prepubertal (PPP)
    b) Localised early-onset (previously LJP) (LEOP)
    c) Generalised early-onset (previously GJP and RPP) (GEOP)

3) Necrotising periodontitis

There is considerable overlap within the classification defined above. For example, a systemic disease such as diabetes may contribute to the development of a non-responsive, so-called "refractory" periodontitis. HIV-associated periodontitis can be classified as periodontitis associated with systemic disease, although it usually manifests as a necrotising ulcerative periodontitis. The age of onset is difficult to determine retrospectively making it difficult to differentiate between GEOP and AP. Furthermore, these two forms of periodontitis often have the same clinical presentation, again making differentiation difficult.

Definitions of Adult and Early-onset Periodontitis.

Prepubertal periodontitis (PPP) affects pre-teenage children and can be localised or generalised (Page et al., 1983b). Localised prepubertal periodontitis affects children from about 4 years of age with attachment loss and alveolar bone loss around the primary molars and incisors only. There is moderate plaque and calculus accumulation with moderate signs of gingival inflammation and bleeding on probing at the diseased sites. The generalised form affects the primary dentition as soon as it erupts and there is severe generalised attachment loss and alveolar bone loss, frequently leading to premature exfoliation of teeth in the primary dentition. It is associated with severe marginal and attached gingival inflammation. Unlike the localised form of prepubertal periodontitis, the generalised form is frequently associated with host defects and/or systemic conditions (Page et al., 1983b; Watanabe, 1990) such as neutrophil dysfunction

(Page et al., 1983b; Schenkein & Van Dyke, 1994), leukocyte adhesion deficiency (Meyle, 1994), Papillon Lefevre syndrome (Hart & Shapira, 1994).

Localised early-onset periodontitis (LEOP) has an age of onset between puberty and 25-30 years (Baer, 1971; Schenkein & Van Dyke, 1994). There is attachment loss of 4mm or more in at least two permanent first molars and incisors; at least one first molar must be affected. To be classified as LEOP, there cannot be attachment loss of 4mm or more affecting more than two teeth other than first molars and incisors.

Generalised early-onset periodontitis (GEOP) affects people below 35. In this form attachment loss of 4mm or more affects at least eight teeth, with three or more non- molar or incisor teeth involved (Page et al., 1983a; Schenkein & Van Dyke, 1994).

Adult periodontitis (AP) is found in subjects 35 years and over. It affects all teeth and is not limited to a few particular teeth.

Prevalence of Early-onset periodontitis

Most studies in adolescents report prevalence estimates of LJP below 1% (Papapanou 1996). Löe & Brown (1991) estimated the prevalence of LJP at 0.53% and GJP at 0.13%. Studies in Europe have reported a prevalence for LJP and GJP between 0.06% and 0.5% (van der Velden et al., 1989; Saxén et al., 1980; Neely et al., 1992). The incidence of LJP in Caucasians has been reported

at 0.1%, 0.2% in Asians and 0.8% for Afro-Caribbeans (Saxby et al., 1984). From a number of studies it has become evident that EOP patients are particularly prone to further deterioration especially at a young age and the deterioration appears to be more marked at the initially affected sites (Albandar et al., 1991, 1993; Källestal et al., 1991). Longitudinal observations in the absence of any conventional oral hygiene or dental care have revealed a relatively small subset (8%) that experienced extremely rapidly progressive disease and could represent early-onset periodontitis, in contrast to the majority (81%), which probably represented adult periodontitis (Löe et al., 1986). EOP is a rare form of periodontal disease with LEOP affecting 1 or 2 in a 1000 and GEOP even rarer.

Prevalence of adult periodontitis

Although there have been many studies examining AP prevalence, there are difficulties in comparing them (Papapanou, 1996). Frequently the rapidly progressing disease in early to late twenties has been included in AP, rather than being classified separately as it is now. The 1976-77 North Carolina survey found that incipient periodontitis occurred among Caucasians mostly after the age of 40, whilst established periodontitis was uncommon before 60 (Hughes et al., 1982). Brown et al. (1989) analysing the data from a 1981 US survey reported the prevalence of periodontitis as 36%, and this increased with age. Reporting the 1985-86 survey, Brown et al. (1990) found that on average 13.4% of subjects had pockets of 4-6mm, which increased with age (5.7% at 18-24 years to 18.1% at 55-65 years). Advanced disease (pockets over 7mm) were

found in an average 0.6% of the study population, again increasing with age. The authors also noted that men were 1.5 times more likely to have deeper pockets than women and Afro-Caribbeans twice as likely as Caucasians. Jenkins et al. (1989) reported that of 800 dentate subjects aged between 16-73 years, 14.4% were affected by bone loss and 1% by generalised advanced bone loss. In their study of a Swedish population, Söder et al. (1994) found that 83% had no pocketing over 5mm, 5% had a 5mm pocket on one tooth, 7% on 2-5 teeth, 2% 6-9 teeth and 3% greater than 10 teeth. A study on a Greek population (Diamanti-Kipioti et al., 1995) reported that between 8% and 18% of their subjects had bone loss of more than 6mm, which increased with age as well.

Therefore it appears that severe forms of AP affect a considerable number, but probably not more than 10% of the population (Papapanou, 1996; Brown & Löe, 1993). This percentage increases with age and appears to reach a peak at the age of 50-60 years (Papapanou, 1996). Increased tooth loss occurring after this age may account for the subsequent decline in prevalence. Studies in elderly populations have supported the conclusion of Papapanou (1996) finding that moderate attachment loss was frequent and widespread but severe disease affected relatively limited proportions (Locker & Leake, 1993; Beck et al., 1990).

Therefore the current view is that periodontal disease is subject related, with a significant subset of individuals within a given population suffering from advanced periodontal destruction.

Risk factors

The term "risk factor" may indicate an aspect of personal behaviour or life-style, an environmental exposure, or inborn or inherited characteristic, which is known to be associated with disease-related conditions. A number of longitudinal studies have shown that smoking is a true risk factor for periodontal disease (Grossi et al., 1994, 1995; Ismail et al., 1990; Locker & Leake, 1993) and that certain microorganism species, and angular bony defects are risk factors (Haffajee et al., 1991a; Beck, 1994; Papapanou et al, 1989; Papapanou & Wennström, 1991). It is unclear if ageing per se is a risk factor or if its effect is due to the prolonged exposure of older subjects to true aetiological factors. Papapanou (1996) identified three main risk factors; smoking, diabetes mellitus and HIV infection. He also noted two other factors which may contribute; periapical pathology and malocclusion. However, different factors may be of importance in distinct population groups. Hence, race (Beck et al., 1990) or age (Grossi et al., 1994) appear to be significant determinants of the interaction between certain factors and disease expression.

1.1.3 Models of pathogenesis

Three models of pathogenesis have been described to explain destruction of the periodontium: the continuous paradigm, the random burst theory and the asynchronous multiple burst hypothesis.

The continuous paradigm postulates slow, constant and progressive destruction, and is supported by cross-sectional studies (Russell, 1967; Löe et al., 1978). The random burst theory proposes short periods of destruction punctuated by periods of resolution occurring randomly in time and at random sites within the subject (Socransky et al., 1984). By contrast, the asynchronous multiple burst hypothesis proposes that destruction occurs within a defined time frame and then resolution or remission follows. This hypothesis suggests that many sites would show bursts of activity over a limited period of time and then these sites would become inactive indefinitely. None of these proposed mechanisms can be established or refuted by presently available data (Williams & Paquette, 1997). However assessment of progression by pocket depth or attachment will, almost by definition, lend themselves to the detection of bursts during longitudinal trials.

1.1.4 Stages of pathogenesis

The Kinane and Lindhe (1997) revision of the 1976 classification of Page and Schroeder is outlined below.

1) Initial stages

Normal "clinically healthy" gingiva has been described above. Within 24 hours of plaque accumulation, the blood vessels beneath the junctional epithelium dilate and the resulting increased permeability allows the ingress of fluid and exudate into the tissues. GCF volume increases with concomitant increase in inflammatory and immune molecules. Löe et al. (1965) showed the increase in

GCF volume was proportional with gingival inflammation. Leukocytes, mainly neutrophils and lymphocytes, migrate into the tissues and accumulate in the junctional epithelium and around blood vessels, mediated by expression of adhesion molecules and a chemoattractant gradient from host and microbial factors. Lymphocytes are retained in the tissues on contact with antigens, cytokines and adhesion molecules. Within 2 to 4 days the cellular response is well established

2) Early gingivitis

After approximately seven days the signs of early gingivitis become apparent. An increase in blood vessels results from the opening up of previously inactive capillary beds. Lymphocytes and neutrophils are the predominant cellular infiltrate, with at this stage very few plasma cells (<10%). Apoptosis of fibroblasts starts to make room for the inflammatory infiltrate (Takahashi et al., 1995).

3) Established gingivitis

The progression of early gingivitis leads to further enhancement of the inflammatory state and established gingivitis. There is an increase in fluid exudation and leukocyte migration. The classical Page & Schroeder lesion (1976) is rapidly dominated by plasma cells but in humans it may take from 3 to 4 weeks (Payne et al., 1975) to over six months (Brecx et al., 1988) for this to occur. A large number of plasma cells are present in the coronal connective tissue and around blood vessels, comprising between 10 to 30% of the cellular

infiltrate. There is some tissue destruction, mainly collagen loss, and proliferation of the dentogingival epithelium in an attempt to maintain epithelial integrity and a barrier to microbial entry. The pocket epithelium loses its attachment to the tooth and has a heavy leukocyte infiltrate. The established lesions can remain at this stage as a stable condition (Lindhe et al., 1975; Page et al., 1975) or progress to periodontitis. The reasons for progression are unclear but may result from an increase in plasma cells in the lesion (Liljenberg et al., 1994).

4) Periodontitis

The pocket depth increases as the epithelium moves apically, establishing an "anaerobic niche". Alveolar bone loss and fibre damage result from inflammatory and immunopathologic tissue damage. At this stage plasma cells may be greater than 50% of the cellular infiltrate.

1.1.5 Differences between AP and GEOP

Longitudinal observations in the absence of any conventional oral hygiene or dental care have revealed a relatively small subset (8%) that experienced extremely rapidly progressive disease and could represent early-onset periodontitis, in contrast to the majority (81%), which probably represent adult periodontitis (Löe et al., 1986). Given the widely accepted view that the initiation of periodontitis is bacterial (Kinane & Lindhe, 1997), the vastly different rates of progression suggest that there may be corresponding aetiological or pathogenic differences between early-onset periodontitis and

adult periodontitis, either in the causative bacteria or in the effectiveness with which the host resists the infection (Ranney, 1993). The short time manifestation in early-onset periodontitis patients of clinically detectable lesions is generally interpreted as being an expression of either particular or aggressive causative agents or high levels of susceptibility of the individual patient, or a combination of the two. Host susceptibility may result from systemic disease (Ranney, 1993), immunological response (Ebersole, 1990), neutrophil dysfunction (Schenkein & Van Dyke, 1994) or from genetic aspects of the host (Tonetti & Mombelli, 1997). One aspect of this thesis is to examine the differences in the microflora between AP and GEOP patients. A review of the microflora of these forms of periodontal disease is to be found in section 1.4.

1.2 Microbial origins of pathogenesis

Currently the primary cause of periodontitis is considered to be bacterial infections of long standing, the composition of which may vary from individual to individual and to a lesser extent from site to site (Listgarten, 1986).

1.2.1 Dental Plaque

Dental plaque forms a microbial biofilm on colonised surfaces (Marsh & Bradshaw, 1995). Biofilms are defined as "matrix-enclosed bacterial populations adherent to each other and/or to surfaces" (Costerton et al., 1994) and can be considered as ecological communities that have evolved to permit survival of the community as a whole (Costerton et al., 1995). This structure provides a means by which the different bacterial species can benefit from each

other and can offer a measure of protection from the host defences (Darveau et al., 1997). The dental plaque biofilm has adapted to grow in a highly specialised ecological niche. It appears that several members of the biofilm community participate in extending the expanding biofilm along the tooth surface.

The dental plaque associated with the periodontally healthy tooth is predominantly that of early supragingival plaque. The bacterial load associated with gingival health is relatively low (10^2-10^3 isolates typically) (Newman et al., 1978; Slots, 1977; Tanner et al., 1996). It is characterised by a predominantly coccoid microbiota, including many Gram-positive species especially streptococci and actinomyces, with about 15% Gram-negative species (Newman et al., 1978; Slots 1977; Tanner et al., 1996). Cultural studies of such plaques indicate that this microbiota is composed predominantly of facultative bacteria (Listgarten, 1976).

The gingivitis-associated microbiota are characterised by a marked increase in the microbial mass (10^4-10^6 organisms) as well as a corresponding increase in the proportion of Gram-negative bacteria, motile rods and filaments (15-50%) (Tanner et al., 1996, Slots et al., 1978).

In adult periodontitis, an abundant, complex microbiota is observed in periodontal pockets, with an increased total microbial load (10^5-10^8 microorganisms) (Tanner et al., 1996). This bacterial population is predominantly Gram-negative, and includes a large proportion of spirochetes

preferentially distributed on the periphery, or tissue side, of the microbial mass. Cultural studies indicate that this microbiota is predominantly anaerobic (Listgarten, 1976). Dark-field microscopy studies have produced similar results (Listgarten & Hellden, 1978).

1.2.2 Formation of supragingival dental plaque

Within minutes after a tooth surface is freshly cleaned, a pellicle forms that consists of proteins and glycoproteins found in saliva and crevicular fluid. Pellicle formation, in addition to enhancing initial bacterial colonisation, provides surfaces for additional bacterial attachment (Skopek & Liljemark, 1994). Within hours the first bacteria colonise the surface, and are mostly Gram-positive, facultative cocci and coccobacilli; mainly Streptococcus and Actinomyces species. Veillonella species, and Gram-negative anaerobic cocci are also early colonisers.

The earliest foci of bacterial accumulation are localised to surface pits and fissures but can also be found on isolated protected portions of the smooth surfaces. In time the bacteria spread over larger sections of the smooth surfaces, with thicker accumulations at protected sites e.g. interdental surfaces and gingival margins. Plaque grows in thickness primarily through cell division of adherent bacteria. During the first day the surface is gradually covered by colonies of dividing bacteria that initially spread laterally along the tooth surface. Once the available surface is covered, the proliferating bacteria begin to grow away from the tooth in the form of columnar microbial colonies (Listgarten et

al., 1975). Around day 3 filamentous bacteria can be found on the surface of the predominantly coccoid plaque. Several species of coccoid bacteria are able to aggregate with the filamentous bacteria (Listgarten et al., 1975). Recently it has been shown that these filamentous bacteria may be *Fusobacterium* species, and that they coaggregate with all other oral bacteria (Whittaker et al., 1996). Therefore, it has been proposed that this organism plays a major role in plaque formation. Competitive growth among the predominantly coccoid microbial colonies continues for approximately 1 week. At that time filamentous bacteria begin to penetrate the coccoid plaque from the surface, gradually replacing the coccoid microbiota with a predominantly filamentous microbiota. The process may continue for approximately 2 more weeks. By then the columnar microbial colonies have disappeared and been replaced with a dense mat of filamentous bacteria orientated more or less perpendicularly to the colonised surface. This new structural organisation remains relatively unchanged over time and can be considered as the typical, relatively stable structure of mature, supragingival dental plaque (Listgarten et al., 1975, Listgarten, 1976). Plaque doubling times are rapid in early development and slower in more mature films (Weiger et al., 1995).

1.2.3 Formation of subgingival dental plaque

The undisturbed growth of supragingival plaque gradually results in soft tissue alterations in the adjacent gingiva. Beginning within a few days of undisturbed plaque formation, the gingival margin begins to show typical inflammatory changes including redness and swelling. The latter changes result in the creation

of a deepened gingival sulcus, which provides a relatively anaerobic environment for the development of an anaerobic microbiota. Anaerobic bacteria that colonise this subgingival region include motile rods and spirochaetes. They are able to increase their mass by contributing to the deepening of the sulcus, thereby increasing the volume of their ecological niche. Because many of the subgingival organisms are motile, the structural organisation is quite different from that supragingivally.

A relatively thin layer of adherent bacteria covers the tooth surface. Rods and filaments tend to be arranged in a palisading pattern, with the long axis of the cells perpendicular to the tooth surface. Unique bacterial aggregates, resembling test-tube brushes, can be found attached to the adhering plaque and extending into the space between the bacterial layer and the adjacent soft tissue wall. The "bristles" of these test-tube brush formations are Gram-negative filamentous bacteria, probably *Fusobacterium* species (Whittaker et al., 1996). The bulk of the subgingival microbiota consists of a complex mixture of predominantly anaerobic bacteria that surround and cover the test-tube brush formations. The lack of well-defined microbial colonies in this environment may be due to the high degree of motility of the resident bacteria. The peripheral region of the subgingival microbiota is composed of a high concentration of spirochetes that are in direct contact with the gingival tissue wall as well as the apical lining of the sulcus or pocket (Theilade, 1985). Sometimes layers of leukocytes, mostly neutrophils that have migrated out of the junctional epithelium, separate the bacterial mass from the sulcular or pocket epithelium.

The bottom of the sulcus or pocket is formed by the coronal, desquamative surface of the junctional epithelium, which is attached to the tooth surface on one side and to the gingival connective tissue on the other (Schroeder, 1977). This portion of the junctional epithelium is subject to bacterial as well as mechanical injuries, which may result in enlarged intercellular spaces and vertical tears in the epithelium. These alterations in the integrity of the junctional epithelium allow a gradual apical colonisation of the tooth surface by coccoid cells and rods (Schroeder & Listgarten, 1977; Vrahopoulos et al., 1992a, b). Irregularities in the root surface may shelter plaque microorganisms and contribute to their retention at such sites. The tendency for bacteria to colonise tooth surfaces freshly exposed because of disruptions in the junctional epithelium leads to a gradual deepening of the sulcus or pocket.

Thus, a distinctive subgingival microbiota, predominantly composed of Gram-negative, anaerobic bacteria, including a number of motile species, becomes established in the gingival sulcus between 3-12 weeks after the beginning of supragingival plaque formation. The establishment of this subgingival microbiota is dependent on a series of inter-related events: the successive colonisation of the tooth surface by different bacterial populations. Each of these microbial populations appears to facilitate the colonisation of this region by the next wave of bacterial settlers, with the ultimate establishment in the subgingival region of a predominantly anaerobic, Gram-negative microbiota. Most bacterial species currently suspected of being periodontal pathogens are anaerobic, Gram-

negative species whose main ecological niche is the subgingival region. In this protected environment they are in an excellent position to participate in the destruction of periodontal tissues, with the resulting maintenance and expansion of their subgingival habitat.

Subgingival plaque increases its growth environment by causing epithelial cells and attachment level to move apically, thereby creating deeper pockets. The mechanism by which this occurs is unknown but almost certainly involves combating the innate host defence system. Although gingival crevicular fluid (GCF) is the main nutritional component of the subgingival microbiota, it contains a potent array of host defence products. It appears that a combination of bacteria rather than just one act co-ordinately to further dental plaque biofilm growth. Bacterial biofilms are very resistant to removal and can be resistant to antibiotics (Anwar et al., 1992) as well as opsonisation and complement-mediated phagocytosis and killing (Jensen et al., 1990). The high level of resistance of biofilm bacteria probably involves limited access and the formation of microcolonies that are shielded from the external environment (Lawrence et al., 1991). The resistance of subgingival biofilms to normal host defences has important consequences for the patient and for periodontal therapy. Subgingival biofilms cannot be removed by daily oral hygiene methods, including subgingival irrigation, or by the use of antimicrobial agents in oral rinses or tooth pastes. Physical removal is essential. It is for this reason that scaling and root planing is an essential component of all forms of successful treatment for

periodontitis. Because of the nature of biofilms, this requirement is unlikely to change (Darveau et al., 1997).

1.3 Treatment of periodontal disease

Regardless of the type of periodontal disease, the sequence of treatment for most patients generally begins with establishing good oral hygiene and a thorough debridement, usually scaling and root planing. Scaling is defined as "instrumentation of the crown and root surfaces of the teeth to remove plaque, calculus, and staining". Root planing is defined as "a definitive treatment procedure designed to remove cementum or surface dentine that is rough, impregnated with calculus, or contaminated with toxins or microorganisms" (The American Academy of Periodontology, 1989).

There is considerable evidence supporting scaling and root planing (SRP) as an essential and effective component of therapy for periodontal disease (Cobb, 1996). The clinical benefits of SRP are derived from the removal of the subgingival microflora and therefore a delay in the re-population of pathogenic microbes allowing some healing to take place (Mousques et al., 1980). However, it appears that the subgingival microflora has supragingival origins as the quantity, composition and rate of subgingival plaque recolonisation is, to some degree, dependent upon supragingival plaque accumulation (Pedrazzoli et al., 1991; Magnusson et al., 1984; Sbordone et al., 1990a). Consequently, effective control of the supragingival plaque combined with frequent subgingival therapy is critical for long-term control of periodontitis.

1.3.1 The effect of scaling and root planing on pocket depth

The effect of SRP is generally related to initial pocket depth. At sites with an initial PD of 1 to 3mm, there seems to be a very slight gain in PD, if any, and an attachment loss of about 0.34mm (Hill et al., 1981; Pihlstrom et al., 1981; Nordland et al., 1987). Pockets with an initial depth of 4 to 6mm reduced in depth by an average of 1.3mm and gained 0.55mm attachment (Hill et al., 1981; Pihlstrom et al., 1981; Nordland et al., 1987; Loos et al., 1989; Badersten et al., 1984a; Pedrazzoli et al., 1991). Deep sites of 7mm and greater gained the most attachment, on average 1.2mm and reduced in depth by 2.16mm (Hill et al., 1981; Pihlstrom et al., 1981; Nordland et al., 1987; Loos et al., 1989; Becker et al., 1988; Mousques et al., 1980; Renvert et al., 1985; Claffey et al., 1988).

1.3.2 Healing after scaling and root planing

After treatment, histologic studies (Waerhaug, 1978) described the regeneration of the root-epithelial surface as a long-junctional epithelial attachment, which precludes the formation of a new connective tissue attachment. Re-establishment of the new epithelium appears to occur within one to two weeks (Waerhaug, 1978). Concomitant with the formation of the new attachment are gradual reductions in the clinical inflammation, that appear correlated to reductions in the inflammatory cell infiltrate, and GCF (Tagge, 1975; Biagini et al., 1988).

1.3.3 Non-responding sites

Although SRP usually brings a reduction in PD, there are some sites that do not respond to therapy (Badersten et al., 1985a, b; Claffey & Egelberg, 1994), which ranged from 10 to 22% of sites in patients in these studies. Further more these non-responder sites seem to be clustered within a small percentage of patients (Grbic & Lamster, 1992; Claffey & Egelberg, 1995).

1.3.4 Effect of scaling and root planing on bleeding on probing

Bleeding from the base of the pocket has traditionally been used as an indicator of disease activity. However, studies have reported weak correlations between BOP and disease progression (Badersten et al., 1985c, 1990; Claffey & Egelberg, 1995; Lang et al., 1986), and Lang et al. (1990) suggested that the absence of bleeding is a better indicator of stability. Regardless of the lack of correlation between BOP and risk for further attachment loss, it would appear that SRP will predictably reduce the level of inflammation, with a mean reduction of 57% (Cobb, 1996).

1.3.5 Effect of scaling and root planing on the microflora

Studies designed to determine the effect of scaling and root planing on the sub-gingival microflora have consistently reported significant reductions in the percentage of motile rods and spirochaetes, *P. gingivalis*, *A. actinomycetemcomitans*, and other Gram-negative anaerobic micro-organisms, and a concomitant increase in the percentage of cocci and non-motile microbes (Slots et al., 1979; Magnusson et al., 1984; Hinrichs et al., 1985; Lavanchy et al.,

1987; Loos et al., 1989; van Winkelhoff et al., 1988; Southard et al., 1989; Shiloah & Patters, 1996; Renvert et al., 1990a; Lowenguth et al., 1995; Rosenberg et al., 1989; Hakkarainen et al., 1986). Several of these studies note that improvement in clinical parameters such as decreased probing depth, and decreased bleeding are associated with the decrease in the above (Hakkarainen et al., 1986; Slots et al., 1979). Mombelli et al. (1994a, b) noted that it was much more difficult to eradicate organisms from deep bleeding pockets. The reasons why in some cases a given bacterium is below detection level after treatment and in others is not, are not well understood (van der Velden & Schoo, 1997).

1.3.6 Repopulation of pockets

The microbes which repopulate the pocket may represent residual organisms following SRP (Renvert et al., 1990a) or the downgrowth of organisms from supragingival plaque (Waerhaug, 1978). *A. actinomycetemcomitans* seems to be especially difficult to eradicate from pockets (Renvert et al., 1990a), which may be due to its ability to invade the periodontal tissues (Christersson et al., 1987a, b). However, Sbordone et al. (1990a) showed that all their study organisms had similar levels of reduction and the re-colonisation of the subgingival plaque was taking place 21 days after treatment and reached pre-treatment levels at about 60 days. Several studies have demonstrated that the microbial re-population of subgingival pockets can be severely inhibited by continual and effective oral hygiene (Dahlén et al., 1992; Katsanoulas et al., 1992; McNabb et al., 1992). As mentioned above it is more difficult to eradicate the microflora of deep bleeding pockets.

1.4 Review of the microbiology of periodontal disease

Studies in both humans and animals have shown that periodontal inflammation and destruction of periodontal tissues are initiated and supported by the bacteria of dental plaque. The concept of microbial specificity in the aetiology of periodontal disease has emerged in the last three decades (Loesche, 1976, Socransky, 1977). Previously it was believed that periodontal disease resulted from the gross accumulation of dental plaque. This hypothesis explained previous clinical experiences when investigators linked the universal presence of gingival inflammation and periodontal pocket formation with apparently ubiquitous presence of abundant plaque (Tanner, 1988). Loesche (1976) suggested that periodontal disease be considered as non-specific because:

1) The lack of bacterial invasion. Bacterial invasion has been demonstrated, but it does not appear, as yet, to be part of an acute phase of disease progression.
2) Their apparent non-specific nature.
3) Their chronicity, and
4) Their universality.

This non-specific theory has been disregarded because the 300-400 bacterial species in the oral cavity show different characteristics and therefore must play different roles (Dahlén, 1993). Although periodontal diseases are polymicrobial infections, cross-sectional and longitudinal studies of the predominant cultivable microflora reveal that only a small number of species are associated with human

periodontal disease (Moore et al., 1983; Theilade, 1986; van Winkelhoff et al., 1988; Haffajee & Socransky, 1994).

The specific plaque hypothesis suggests that a specific agent may be responsible for periodontal destruction. The concept of bacterial specificity has been further supported by clinical observations, by therapeutic effect of control of these bacteria, and by experimental models of periodontitis in both gnotobiotic and conventionally maintained animals. However, the continued finding of increased numbers of a range of species in periodontitis patients, the presence of suspected agents in inactive sites, the failure of "specific" antibiotics to stop disease progression, and the absence of these agents in some active sites indicates that the specific plaque hypothesis may not be valid (Tanner, 1988).

In the last few years it has become clear that a number of organisms are involved in the disease process. A number of proposed pathological agents are not capable of initiating a mono-infection (Mayrand & McBride, 1980; Dahlén et al., 1989b), but if these (*P. gingivalis*, *P. intermedia*, *P. micros*) are excluded, the microbial mixture loses its capacity to produce experimental infections. This theory has been supported by further microbiological findings especially those reporting an increase in number of bacterial species in periodontitis patients (Moore, 1987) and bacterial cluster patterns that may be related to disease (Socransky et al., 1998). Even so only three organisms have been designated aetiological agents: *A. actinomycetemcomitans*, *P. gingivalis* and *B. forsythus* (Consensus report, American Academy of Periodontology 1996).

Given their preponderance in subgingival plaque, it is unsurprising that a variety of Gram-negative organisms have been implicated in the aetiology of periodontal disease. These species include *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *Capnocytophaga* species, *B. forsythus*, *F. nucleatum* & *C. rectus*, as well as certain Gram-positive species such as *Eubacterium* (Moore et al., 1983, Moore, 1987; Haffajee & Socransky, 1994). A more complete list is shown in table 1.1 (adapted from Darveau et al., 1997).

Periodontal disease occurs in an area inhabited by many bacteria, and there are many problems in identifying specific aetiological agents. Numerous microbial species have been identified in supra- and sub-gingival plaques, and 30 to 100 may be recovered from a single site. Many of these species are difficult or impossible to culture and identify. Even though there is a variety of methods available to identify plaque bacteria that do not rely on bacterial viability, there is no current method that is able to identify and accurately quantitate all the bacterial species present in subgingival plaque. Sites in an individual do not actively progress at all times with respect to attachment loss, and the flora at these sites may not reflect a "disease-associated" flora. Different sites in a subject have their own individual flora and these sites may vary in their disease activity depending on the bacteria colonising that site. It is likely that hosts differ in their threshold susceptibility, and what may cause disease in one subject may not have an effect in another. Bacterial pathogens may exist in a carrier state and are sometimes compatible with health. Bacterial species present in

Table 1.1 Species commonly found in periodontal pockets

P. gingivalis
A. actinomycetemcomitans
P. intermedia
B. forsythus
Black-pigmented *Bacteroides*
T. denticola
Other *Spirochaetes*
F. nucleatum
C. rectus
Capnocytophaga species
E. corrodens
Eubacterium species
Selenomonas species
P. micros
Streptococcus species
Veillonella species
Actinomyces species
Enteric rods
H. pylori
Herpes group viruses

lower numbers in plaques associated with health, are likely to play little role in progressive disease. However opportunistic species may grow as a result of the disease rather than being the cause. It is becoming increasingly apparent that progressive periodontitis is a mixed infection, the result of a combination of bacteria rather than one specific agent. The situation is further compounded because most bacteria in subgingival plaque have a number of clonal types or subspecies, and these do not display the same level of virulence. The inability to distinguish virulent from avirulent clonal types has hindered understanding of the role of the microflora in the disease process. Inadequate reproducibility and measurement errors make it difficult to associate specific microorganisms with active disease. For example, the physical constraints of a pocket may make it difficult to obtain a representative sample.

1.4.1 Criteria for defining periodontal pathogens

Given the range and nature of the problems with identifying periodontopathogenic bacteria, a number of criteria have been defined (Socransky, 1977; Socransky & Haffajee, 1992). The criteria include association, elimination, animal pathogenicity, host responses, and production of virulence factors. The criterion of association requires that the suspected pathogenic species be more frequently detected and at higher levels in cases than controls. For example the species should be higher in actively progressing sites than in healthy sites, gingivitis sites, non-progressing sites, or sites showing improvement. Longitudinal assessment might also show an increase in the species prior to or concomitant with measured disease progression. The

fundamental basis of elimination studies is that treatment administered to subjects with a given form of disease should influence both the clinical status of the disease and members of the associated microbiota. It is reasonable to expect that successful therapy will diminish the level of a pathogen, improve the clinical situation, and halt disease progression. Failure to eliminate or diminish the level of the pathogen should be linked with a lack of clinical improvement and may ultimately lead to further progression at that site or in that subject. Testing of pathogenicity in animal model systems continues to be used to further support or refute possible pathogens. In spite of concerns with animal model systems, they can provide additional evidence of roles for certain species in disease and are particularly useful in defining virulence factors. The host response can also be used to discriminate periodontal pathogens. A periodontal pathogen that causes destructive periodontal disease often will elicit an elevated immunological response, either locally or systemically. If a species (or its antigens) gains access to underlying periodontal tissues and causes damage, it seems likely that the host will produce antibodies or a cellular immune response directed at that species. Thus the host response could act as a pointer to the pathogen(s). In certain circumstances the pathogen may diminish aspects of the host immune response. Finally the ability of certain species to produce virulence factors has been used to support possible roles of such species in periodontal diseases. The production of unique biochemical determinants by pathogens may be important in disease and an indicator of the potential of the species to contribute to disease progression.

Using these criteria the role of a number of proposed pathologic agents in AP and GEOP will be discussed in the next section. However only those criteria relevant to this section will be discussed. As a number of excellent reviews have extensively covered virulence factors and animals studies for these agents (Holt et al., 1999; Fives-Taylor, 1999; Haffajee & Socransky, 1994), these will not be discussed in this review. However some virulence factors will be mentioned where relevant. In addition, since part of this thesis is concerned with the humoral immune aspects of periodontal disease, this criterion will be discussed separately and in some detail.

1.4.2 Microbiology of Adult Periodontitis

This section reviews the possible microorganisms that may be aetiological agents in Adult periodontitis. The change in definitions of periodontal disease over the years has resulted in many papers grouping GEOP and AP together. Where possible the 1996 AAP guidelines have been applied to papers to separate the two groups. If this has not been possible and the paper is thought to be relevant it has been included in the AP section.

Table 1.1 shows the species/organisms that are commonly isolated form periodontal pockets and which may be aetiological agents. Each species/organism will be reviewed in turn.

Porphyromonas gingivalis

Porphyromonas gingivalis is a Gram-negative anaerobic non-motile asaccharolytic (non-carbohydrate fermenter) short or coccal rod. It is a member of the black-pigmented anaerobes group. The normal habitat of *P. gingivalis* is the oral cavity and most likely the gingival sulcus (Olsen et al., 1999). The organism is rarely found in supragingival plaque or outside the oral cavity (van Winkelhoff et al., 1988).

A large number of studies have investigated the role of *P. gingivalis* in periodontal disease. There is no need to list all the studies in the text of this thesis. Instead a number of studies have been listed in table 1.2 and referred to where appropriate. Some studies report an absence or very low prevalence (<10%) in healthy subjects (Table 1.2). Others report high levels in subjects without "clinical evidence of periodontal disease" (Mombelli et al., 1998) (Al-Yahfoufi et al., 1994; Dahlén et al., 1992; Gmür & Guggenheim, 1994; Kojima et al., 1993; Di Murro et al., 1987; McNabb et al., 1992). Generally in AP patients, the prevalence and levels of *P. gingivalis* are raised compared to health and gingivitis (table 1.2). *P. gingivalis* may be found in 13% to 100% of patients and 33% to 81% of sites (Table 1.2). The prevalence of *P. gingivalis* is linked to age and seems to increase as age increases (Rodenburg et al., 1990).

P. gingivalis correlates well with pocket depth and comprises a considerably higher proportion of the subgingival microbiota in deeper pockets (table 1.2). It has been linked with bleeding on probing (Albandar et al., 1990; Socransky et

Table 1.2 *P. gingivalis* in AP: Association and effect of therapy

Criterion		References
Increased prevalence in disease	Increased in AP subjects	Slots et al., 1979; Spiegel et al., 1979; Moore et al., 1983; White et al., 1981; Grossi et al., 1994; Tanner et al., 1979; Zambon et al., 1981, 1985; Riviere et al., 1996b; Mombelli et al., 1998; Riggio et al., 1996; Wikstrom et al., 1993; Rodenburg et al., 1990; Slots et al., 1986; Slots, 1986; Loesche et al., 1985; Savitt & Socransky, 1984; Riviere et al., 1996b; Slots et al., 1991; Africa et al., 1985; van Winkelhoff et al., 1986; Simonson et al., 1992; van der Weijden et al., 1994; Ali et al., 1996; Socransky et al., 1991; Wolff et al., 1993; Papapanou et al., 1993; Gunaratnam et al., 1992; Christersson, 1992; Kornman et al., 1991; Mombelli et al., 1991a; Kojima, 1993; Ali et al., 1992; Haffajee et al., 1988a; Mombelli et al., 1995; Ashimoto et al., 1996; Söder et al., 1993; von Troil-Linden et al., 1995
	Decreased in health/ gingivitis	Okuda et al., 1988; White et al., 1981; Tanner et al., 1998a; Spiegel et al., 1979; Adler et al., 1995; Savitt & Socransky, 1984; van Winkelhoff et al., 1986
	Presence correlates with deep sites	Ali et al., 1996; Socransky et al., 1991; Wolff et al., 1993; Wikstrom et al., 1993; Beck et al., 1992;

		Albandar et al., 1990; Haffajee et al., 1991b; Christersson et al., 1992; Mombelli et al., 1991a; Kigure et al., 1995; Kojima, 1993
	Increased in progressing/ active sites	Slots et al., 1986a; Albandar et al., 1990; Dzink et al., 1985, 1988; Moore et al., 1991; Slots et al., 1986; Tanner et al., 1987a; Haffajee 1991b
Response to treatment	Decrease after SRP	Renvert et al., 1997; Danser et al., 1996; Hellstrom et al., 1996; Haffajee et al., 1997a; Nieminen et al., 1995; Preber et al., 1995; Shiloah & Patters, 1994; Sigurdsson et al., 1994; Socransky & Haffajee 1993; Rosenburg et al., 1993; Wikstrom et al., 1993; Ali et al., 1992; Goodson et al., 1991; Maiden et al., 1991; Mombelli et al., 1995; Renvert et al., 1990a; Loesche et al., 1985; Slots et al., 1985; Simonson et al., 1992; Haffajee et al., 1995, 1988a
	Decrease after surgery	Danser et al., 1996; Nieminen et al., 1996; Mombelli et al., 1995; Sigurdsson et al., 1994; Rosenburg et al., 1993; Ali et al., 1992; Renvert et al., 1990c
	Decrease after antibiotics	Flemmig et al., 1998; Haffajee et al., 1996; Nieminen et al., 1996; Renvert et al., 1990a; Lowenguth et al., 1995; Kornman et al., 1994; Rosenburg et al., 1993; van Steenberghe et al., 1993; van Winkelhoff et

		al., 1992; Goodson et al., 1991; Maiden et al., 1991; Pedrazzoli et al., 1992; Walker et al., 1990; Kulkarni et al., 1991; Gusberti et al., 1988; Loesche et al., 1984

al., 1991; Christersson et al., 1992; Mombelli et al., 1991a; Kojima et al., 1993), suppuration (Socransky et al., 1991), and bone loss (Tanner et al., 1984; van der Weijden et al., 1994). In active/progressing sites *P. gingivalis* is detected with a much higher frequency and in greater numbers (Table 1.2). In spite of the overwhelming evidence implicating *P. gingivalis* as a pathogen in periodontal disease, studies by Moore et al. (1983) and Kojima et al. (1993) reported that the presence of *P. gingivalis* was not the cause of the disease process but a result of it.

Improvement in clinical condition is related to reduction or elimination of *P. gingivalis* (Haffajee et al., 1997a; Newman et al., 1994). Scaling and root planing (SRP) has been shown to be very effective in reducing the levels of *P. gingivalis* in sites, less so in patients, but not very effective in eliminating the organism (Table 1.2). After SRP, the presence of *P. gingivalis* correlates with probing depth and bleeding on probing (Wikström et al., 1993).

Surgery is equally as effective in reducing *P. gingivalis*, though does not generally eliminate the organism (Table 1.2). Both systemic and locally applied antimicrobials effectively reduce *P. gingivalis* levels, but as above, do not eliminate the organism (Table 1.2).

An increased number of *P. gingivalis* is linked to the progression of sites after treatment (Tanner et al., 1987a; Socransky & Haffajee et al., 1993). Refractory subjects also harbour high levels of *P. gingivalis* (Walker & Gordon, 1990; Choi

et al., 1990), but not relapsing sites (Slots et al., 1986). An absence or low prevalences and proportions indicate non-progression and continued health (Beck, 1994; Wennström et al., 1987; Bragd et al., 1987; Machtei et al., 1997; Page & Beck, 1997).

The high occurrence of *P. gingivalis* in AP lesions, together with the organism's pathogenic potential have resulted in the organism being named as an aetiological agent in periodontal disease, especially AP (Consensus report, American Academy of Periodontology, 1996). If *P. gingivalis* is an endogenous organism, treatment will not eradicate it. Perhaps the goal of treatment should be its reduction below a certain level, maybe that detectable by culture or DNA probe, as its absence in these tests seems to be the best indication of disease remission.

Actinobacillus actinomycetemcomitans

A. actinomycetemcomitans is a small, non-motile, Gram-negative saccharolytic capnophilic round-ended rod. It is thought to be one of the main aetiological agents in LJP (Zambon, 1985; Slots et al., 1980; Slots & Ting, 1999). In younger patients it has been extensively studied, but less so in adults. As age increases its prevalence decreases (Rodenburg et al., 1990; Slots et al., 1990a).

There are also a great number of studies which have examined the role of *A. actinomycetemcomitans* in adult periodontal disease. For the sake of brevity, a

number of references have also been placed in a table and referred to in the text (Table 1.3).

Most periodontally healthy adults show low or no detectable levels of subgingival *A. actinomycetemcomitans* (Table 1.3), although in some study populations it has also been detected with considerable frequency in minimally diseased subjects (Al-Yahfoufi et al., 1994; Dahlén et al., 1989a; Gmür & Guggenheim, 1994; McNabb et al., 1992). However it has been linked to AP (Table 1.3), and its prevalence ranges from 10% to 100% of patients and 35% to 45% of sites (Table 1.3). Zambon (1985) reported that 36% of the population carry *A. actinomycetemcomitans* normally and that the prevalence in AP was only slightly higher than that of the normal population, but also much lower than that of early-onset periodontitis.

A number of reports indicate that the presence of *A. actinomycetemcomitans* correlates with active/progressing sites (Table 1.3), deep pockets (Table 1.3), bleeding on probing (Socransky et al., 1991; Dzink et al., 1985; Ebersole et al., 1994b; Mombelli et al., 1994b), and suppuration (Socransky et al., 1991). Other authors have found no such correlations (Albandar et al., 1990; Skaar et al., 1992).

There are five known serotypes of *A. actinomycetemcomitans*, a-e (Asikainen et al., 1991; Gmür et al., 1993). Although patients usually only have one serotype (Ebersole et al., 1994b), there are mixed reports about which is more prevalent,

Table 1.3 *A. actinomycetemcomitans* in AP: Association and effect of treatment

Criterion		References
Increased prevalence in disease	Increased in AP subjects	Savitt & Socransky, 1984; Grossi et al., 1994; Mombelli et al., 1994a; Bonta et al., 1985; Cao et al., 1990; Mombelli et al., 1998; Rodenburg et al., 1990; van der Weijden et al., 1994; Ali et al., 1996; Wolff et al., 1993; Papapanou et al., 1993; Gunaratnam et al., 1992; Riggio et al., 1996; Haffajee et al., 1988a; Christersson et al., 1992; Kornman, 1991; Slots et al., 1980, 1990a; Zambon et al., 1983b; Mombelli et al., 1995
	Decreased in health/ gingivitis	Tanner et al., 1998; Cao et al., 1990; Slots & Ting, 1999
	Presence correlates with deep sites	Wolff et al., 1993; Slots et al., 1980; Tanner et al., 1979; Socransky et al., 1991; Ebersole et al., 1994; Mombelli et al., 1994a
	Increased in progressing/ active sites	Slots et al., 1986a, b; Tanner et al., 1987a; Dzink et al., 1983, 1985, 1988
Response to treatment	Decrease after SRP	Flemmig et al., 1998; Renvert et al., 1997; Nieminen et al., 1996; Preber et al., 1995; Mombelli et al., 1994a, b; Shiloah et al., 1994; Sigurdsson et al., 1994; Rosenburg et al., 1993; Wikstrom et al., 1993; Goodson et al., 1991; Maiden et al., 1991; Listgarten et al., 1991; Renvert et al., 1990 a, c

	Decrease after surgery	Rosenburg et al., 1993; Danser et al., 1996; Nieminen et al., 1996; Haffajee et al., 1988b; Mombelli et al., 1995; Sigurdsson et al., 1994; Renvert et al., 1990c
	Decrease after antibiotics	Flemmig et al., 1998; Nieminen et al., 1996; Renvert et al., 1996; Kornman et al., 1994; Muller et al., 1993; van Winkelhoff et al., 1992

serotypes a or b, though b seems to be more commonly found (Haffajee et al., 1984; Asikainen et al., 1997; Saarela et al., 1992; Asikainen et al., 1991; Zambon et al., 1983a). Serotype c is related to health (Asikainen et al., 1991). Interestingly Mombelli et al. (1994a) reported that in their study population, the majority of patients showed only limited numbers of positive samples and then low counts. A small number of patients displayed a high number of positive samples and high counts. This suggests that there are some subjects who are more susceptible to infection by *A. actinomycetemcomitans* or that there is variation in virulence and pathogenic potential (Mombelli et al., 1998).

Treatment of *A. actinomycetemcomitans* infected patients is difficult especially deep pockets and high counts of *A. actinomycetemcomitans* (Mombelli et al., 1994b; Renvert et al., 1990b, 1996; Bragd et al., 1987; van Winkelhoff et al., 1992; Christersson et al., 1985). SRP reduces the levels of *A. actinomycetemcomitans* (Table 1.3), but is very often quite ineffective with only minimal reduction reported. This failure of SRP may reflect the ability of *A. actinomycetemcomitans* to invade gingival tissue (Saglie et al., 1985; Christersson et al., 1987a, b; Meyer et al., 1991).

Periodontal surgery, while more effective than SRP, also often fails to control effectively subgingival *A. actinomycetemcomitans* (Table 1.3). Although conventional surgery reduces *A. actinomycetemcomitans* numbers better than SRP, resective surgery seems to be more effective in eliminating *A. actinomycetemcomitans* (Ali et al., 1992), as any infected tissue is removed

rather than providing a nidus for re-infection. Systemic antibiotics have the potential to eliminate *A. actinomycetemcomitans* from pockets and gingival tissue. Combined with SRP and/or surgery they may markedly reduce *A. actinomycetemcomitans* infection (Table 1.3). Locally applied antibiotics have had mixed reports, but seem generally less effective than systemic antibiotics (Hitzig et al., 1997; van Steenberghe et al., 1993; Goodson et al., 1991; Maiden et al., 1991). Although one report by Lowenguth et al. (1995) eliminated *A. actinomycetemcomitans* from their patient population.

Given the virulence of *A. actinomycetemcomitans* some authors have suggested elimination of *A. actinomycetemcomitans* should be the goal of any periodontal therapy, as failure to eliminate *A. actinomycetemcomitans* can lead to continued attachment loss or reduced healing (Zambon, 1996). However, it should be borne in mind that elimination probably only means a reduction below the level of detection of the microbiological test used. *A. actinomycetemcomitans* will probably still be present in the pocket or tissue, albeit at a reduced level below that required to cause disease. Higher levels of *A. actinomycetemcomitans* have been reported in sites that continue to lose attachment after treatment (Haffajee et al., 1995), and in relapsing patients (Kornman, 1991). Very low levels of *A. actinomycetemcomitans* (<0.01%), while difficult to achieve, are commensurate with non-progression of periodontitis (Bragd et al., 1987) and the absence of *A. actinomycetemcomitans* is a good predictor for no further attachment loss (Wennström et al., 1987).

A. actinomycetemcomitans may be a major pathogen in AP, but probably only in a subset of susceptible patients (Mombelli 1994a; Slots & Ting, 1999). Given its decreasing prevalence with age, it is likely that other organisms are more involved in the disease process.

Prevotella intermedia

P. intermedia is also a member of the black-pigmented anaerobes group. It is a Gram-negative, short round-ended rod. The organism is generally detected in higher frequencies and proportions in adult periodontitis sites and subjects ranging from 29% to 100% of subjects and 13% to 88% of sites than in health and gingivitis (Table 1.4). Similarly *P. intermedia* has been detected in some study populations with minimal disease (Al-Yahfoufi et al., 1994) and at similar levels in healthy subjects (Grossi et al., 1994; Slots et al., 1990a).

Its presence correlates with an increase in disease severity especially pocket depth (Table 1.4). In addition, *P. intermedia* has been associated with bleeding on probing (Socransky et al., 1991; Dzink et al., 1985; Mombelli et al., 1991a), suppuration (Socransky et al., 1991), and bone loss (van der Weijden et al., 1994). Again not all studies report these correlations (Christersson et al., 1992; Albandar et al., 1990).

Both the frequency of detection and levels of *P. intermedia* are increased in active/progressing periodontitis lesions (Table 1.4). *P. intermedia* has been

Table 1.4 *P. intermedia* in AP: Association and effect of treatment

Criterion		References
Increased prevalence in disease	Increased in AP subjects	Wikstrom et al., 1993; Rodenburg et al., 1990; Slots et al., 1986a; Loesche et al., 1985; Savitt & Socransky, 1984; Slots et al., 1991; Africa et al., 1985; van Winkelhoff et al., 1986; Dibart et al., 1998; Moore et al., 1983; van der Weijden et al., 1994; Ali et al., 1996; Socransky et al., 1991; Dahlen et al., 1992; Wolff et al., 1993; Papapanou et al., 1993; Gunaratnam et al., 1992; Haffajee et al., 1988a; Christersson et al., 1992; Kornman et al., 1991; Mombelli et al., 1991a; Adler et al., 1995; Slots et al., 1990a
	Presence correlates with deep sites	Beck et al., 1992; Haffajee et al., 1991b; Ali et al., 1996; Socransky et al., 1991; Wolff et al., 1993; Mombelli et al., 1991a
	Increased in progressing/ active sites	Slots et al., 1986 a, b; Dzink et al., 1988; Tanner et al., 1987a; Moore et al., 1991; Dzink et al., 1985
Response to treatment	Decrease after SRP	Ali et al., 1992; van Winkelhoff et al., 1988; Renvert et al., 1990a; Wikstrom et al., 1993; Bragd et al., 1987; Socransky et al., 1993
	Decrease after surgery and/or antibiotics	van Steenberghe et al., 1993; Ali et al., 1992; Kulkarni et al., 1991; Loesche et al., 1991; Haffajee et al., 1988b, 1984; Pedrazzoli et al., 1991; Socransky et al., 1993

detected attached to epithelial cells in increased numbers at diseased sites (Dzink et al., 1989; Dibart et al., 1998).

In response to treatment, clinical improvement is generally accompanied by a concomitant decrease in *P. intermedia* (Table 1.4). Studies investigating the effect of SRP on the frequency of detection and levels of *P. intermedia* report a decrease after treatment (Table 1.4), although Loesche et al. (1985) reported that SRP had no effect on *P. intermedia* levels. Mombelli et al. (1991a) suggested that they would not expect the levels of *P. intermedia* to change as much as other organisms because *P. intermedia* seems to be more of a commensal. Similarly studies employing surgical methods and/or antibiotics also report lower *P. intermedia* with clinical improvement (Table 1.4).

In recurrent or refractory patients, continued or recurring high levels of *P. intermedia* have been reported (Slots et al., 1986; Tanner et al., 1987a; Kornman, 1991). An absence of *P. intermedia* or low levels in the microflora (<2%) are associated with continued periodontal health (Wennström et al., 1987; Beck et al., 1992; Bragd et al., 1987). Dahlén et al. (1992) showed that continuing good oral hygiene can keep the levels of *P. intermedia* low in periodontal pockets.

Strains of *P. intermedia* that show identical phenotypic traits have been separated into two species, *P. intermedia* and *P. nigrescens* (Shah & Gharbia, 1992). This distinction makes earlier studies of this species difficult to interpret, since data from the two species may have been pooled.

P. intermedia is a likely candidate for a periodontopathogenic organism, but to a lesser extent than *P. gingivalis*.

Bacteroides forsythus

B. forsythus is a slow growing, Gram-negative, anaerobic, spindle-shaped, fusiform organism, and also belongs to the black-pigmented anaerobes group. It was reported by Tanner et al. (1979) as a "fusiform" Bacteroides and classified as *B. forsythus* in the mid-eighties (Tanner et al., 1986). There are surprisingly few reports about the pathogenicity of *B. forsythus* in periodontal disease, even though it has been named an aetiological agent (Consensus report, American Academy of Periodontology, 1996). *B. forsythus* has been associated with AP (Gmür et al., 1989; Tanner et al., 1998a; Haffajee et al., 1988a; Lai et al., 1987), although Lai et al. (1987) showed that very similar levels of *B. forsythus* were found in gingivitis and AP sites. There is great variation in its prevalence, from 10% (Haffajee et al., 1988a) to 100% (Christersson et al., 1992). The organism has been strongly associated with active/progressing lesions (Grossi et al., 1994; Dzink et al., 1983, 1988; Tanner et al., 1987a, 1989, 1998a; Dibart et al., 1998) and its numbers increase with increasing pocket depth (Socransky et al., 1991; Dzink et al., 1985; Christersson et al., 1992; Machtei et al., 1997). Its presence has also been linked with bleeding on probing (Tanner et al., 1989; Christersson et al., 1992) and bone loss (Tanner et al., 1984).

A few studies have shown that clinical improvement after either SRP or surgery and/or antibiotics is accompanied by a decrease in frequency of detection and levels of *B. forsythus* (Haffajee et al., 1995, 1997a, 1988c; Socransky & Haffajee, 1993). Sites which lost attachment had high levels of the organism (Haffajee et al., 1995; Socransky & Haffajee, 1993) and it has been associated with further attachment loss after treatment (Tanner et al., 1987a).

There are so few reports about *B. forsythus*, it is difficult to assign a role for it in AP. This presumably reflects the difficulty of culturing the organism. Further studies are required that do not rely on its viability to determine its role in AP.

Other Black-pigmented Anaerobes

Other members of this group include *B. fragilis*, *B. gracilis*, *B. ureolyticus*, and *P. melaninogenica*. *B. gracilis* has been linked to progressing sites (Tanner et al., 1987b), but in general this group is not particularly pathogenic. After treatment numbers seem to decrease (Pedrazzoli et al., 1991), although slight increases in *P. melaninogenica* (Haffajee et al., 1988c), and *B. fragilis* and *B. ureolyticus* (Haffajee et al., 1997a) have been reported. As a group these bacteria are not good predictors of attachment loss (Macfarlane et al., 1988).

Spirochaetes

Spirochaetes are a group of motile helical rods with tight regular or irregular spirals and flagella. They are fastidious and very difficult to culture. Oral spirochaetes belong to the genus *Treponema*, and there are currently four named

human species; *T. denticola*, *T. vincentii*, *T. pectinovarum*, and *T. socranskii*. Other strains representing a number of additional species have been isolated but not yet fully characterised (Tanner et al., 1994).

Spirochaetes are likely aetiological agents in ANUG (Loesche et al., 1982; Listgarten & Socransky, 1964; Riviere et al., 1991). Their role in AP is less clear due to their difficulty to culture. The use of microscopy in periodontal studies has identified small, medium and large spirochaetes, but cannot identify individual species. Thus the role of pathogenic species may have been obscured by non-pathogenic species (Haffajee & Socransky, 1994). Healthy sites exhibit few, if any, spirochaetes, gingivitis sites low to moderate levels, and deep pockets harbour many (Riviere et al., 1995, 1996a). Spirochaete numbers are generally increased in AP subjects and are often found with a very high prevalence (60-100%) (Loesche et al., 1985; Savitt & Socransky, 1984; Riviere et al., 1992, 1995, 1996a; Slots et al., 1991; Moore et al., 1983, 1991, 1987; Armitage et al., 1982). Spirochaetes have been linked with increasing pocket depth, bleeding on probing and bone loss (Omar et al., 1991; Tanner et al., 1984; Savitt & Socransky, 1984).

Pathogen-related oral spirochaetes (PROS) comprise the major proportion of spirochaetes (Riviere et al., 1995). These organisms have been found at healthy and gingivitis sites (Riviere et al., 1996a). *T. denticola* also comprises a large proportion of spirochaetes (Barron et al., 1991; Riviere et al., 1995). This spirochaete is generally found in much higher numbers in AP patients compared

to health and gingivitis (Simonson et al., 1992; Riviere et al., 1996b). *T. denticola* can be absent from healthy patients (Riviere et al., 1995), and also linked to initial periodontitis (Riviere et al., 1997). High levels of spirochaetes have been reported in refractory patients (Walker et al., 1993).

Periodontal therapy reduces the prevalence and proportion of spirochaetes. SRP has been shown to decrease significantly the level of spirochaetes including *T. denticola* (Loesche et al., 1985, 1992b; Macfarlane et al., 1988; Listgarten & Levin, 1981; Simonson et al., 1992; Katsanoulos et al., 1992; Haffajee et al., 1997a; Slots et al., 1985). Improved clinical condition is linked with reduction in *T. denticola* levels (Simonson et al., 1992; Haffajee et al., 1997b). Surgical and antimicrobial therapy has a similar effect on *T. denticola* and other spirochaetes (Mombelli et al., 1995; Walker & Gordon, 1990; Kulkarni et al., 1991; Loesche et al., 1984, 1991; Lundstrum et al., 1984; van Oosten et al., 1986; Gusberti et al., 1988; Quee et al., 1987). However MacFarlane et al. (1988) reported that spirochaetes are poor predictors of future disease activity.

From these studies it is clear that spirochaetes play a major role in the pathogenesis of AP. However confusion exists over which species are involved, and again further investigation is required using non-culture reliant techniques able to distinguish species.

Fusobacterium nucleatum

F. nucleatum is a Gram-negative, anaerobic, spindle-shaped rod that has three subspecies; nucleatum, vincentii and polymorphum. The species is often the most common isolate in AP subgingival plaque samples (Tanner et al., 1989; Haffajee et al., 1988a), occurring in 80-100% of AP patients (Wikström et al., 1993; Savitt & Socransky, 1984; Slots et al., 1991; Papapanou et al., 1993; Mombelli et al., 1995) and approximately 7-10% of isolates (Dzink et al., 1985, 1988). Commonly it is found in increased numbers in AP subjects compared to health and gingivitis (Grossi et al., 1994; Moore et al., 1983; Savitt & Socransky, 1984; Lippke et al., 1991) and active sites (Dzink et al., 1988; Tanner 1987a; Moore et al., 1991). Its presence may correlate with pocket depth (Dzink et al., 1985), but Albandar et al. (1990) suggested that *F. nucleatum* did not correlate with attachment loss, pocket depth nor bleeding on probing. Tanner et al. (1984) linked *F. nucleatum* with bone loss, but found similar proportions in active and inactive sites. The organism has also been correlated with refractory periodontitis (Haffajee et al., 1988c; Walker et al., 1993).

Wikström et al. (1993) reported an increase in *F. nucleatum* levels after SRP, whereas Haffajee et al. (1997a) reported similar levels. However surgery and/or antibiotics are effective in reducing *F. nucleatum* levels (Mombelli et al., 1995; Kulkarni et al., 1991; Haffajee et al., 1988b).

Overall, *F. nucleatum* is a likely candidate for AP and has been suggested to play a major role in the framework of the plaque biofilm (Whittaker et al., 1996).

Campylobacter rectus

C. rectus is a Gram-negative, anaerobic short motile vibrio and was previously classified as *Wolinella recta.* Studies have reported the prevalence of the organism to be from 15% to 81% of AP patients and 24% to 94% of sites (Rams et al., 1993; Wikström et al., 1993; Riviere et al., 1996b; Slots et al., 1991; Dahlén et al., 1992; Papapanou et al., 1993; Gunaratnam et al., 1992; Kornman, 1991; Mombelli et al., 1995). It has been found with both higher frequency and numbers in AP subjects compared to health and gingivitis (Lai et al., 1992; Riviere et al., 1996b; Moore et al., 1983; Haffajee et al., 1991c; Lippke et al., 1991; Socransky et al., 1991; Dibart et al., 1998; Moore et al., 1987; Tanner et al., 1989, 1998a). Grossi et al. (1994) and Dahlén et al. (1992) found no differences between healthy and periodontitis subjects. *C. rectus* has been associated with active disease (Rams et al., 1993; Albandar et al., 1990; Dzink et al., 1988; Tanner et al., 1987a, b, 1989, 1998a; Haffajee et al., 1991b; Moore et al., 1991; Dzink et al., 1985). Its presence has also been correlated with bleeding on probing (Tanner et al., 1989; Albandar et al., 1990), deep pockets (Albandar et al., 1990; Dzink et al., 1985), and bone loss (Tanner et al., 1984).

In response to effective treatment, whether it be SRP, surgery or antibiotics, the levels of *C. rectus* decrease (Rams et al., 1993; Wikström et al., 1993; Tanner et al., 1987b; Haffajee et al., 1988b, 1984; Mombelli et al., 1995), but high levels remain in active/non-responder sites (Haffajee et al., 1988b). Its levels may also be kept low by good oral hygiene (Dahlén et al., 1992). However the presence of *C. rectus* is linked to the recurrence of disease (Lai et al., 1992; Haffajee et

al., 1991b) but it is a poor predictor of further attachment loss (Rams et al., 1993).

C. rectus would seem to be an important pathogen in AP.

Capnocytophaga species

Capnocytophaga species are Gram-negative, long fusiform bacteria, and include *C. ochracea*, *C. sputigena*, and *C. gingivalis*. The genus has been found in 12% to 94% of AP patients and 27% to 88% of sites (Mombelli et al., 1995; Wikström et al., 1993; Ali et al., 1996; Papapanou et al., 1993; Gunaratnam et al., 1992; Kornman, 1991). However there seems little difference between health and disease (Dahlén et al., 1992; Papapanou et al., 1993) or the levels are higher in health (Grossi et al., 1994; Haffajee et al., 1991b, c; Dzink et al., 1985). Haffajee et al. (1991b) reported that *C. ochracea* was related to a decreased risk of disease progression, even though it has been found with high frequency in refractory patients (Kornman, 1991).

There are mixed reports regarding the outcome of treatment. Levels after SRP or surgery may increase (Mombelli et al., 1995), stay the same (Wikström et al., 1993; Haffajee et al., 1997a), or decrease (Ali et al., 1992) with clinical improvement.

The species seems to be associated with health or gingivitis (Darveau et al., 1997) rather than AP. The high numbers perhaps indicate a commensal nature to the species.

Eikenella corrodens

E. corrodens is a Gram negative, capnophilic, asaccharolytic, regular small rod with blunt ends. It has been recognised as a pathogen in other diseases (Haffajee & Socransky, 1994). It can be found in as low as 10% and as high as 70% of subgingival AP plaque samples (Kornman, 1991; Wikström et al., 1993; Savitt & Socransky, 1984; Riviere et al., 1996b; Papapanou et al., 1993; Wolff et al., 1993) though it is often present in healthy subjects (Chen et al., 1992). *E. corrodens* has been reported to be more frequently detected in AP patients (Savitt & Socransky, 1984; Dzink et al., 1985; Chen et al., 1989; Riviere et al., 1996b; Chen et al., 1992) and active sites (Dzink et al., 1988; Tanner et al., 1987a, b). One report finds a negative correlation between the organism and clinical measurements, such as pocket depth and bleeding on probing (Albandar et al., 1990).

After successful SRP, Haffajee et al. (1997a) found little change in the prevalence and numbers of *E. corrodens*, but Wikström et al. (1993) reported a significant increase in *E. corrodens* which correlated with the pocket depth after treatment. Following surgery and/or antibiotics levels of *E. corrodens* are much reduced (Tanner et al., 1987b; Haffajee et al., 1984).

E. corrodens is not one of the predominant periodontopathogenic organisms but may play a limited role in periodontitis (Chen et al., 1992).

Eubacterium species

Eubacterium species are Gram-positive, strictly anaerobic, small, pleomorphic rods. They can be difficult to culture. The most common members of the species are *E. nodatum*, *E. brachy*, and *E. timidum*. These species have been associated with AP (Moore et al., 1983; Uematsu et al., 1992) and active lesions (Moore et al., 1991). More recently Grossi et al. (1994) found a slight decrease in *Eubacterium* species in AP patients. Haffajee et al. (1997a) reported only a slight decrease in numbers after successful SRP. *Eubacterium* species appear to be promising candidates as periodontal pathogens, but efforts to investigate the species have been hampered by its difficulty to grow in culture. Further investigation is required with modern diagnostic microbiological techniques.

Selenomonas species

Selenomonas species are Gram-negative, curved, saccharolytic rods, and in a few reports have been linked with AP (Tanner et al., 1989; Lai et al., 1989), but more so early disease progression (Tanner et al., 1998a; Moore et al., 1991). Tanner et al. (1998a) reported an increase in *S. noxia* in gingivitis compared to health and in AP over gingivitis. After successful SRP, Haffajee et al. (1997a) reported similar levels. Little seems to be known about this species and further study is required to determine its role in AP.

Peptostreptococcus micros

P. micros is a Gram-positive, anaerobic, small asaccharolytic coccus. It has been found more frequently and in higher numbers in AP (Moore et al., 1983), but mainly at sites of periodontal destruction (Tanner et al., 1987a; Moore et al., 1991; Haffajee et al., 1991c; Dzink et al., 1988; Rams et al., 1992). High levels are found in active sites after surgical and antibiotic therapy (Haffajee et al., 1988b) and *P. micros* is linked to disease progression (Haffajee et al., 1991b). Thus *P. micros* would seem to be a promising candidate for a periodontal pathogen in AP.

***Streptococcus* species**

Streptococcus species are commonly found in the oral cavity, and include *S. mutans* group, *S. salivarius* group, *S. milleri* group, and *S. oralis* group. Although the species may be aetiological agents of disease elsewhere in the body, these streptococci seem to be more associated with health than AP (Dzink et al., 1988; Tanner et al., 1989; Haffajee et al., 1991c). One member of the *S. milleri* group, *S. intermedius*, has been linked with active sites in AP and refractory patients (Tanner et al., 1987a; Dzink et al., 1988; Magnusson, 1991; Walker et al., 1993; Haffajee et al., 1988a, c). Although the data is limited, it appears that only *S. intermedius* may be associated with periodontal disease.

Veillonella species & *Actinomyces* species

These two species are associated with health (Tanner et al., 1998a), gingivitis sites (Tanner et al., 1989) or inactive sites (Dzink et al., 1985, 1988). As the pocket depth increases levels of *V. parvula* decrease (Socransky et al., 1991) and increased levels of *V. parvula* are associated with decreased risk of disease progression (Haffajee et al., 1991c). After periodontal therapy, SRP, surgery or antimicrobials, levels of *V. parvula* increase (Haffajee et al., 1988b, 1997a), presumably in relation to the decreased pocket depth.

Enteric rods

These are non-oral Gram-negative facultatively anaerobic rods commonly found in the intestinal system. Ali et al. (1996) showed that roughly 60% of AP patients were positive for these organisms, and Slots et al. (1990b) that *Enterobacteriaceae*, *Pseudomonadaceae*, and *Acinetobacter* species were cultured from 14% of patients. These species frequently constituted a major part of the culturable subgingival flora. Their role in periodontal disease is yet to be determined, but they are pathogenic at other body sites (Slots et al., 1990b).

Helicobacter pylori

H. pylori is a Gram-negative micro-aerophilic bacterium which is recognised as being an aetiological agent of chronic gastritis, peptic ulcer disease and gastric cancer (Lee et al., 1993; Forman et al., 1991). A recent study by Riggio & Lennon (1999) demonstrated the presence of *H. pylori* in 33% of subgingival

plaque samples from deep AP pockets. Its role, if any, in periodontal disease is yet to be determined.

Herpes group viruses

Recent reports from Slots' group have shown that Human Cytomegalovirus (HCMV), Epstein-Barr virus -1 & -2 (EBV), Herpes Simplex virus (HSV), Human Papilloma virus (HPV) and Human Immunodeficiency virus (HIV) are detectable in periodontal subgingival plaques (Parra & Slots, 1996; Contreras & Slots, 1996; Contreras et al., 1999). HCMV has been detected in 60% of periodontal patients and 30% of gingivitis patients, and is the most common. EBV has been found in 30% of periodontal patients, HSV 20%, HPV 17%, and HIV 7%. These were not found in gingivitis patients (Parra et al., 1996). There is an increase in positive sites for all these viruses with increasing pocket depth (Contreras & Slots, 1996). Contreras & Slots (1996) suggested that the viruses may impair host defences, promote attachment and colonisation of periodontal pathogens, cause cytopathic effects, alter the inflammatory mediator and cytokine response, and cause tissue damage. In a later paper this group showed associations between EBV-1 and HCMV with *P. gingivalis*, *P. intermedia*, *B. forsythus*, *T. denticola*, and *P. nigrescens* in subgingival plaque samples (Contreras et al., 1999). HSV and EBV-2 showed no significant associations. The role of these viruses in periodontal disease is promising, but much remains to be investigated.

1.4.3 Microbiology of Generalised Early-onset Periodontitis

GEOP has previously been called rapidly progressive periodontitis (RPP), generalised juvenile periodontitis (GJP), and post juvenile periodontitis. A review of the microbiology literature is complicated because of the many different criteria that have been used to define this disease entity in the past and very often patients with this form of periodontitis have been included as part of an adult periodontitis group. Subjects diagnosed as RPP or GJP have been included in this section.

Porphyromonas gingivalis

P. gingivalis has also been implicated in GEOP in a number of studies (Vandesteen, 1984; Kamagata et al., 1989; Abu Fanas et al., 1991; Nishimura et al., 1990; Masunaga et al., 1990; Kamma et al., 1994, 1995; Conrads & Brauner, 1993; Listgarten et al., 1995; Sasaki et al., 1989; López et al., 1995; 1996; Moore et al., 1982; Loesche et al., 1985), and is often the predominant organism (Vandesteen, 1984; Kamagata et al., 1989; Abu Fanas et al., 1991; Nishimura et al., 1990; Kamma et al., 1994; López et al., 1995). Wilson et al. (1985) found that 8 to 16 percent of the cultivable microflora was *P. gingivalis* and suggested that *P. gingivalis* was involved in the aetiology of GEOP. Similarly Albandar and co-workers (1997) found that *P. gingivalis* was significantly associated with generalised disease in 13 to 19 year olds and was found in much higher levels in GEOP patients than healthy controls. In their study of Chilean GEOP subjects, López et al. (1995) found *P. gingivalis* in 80% of patients, 80% of affected sites

and in only 10% of unaffected sites. Kamma and colleagues (1994) investigated the presence of *P. gingivalis* in 73 GEOP lesions in ten patients and reported that in pockets over six millimetres deep *P. gingivalis* predominated. Similarly Loesche et al. (1985) reported significantly higher proportions of *P. gingivalis* (as well as *P. intermedia* and *B. melaninogenicus*) in GEOP subjects. Conversely Ou Yang (1994) found a negative correlation with numbers of *P. gingivalis* subgingivally and pocket depth. Williams et al. (1985) did not detect *P. gingivalis* in their study. *P. gingivalis* is also frequently isolated with *P. intermedia* (Vandesteen, 1984; Abu Fanas et al., 1991; Masunaga et al., 1990; Conrads & Brauner, 1993), *A. actinomycetemcomitans* (Kamagata et al., 1989; Masunaga et al., 1990; Listgarten et al., 1995), *F. nucleatum* (Nishimura et al., 1990) or a combination of these including *B. forsythus* (Kamma et al., 1994, 1995; Sasaki et al., 1989).

Abu Fanas et al. (1991) used antibiotics in the treatment of GEOP patients and had significant reductions in bleeding on probing and pocket depth with a significant reduction in mean percentage of black-pigmented *Bacteroides*. Masunaga et al. (1990) linked a reduction in pocket depth to the reduction of numbers of *B. gingivalis* and *B. intermedia*.

Actinobacillus actinomycetemcomitans

A number of researchers have found *A. actinomycetemcomitans* in GEOP patients (Kamagata et al., 1989; van Winkelhoff et al., 1989; Masunaga et al., 1990; Müller et al., 1993, Nakagawa et al., 1996; Listgarten et al., 1995; Kamma

et al., 1995; Sasaki et al., 1989; Sbordone et al., 1990b; Matsue et al., 1990; López et al., 1995). The frequency and numbers of the organism are increased, with López et al. (1995) finding *A. actinomycetemcomitans* in 60% of GEOP patients and 50% of affected sites. However, Vandesteen (1984), Williams et al. (1985), Moore et al. (1982) and López et al. (1996) could not detect *A. actinomycetemcomitans* in GEOP patients, and, although *A. actinomycetemcomitans* is present in some patients, other organisms such as *P. gingivalis*, *P. intermedia*, *F. nucleatum* and *B. forsythus* are found with greater frequency and numbers (Kamagata et al., 1989; Nishimura et al., 1990; Masunaga et al., 1990; Sasaki et al., 1989). Listgarten et al. (1995) reported that serotype b was the most common, followed by serotype a and then serotype c.

van Winkelhoff et al. (1989) demonstrated that the elimination of *A. actinomycetemcomitans* by hygiene phase therapy and antibiotics from ten GEOP patients resulted in clinical improvement and that the one patient who was still *A. actinomycetemcomitans* positive after treatment did not improve clinically. Conversely, Masunaga et al. (1990) reported that the levels of *A. actinomycetemcomitans* were not changed by scaling and root planing, and the reduction in pocket depth was due to the elimination of *Bacteroides intermedius* (*P. intermedia*) and *Bacteroides gingivalis* (*P. gingivalis*).

Prevotella intermedia

P. intermedia has also been associated with GEOP. It has been found at a higher prevalence and in higher numbers (Vandesteen, 1984; Kamagata et al., 1989;

Abu Fanas et al., 1991; Masunaga et al., 1990; Kamma et al., 1994; Conrads & Brauner, 1993; Moore et al., 1982; López et al., 1995, 1996; Loesche et al., 1985), and it can be the predominant organism (Williams et al., 1985; Sasaki et al., 1989). In two studies by López et al. (1995, 1996) they reported that *P. intermedia* was found in all GEOP patients and sites in one group and in 40% of similar patients and sites in another group. Moore et al. (1982) found *P. intermedia* to make up 2.9% of the flora in young adult humans with severe periodontal disease, which was an increase compared to healthy subjects. *P. intermedia* was shown by Albandar et al. (1997) to be of higher prevalence and proportions in progressing sites in 13 to 19 year olds. Masunaga et al. (1990) also demonstrated that the reduction in *B. intermedius* levels (as well as *B. gingivalis*) was required for a reduction in pocket depth.

The majority of studies on the microbiology of GEOP have been directed towards *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* detection and there are far fewer investigations which have addressed the role/presence of other periodontal pathogens.

Other organisms in Generalised Early Onset Periodontitis

Kamma et al. (1994, 1995), Sasaki et al. (1989), and Listgarten et al. (1995) detected *B. forsythus* in GEOP patient's subgingival plaque samples and reported a maximal detection frequency of 53.4 percent (Kamma et al., 1994). *F. nucleatum* has also been detected in GEOP patients and sometimes at high levels (Abu Fanas et al., 1991; Nishimura et al., 1990; Kamma et al., 1994, 1995;

López et al., 1995; Moore et al., 1982). López et al. (1995) found a greater prevalence of *F. nucleatum* in affected patients and sites compared to health. In their comprehensive study, Moore et al. (1982) reported that 83% of sites were positive for *F. nucleatum*, but this prevalence was very similar to healthy and gingivitis patients. Abu Fanas et al. (1991) reported a reduction of the mean percentage of *F. nucleatum* with a reduction in bleeding on probing and pocket depth.

Other organisms have been detected or isolated from subgingival plaque samples of GEOP patients, and include *Campylobacter* species (López et al., 1995) including *Campylobacter rectus* (Moore et al., 1982), *Eikenella corrodens* (Masunaga et al., 1990; López et al., 1995), Streptococci (Kamma et al., 1994), other *Bacteroides* species (Abu Fanas et al., 1991; Williams et al., 1985; Loesche et al., 1985), *Capnocytophaga* species (Kamma et al., 1995; López et al., 1995), *Eubacterium* species (Moore et al., 1982) and spirochaetes (Kamma et al., 1995; López et al., 1995; Moore et al., 1982). Moore et al. (1982) in their analysis of the GEOP flora reported that there was a significant increase in the levels of *Eubacterium* species, treponemal counts, *Treponema denticola*, large treponemes and *Mycoplasma* species in progressing sites. A later study (Löe & Brown, 1991) reassessed a group of LJP patients 6-7 years after initial diagnosis of LJP/EOP. In these subjects they reported that progressing sites had significantly increased levels of *C. concisus*, *Eubacterium* species, *P. gingivalis* and *Campylobacter*. In their wide ranging studies of Chilean GEOP subjects, López et al. (1995) found spirochetes in 27% of affected sites and 10% of non-affected

sites. They also linked *Capnocytophaga* species with GEOP, which was the most prevalent species in their study. Also in this study, they could find no significant differences in the levels of *E. corrodens* and *Campylobacter* species between healthy and diseased sites. Williams et al. (1985) reported that *Wolinella* species was not associated with disease.

One study (Kamma et al., 1995) examined the relationship between pocket depth and prevalence of periodontal pathogens. In severe lesions they found that *P. gingivalis*, *B. forsythus*, *F. nucleatum*, *A. actinomycetemcomitans* and *Campylobacter* species predominated. In medium lesions *B. forsythus*, *P. gingivalis*, *P. intermedia*, *F. nucleatum* and *Capnocytophaga* species were detected, and in minimal lesions *Streptococcus* species, *Actinomyces* species, *C. ochracea*, *Haemophilus segnis* and *Veillonella parvula* were present.

Often more than one organism has increased prevalence and numbers in GEOP pockets (Abu Fanas et al., 1991; Masunaga et al., 1990; Kamma et al., 1995; Listgarten et al., 1995; López et al., 1995, 1996; Moore et al., 1982).

1.4.4 Overview

Microbial aetiology of periodontal disease is complex. Clearly many species are involved, perhaps in a synergistic manner. Tables 1.5 and 1.6 rank the involvement of the agents on the evidence discussed in AP and GEOP. There is strong evidence to implicate *P. gingivalis* and *A. actinomycetemcomitans* in AP. *B. forsythus*, *P. intermedia*, *C. rectus*, *E. nodatum*, *T. denticola* and other

Table 1.5 Ranking of putative periodontal pathogens in Adult Periodontitis
(adapted from Haffajee & Socransky, 1994)

Very strong	Strong	Moderate	Early stage
A. actinomycetemcomitans	*B. forsythus*	*S. intermedius*	*Selenomonas* sp.
P. gingivalis	*P. intermedia*	*P. nigrescens*	Enteric rods
	C. rectus	*P. micros*	*B. gracilis*
	E. nodatum	*F. nucleatum*	Viruses
	T. denticola	*Eubacterium* species	*H. pylori*
	Other *Treponema* sp.	*E. corrodens*	

Table 1.6 Ranking of putative periodontal pathogens in Generalised early-onset periodontitis

Very strong	Strong	Moderate/Early Stages
A. actinomycetemcomitans	*B. forsythus*	*Eubacterium* species
P. gingivalis	*P. intermedia*	*T. denticola*
	F. nucleatum	*Campylobacter* sp.
		Capnocytophaga sp.

Treponemes are also implicated, but to a lesser degree. In GEOP patients there is evidence to suggest that periodontal infection is also a mixed microbial infection, with strong evidence for involvement of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia*. Other bacteria are probably involved but generally there are too few studies to reach conclusions, and thus further investigation is needed. Obviously these organisms do not occur alone and some studies have suggested groupings (Socransky et al., 1998; Söder et al., 1993). Equally not all are always present (Wolff et al., 1993; Socransky et al., 1991). Tanner et al. (1994) suggested that only 60% of the flora have been cultured, and there are many species of spirochaetes whose role is unclear. There is a need for studies that do not rely on the viability or ability of an organism to grow in culture.

This overall research project includes as one of its aims an investigation of the presence of *P. gingivalis*, *P. intermedia*, *B. forsythus*, *A. actinomycetemcomitans* and *T. denticola* in both AP and GEOP patients using polymerase chain reaction (PCR). In addition the effect of scaling and root planing on the prevalence of these five organisms will be investigated.

1.5 Immunological aspects of periodontal disease

Infections associated with periodontal disease stimulate broad-ranging immune responses, and published studies on antibodies to the major periodontal organisms are considered in this section. The basic concepts of the immune response are well established and the summary below is intended merely to establish a basic background on which to introduce the study objectives for this humoral response investigation before and after treatment.

1.5.1 Basic Concepts

The immune system is a collection of tissues, cells and molecules whose function is to protect the host against infectious agents (Sharon, 1998). Immune responses may be subdivided into two broad divisions, termed innate and adaptive immunity. These two types of immunity differ in certain key properties including specificity and memory.

Innate immunity represents an important first line of defence against infectious agents. This type of immunity is present from birth, is not enhanced by prior exposure, lacks memory and does not display antigenic specificity. Innate immunity entails a number of elements, both cellular and non-cellular. Physical barriers such as the skin and mucous membranes represent a component that infectious agents must breach to gain access to the host. The washing action of fluids such as tears, saliva, urine and possibly gingival crevicular fluid may keep surfaces clear of invading organisms, and may also contain bacteriocidal agents. The normal flora of the body can also act as an effective buffer against infection, by inhibiting the growth of pathogenic organisms by competition for nutrients or production of inhibitors. Phagocytic cells in the blood stream and tissues can destroy invading agents. These include polymorphonuclear leukocytes (PMN's), monocyte/macrophages, and natural killer cells. Finally there are the soluble components. These are a range of molecules that can damage cell walls, aid phagocytosis and cell recruitment or prevent cellular infection, and include lysozyme, cytokines, acute phase proteins, complement components and interferons.

The persistence of an infection in spite of an innate immune response typically leads to induction of an adaptive immune response. Adaptive immune responses are characterised by 1) specificity for the offending antigen(s), 2) memory, which allows a more rapid and heightened response upon re-infection by the same or closely related antigen, 3) diversity, the ability to respond to a wide range of different antigens, and 4) self versus non-self recognition. The adaptive immune response can be subdivided into cell-mediated and humoral immunity. Humoral immunity is mediated by antibodies, whereas cell-mediated immunity involves the direct action of immune cells.

1.5.1.1 Role of B-lymphocytes

The cells mainly involved in adaptive immunity are antigen-presenting cells and lymphocytes. The former include macrophages, dentritic cells in the follicles of lymph nodes, Langerhan's cells of the skin and mucous membranes, and B-lymphocytes. These cells present antigens to B-lymphocytes and T-helper lymphocytes. The activated T-helper cells then produce cytokines that stimulate B-cells to differentiate into antibody producing cells. B-cells are produced in the bone marrow and are programmed to produce only one antibody. They display this on their surface and when bound by the specific antigen, a triggering signal causes the B-cell to develop into an antibody-producing cell. The activated B-cell then proliferates to produce a large number of antibodies by clonal expansion. Clonal expansion not only produces antibody-producing plasma cells but also a proportion of quiescent cells expressing the antibody on

their surface. These memory cells provide a relatively large pool ready to produce a much more rapid and intense antibody response on further antigen challenge.

1.5.1.2 Role of T-lymphocytes

T-cells act by another mechanism to destroy invading viruses, mycobacteria and protozoa that attempt to evade host defences by proliferating inside host cells. T-cells differentiate within the thymus and will only recognise foreign antigen when it is on the surface of a host cell in association with cell surface markers of the major histocompatibility complex (MHC).

Killing of virally infected cells can be achieved by two mechanisms, one involving NK cells and the other cytotoxic T-cells. Apart from a direct effect on infected cells, NK cells can also kill by the process of antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells have a receptor for the Fc portion of antibody molecules and can bind to antibodies directed against virally coded surface antigens.

A sub-population of cytotoxic T-cells produces a wide range of surface receptors similar to the surface antigen receptors on B-cells. These T-cells are programmed to recognise only one antigen when it is found in association with a class I MHC receptor. The cytotoxic T-cell can thus bind to the infected cell and destroy it.

T-helper cells will bind to an antigen for which they are programmed when it is found in association with a class II MHC receptor on the surface of an infected macrophage. They then produce lymphokines re-triggering the microbiocidal mechanisms of the macrophage.

Cytotoxic T-cells are also capable of acting in this way. In a similar fashion to that of B-cells, T-cells are selected and activated by antigen and expanded by clonal proliferation to produce a large clone of activated T-cells and also a pool of quiescent memory cells.

1.5.1.3 Immunoglobulins

Antibodies are glycoproteins that are present in serum and fluids. They bind specifically to foreign antigens and are induced when the lymphoid system interacts with immunogenic materials on or produced by microorganisms.

The immunoglobulin molecule is Y-shaped and consists of four peptide chains, two identical heavy and two identical light chains. The light chains with parts of the heavy chain form the Fab region that has antigen-binding properties and contains considerable variability. The remainder of the heavy chains form the Fc region which is relatively constant and can bind with cell receptors or complement. There are five different types of heavy chain and these define the immunoglobulin classes that exist in humans; IgA, IgD, IgE, IgG and IgM.

IgG is the most important serum immunoglobulin and after prolonged exposure to most antigens, antibody activity is mainly associated with this isotype (75% of serum immunoglobulin). It is the predominant antibody in a secondary immune response. There are four subclasses, IgG1-4, which respond to different antigens. IgG2 is found frequently in response to polysaccharide antigens, whereas IgG1 and IgG3 are primarily directed to proteins and viral antigens, and IgG4 associated with allergic-type reaction. IgG possesses the unique property among immunoglobulin classes of being able to cross the placenta.

IgA is adapted to defend the exposed external and internal surfaces of the body. It is found predominantly in sero-mucous secretions e.g. lung secretions, saliva and secretions of the gastro-intestinal and urinary tract. In serum it is monomeric, but, in these fluids, dimeric with a joining (J) chain and a secretory component. IgA antibodies essentially coat microorganisms and thereby inhibit their adherence to mucosal cells. There are two subclasses, IgA1 and IgA2.

IgM is the first immunoglobulin to appear in the immune response. It consists of five immunoglobulin molecules linked together by J chains and as such has a high capacity to agglutinate/aggregate bacteria. IgM activates the classical complement cascade efficiently.

The exact function of IgD has not yet been determined but may be important in B cell differentiation. IgE is involved in atopic or allergic reactions and also appears in response to parasitic infections.

1.5.2 Avidity

1.5.2.1 Basic concepts

The overall strength of antigen-antibody bonds is known as antibody avidity or functional affinity. The B-cell selection process brings about maturation of IgG antibody affinity which is initially low and increases during subsequent weeks and months (1 to 7 months). A few days after antigenic contact, the antibodies produced still originate from unmutated plasma cells. At the end of the first month, there are mutations in the antibody variable region and an increase in IgG antibody affinity. Avidity maturation seems to be proportional to antigen dose. Low doses of antigen give rise to a more rapid maturation and higher doses to a slower maturation. Therefore, low avidity antibodies are produced during the first stage of infection when a high level of antigens is present (Gutierrez & Maroto, 1996).

There are four main intermolecular forces involved in antibody-antigen interactions; 1) electrostatic forces caused by the attraction between ionic groups with opposite charge, 2) hydrogen bonding, 3) hydrophobic bonding and 4) van der Waals forces. In general the complementary electron cloud shapes on the combining sites of the antibody and the surface determinant antigen enable the two molecules to fit together so that the intermolecular distance becomes very small and the non-specific protein interactions (as above) are considerably increased.

1.5.2.2 Methods of measuring avidity

The measurement of the avidity of specific IgG antibodies for serodiagnosis can be carried out using methods such as agglutination, radio-immunoassay, complement fixation, ELISA, IFA, electroblotting and electrophoresis. It is necessary to denature the reaction and denaturing substances can either be included in the dilution of the serum or applied after the formation of the antigen-antibody complex. Denaturing substances include diethylamine, potassium thiocyanate, guanidine and urea. In this study thiocyanate disassociation was used to determine avidity, as previous studies have shown its efficacy (Mooney et al., 1994, 1995).

1.5.3 Humoral immune response and periodontal disease

1.5.3.1 Introduction and important considerations

Periodontal infection can stimulate an immune response, similar to infections at other sites in the body. This response is also directed towards eliminating and/or neutralising the infectious agent. Humoral immune responses in periodontal disease can provide strong evidence of previous or current infection by a periodontal pathogen. However, before any discussion of the specific humoral immune response in periodontal disease, a number of issues should be addressed.

Firstly the organisms to which the response is being directed must be considered. Microbial aetiology and pathogenesis must be taken into account as bacteria may provoke an immune response but not fulfil other criteria for pathogenesis. For a detailed study of the microbiology of AP see section 1.4.2. In addition

antibodies may be detected to non-oral bacteria and non-bacterial antigens (Berglund, 1971, Mallison et al., 1989). The use of a large battery of micro-organisms, including many of doubtful relevance, may lead to erroneous conclusions or an apparent lack of focus. In this study *P. gingivalis*, *P. intermedia, B. forsythus*, *A. actinomycetemcomitans* and *T. denticola* were chosen for particular attention because of their strong association with adult periodontal disease (Consensus report, American Academy of Periodontology, 1996; Haffajee & Socransky, 1994; Riviere et al., 1995, 1996a, Tanner et al., 1998a).

Secondly, the choice between whole bacterial cells and particular antigens is important (Wilton et al., 1991). In this study it was decided to use whole cells because antibody avidity was to be investigated. The use of single antigen may reflect only the response to that particular antigen and not the whole cell. By using whole cells the global range of interactions against each organism can be determined and, therefore, the overall response may yield information that would be lost by concentrating on a particular antigen, which may or may not be an important antigen.

Thirdly, and related to the last point, there is the matter of antibody function. Demonstrations of an association between host antibody response and periodontal disease may only be academic unless it can be related to the biological function of the antibody. These functions, which include the ability to

opsonise bacteria and the ability to bind strongly to fimbriae, may relate to antibody avidity.

Fourthly, we must consider whether local antibody levels in the GCF or systemic levels or both are of importance; and whether local levels are merely a reflection of serum levels or whether significant antibody production by gingival plasma cells is taking place. This is important in the consideration of subject and site susceptibility to disease onset and progression.

Finally, the assay of these antibodies before and after treatment may provide useful information on the relationship between the titre and avidity and disease progression at both subject and site levels, and their changes following therapy.

1.5.3.2 Response to Gram-positive organisms

Most studies concerned with the humoral immune response to oral microorganisms have tended to concentrate on Gram-negative organisms, but there have been some that have focused on Gram-positive organisms.

An early study by Taubman et al. (1982) reported similar levels of IgG and IgM antibody directed towards *A. naeslundii*, *A. viscosus*, *E. brachy*, *S. mutans* and *S. sanguis* in LEOP, GEOP, AP and healthy subjects. Haffajee et al. (1988c) concluded that relatively abundant constituents of Gram-positive flora in subgingival plaque, e.g. *Actinomyces* species, *S. mutans* and *S. sanguis*, do not provoke a significant humoral immune response even in patients whose flora

contain a large proportion of these organisms. This same group also showed that these bacteria do not contain antigens that cross-react with Gram-negative constituents of the plaque (Taubman et al., 1982; Ebersole 1986).

Studies of these organisms by other groups have also failed to demonstrate increased titres in periodontal disease (Doty et al., 1982; Ranney et al., 1981; Gilmour & Nisengard, 1974). These have generally shown a wide variation and a lack of discrimination between diseased and healthy groups.

It seems that differences in the humoral immune response to Gram-positive bacteria do not appear to occur between periodontitis patients and healthy controls. Both Tew et al. (1985a) and Nisengard & Beutner (1970) reported increased titres to Gram-positive organisms but the use of different methodology may account for these opposing studies. It seems reasonable to suggest that there are no abnormal changes in the humoral immune response to the Gram-positive flora in periodontal disease.

1.5.3.3 Response to Gram-negative organisms

Similar to the microbiological studies, studies of the humoral immune response to Gram-negative organisms have tended to concentrate on *P. gingivalis* and *A. actinomycetemcomitans*. To a lesser extent, antibody titres to other organisms such as *P. intermedia*, spirochaetes, and *Capnocytophaga* species have been investigated, perhaps because of a lack of response or difficulty culturing some species.

P. gingivalis

Mouton et al. (1981) investigated the serum IgG, IgM and IgA antibody response to *P. gingivalis* in various patients groups, including healthy subjects, and age groups. This paper was important in establishing a number of points:

1) Based upon the humoral immune response, *P. gingivalis* was probably aetiologic in periodontal disease.
2) This response was probably protective.
3) Diseased and healthy individuals could be distinguished in terms of their antibody response to this organism.
4) There were indications of differences in the response in different periodontal disease states.

Also this report established that detectable levels of antibody to *P. gingivalis* were found in a significant proportion of healthy adults and that there was a correlation between antibody levels and age. IgG and IgM antibodies were detectable in children as young as six months, and children aged 6-12 years demonstrated significantly higher antibody levels than younger children.

These findings have been confirmed and extended by this and other research groups. Periodontitis patients, especially AP and GEOP, demonstrate higher antibody titres to *P. gingivalis* than healthy patients (Altman et al., 1982; Ebersole et al., 1982; Taubman et al., 1982; Suzuki et al., 1984; Naito et al., 1984; Farida et al., 1986; Gunsolley et al., 1990; Lopatin et al., 1991; Zafiropoulos et al., 1992; Kinane et al., 1993; Mooney & Kinane 1994).

Ebersole et al. (1982) reported that 58% of AP patients and 50% of RPP patients had increased titres compared to health. Eighty five percent of control subjects showed no elevations in antibody to any of the organisms tested and, interestingly, 20% of AP patients also.

This group in a later study (1986) confirmed these findings and suggested that increased antibody titres may reflect colonisation by the organism or pathogenicity. Ebersole et al. (1984b) had previously shown a significant relationship between elevated systemic IgG and the ability to culture the organism from subgingival plaque.

Generally it would appear that there is a positive relationship between serum antibody and *P. gingivalis*. There are, however, a significant number of studies reporting no differences between patients and controls or even lower antibody levels in patients (Baranowska et al., 1989; Tew et al., 1985a; Farida et al., 1986; Doty et al., 1982).

A. actinomycetemcomitans

An early study of antibody response to *A. actinomycetemcomitans* linked this response specifically to LJP by demonstrating the presence of these antibodies in these patients but not in normal subjects (Genco et al., 1980). Ebersole et al. (1980) also showed an association between increased levels and frequency of antibody to *A. actinomycetemcomitans* and LJP. These authors elaborated on this in a later study (Listgarten et al., 1981) in which they showed a significantly

increased level of IgG antibody to *A. actinomycetemcomitans* serotype b in 90% of LJP patients, but only 40% of RPP and 25% of AP patients. These findings of high *A. actinomycetemcomitans* antibody titres in LJP but not RPP and AP patients have been confirmed by more recent reports (Genco et al., 1985; Vincent et al., 1985; Farida et al., 1986; Schenck, 1989; Ebersole et al., 1987a, 1991; Zafiropoulos et al., 1992). Tew et al. (1985a) reported no obvious differences between disease and health in IgG titres to *A. actinomycetemcomitans* and a later report by this group found decreased IgG titres with increased disease severity (Gunsolley et al., 1987). Ebersole et al. (1990) found a correlation between antibody titres and periodontal destruction. In a later study this group (1992) found a positive correlation between titre to *A. actinomycetemcomitans* and the number of teeth infected, but a negative correlation between IgG levels and the proportion of *A. actinomycetemcomitans* in subgingival plaque samples.

The *A. actinomycetemcomitans* titre in AP patients appears similar or slightly higher than that in healthy patients (Ebersole et al., 1982; Genco et al., 1985; Vincent et al., 1985; Farida et al., 1986). Overall *A. actinomycetemcomitans* is a likely aetiological agent in LEOP, less so in GEOP and not in AP.

P. intermedia

Two studies by Ebersole et al. (1986) and Tew et al. (1985a) examined antibody responses to various members of the *Bacteroides* genus. Although elevated responses to other *Bacteroides* species were detected in some patients, especially

P. intermedia, *P. gingivalis* was the most consistent in eliciting an antibody response. Other studies have reported similar antibody responses between AP, GEOP, LEOP and healthy subjects (Doty et al., 1982; Taubman et al., 1982; Naito et al., 1984; Wheeler et al., 1994; Gmür et al., 1985). Zafiropoulos et al. (1992) correlated *P. intermedia* titres with *P. gingivalis* titres and also reported *P. gingivalis* titres greater than *A. actinomycetemcomitans*, which were in turn greater than *P. intermedia* titres in AP subjects. *P. intermedia* seems to elicit little response in disease, which may reflect its opportunistic and commensal nature.

Other Gram-negative organisms

Antibody responses to other Gram-negative organisms have generally failed to demonstrate any consistent and convincing association between these responses and the occurrence and extent of periodontal disease. Vincent et al. (1985), Naito et al. (1984), and Ebersole et al. (1987a, 1988) could not demonstrate any association between antibody levels to *Capnocytophaga* species and particular disease classifications. However Tolo & Schenck (1985) reported increased IgG, IgM and IgA titres to *C. ochracea* and also *E. saburreum*. Increased titres to *F. nucleatum* have been found by a number of authors (Vincent et al., 1985; Naito et al., 1984; Tolo & Schenck, 1985). Antibodies to *E. corrodens* were found to be slightly higher in AP patients than in healthy subjects by Naito et al. (1984). The results would generally indicate that other Gram-negative organisms play little part in the aetiology of periodontal disease, but in a small group of patients they may have a role.

Response to spirochaetes

There have been some studies of antibodies directed towards spirochaetes. Jacob et al. (1982) showed increased titres to *T. denticola* in AP patients compared to health. However, most studies have not shown significant differences (Tew et al., 1985a; Aukhil et al., 1988; Lai et al., 1986; Mangan et al., 1982). This may reflect difficulty in culturing the organisms, a lack of antigenicity by spirochaetes, immunosuppression by spirochaete products (Ebersole & Taubman, 1994), or that spirochaete colonisation is limited to subgingival plaque. Spirochaetes have been shown to be antigenic in other parts of the body and in animals, and are known to invade tissues in ANUG.

1.5.3.4 Avidity and periodontal disease

There have been very few reports dealing with antibody avidity in relation to periodontal disease. Ebersole et al. (1990) studied the increase in avidity in the non-human primate, *Macaca fascicularis*, following immunisation with tetanus toxoid, which they used as a prototype bacterial exotoxin. They found that IgG avidity increased from 0.9M to 1.72M following primary immunisation, and to 2.56M after secondary immunisation. Lopatin et al. (1991) demonstrated that avidity of antibody rose to a similarly high level in rabbits post-immunisation with *P. gingivalis*, but that human antibodies to this organism appear to be of generally low avidity. In this study IgG antibodies to *P. gingivalis* were of significantly higher avidity in periodontitis patients than in controls. Lopatin & Blackburn (1992) and also Mooney et al. (1993) reported increased titres to *P.*

gingivalis in periodontitis patients compared to health, but that the avidity of these antibodies was not significantly higher. In the study by Mooney et al. (1993) *A. actinomycetemcomitans* antibody avidity was lower than that for *P. gingivalis*, and that IgG avidity correlated with titre, but not IgM or IgA. Lopatin et al. (1991) had previously reported no significant relationship between titre and avidity. A later study by Mooney et al. (1994) showed that IgM and IgG avidity was lower in RPP patients when compared to AP. Interestingly AP antibody avidity was significantly higher for *P. gingivalis* antibodies than control subjects but *A. actinomycetemcomitans* antibody avidity was similar. Chen et al. (1991) demonstrated that IgG avidities to *P. gingivalis* were lower in RPP patients than in control subjects. Another recent study of titre and avidity of IgG antibodies to *P. gingivalis* in RPP patients by Whitney et al. (1992) also showed lower avidities in RPP patients than in controls. Sjöström et al. (1992) showed that IgG antibodies in low-titre sera from control subjects were significantly more effective in opsonising *A. actinomycetemcomitans* than IgG antibodies in low-titre sera from RPP patients. This study suggests a crucial link between antibody avidity and function. O'Dell and Ebersole (1995) hypothesised that antibody avidity to *A. actinomycetemcomitans* could help to explain the relationship between the active host response and chronic infection with this pathogen. Their data suggested that both antibody levels and avidity could contribute to variation in host resistance to infection and disease associated with *A. actinomycetemcomitans*. Underwood et al. (1993) have demonstrated that anti-*A. actinomycetemcomitans* antibodies are important in promoting phagocytosis and killing of *A. actinomycetemcomitans*. They suggest that

subjects who develop high levels of highly avid antibodies against this organism may have greater resistance to continued and repeated infection by this pathogen.

It appears that the various forms of disease produce antibodies of differing avidity and suggests that 1) the quality of the immune response may have a bearing on the aetiology, and 2) different organisms may have differing relevance. The low avidity antibodies compared to other antibodies, such as tetanus toxoid or streptokinase (Lopatin & Blackburn, 1992), may result from a failure of biologic function of antibody against bacterial infection (Mooney et al., 1993). Alternatively, Lopatin et al. (1991) suggested that low avidity antibody resulted because *P. gingivalis* and other organisms act as immunoadsorbants. More realistic is the theory of oral tolerance (Lopatin et al., 1991). This suggests that low avidity antibodies arise from either high antigenic doses at the onset of infection, chronic exposure or *in utero* exposure. Early chronic exposure to low doses either *in utero* or in early life might compromise the ability of the host to mount an effective immune response. Children have been shown to have higher avidity to periodontal pathogens than adults, which is suggestive of chronic exposure (Lopatin et al., 1991).

1.5.3.5 Local responses

Most studies investigating the humoral immune response have concentrated on systemic antibody levels. Much less work has been done on the relationship between local antibody levels and local disease status. A number of studies have reported increased antibody levels to pathogens in GCF (Ebersole et al., 1985c;

Naito et al., 1984; Martin et al., 1986; Genco et al., 1985). However Baranowska et al. (1989) found no significant difference in the level of specific IgG to *P. gingivalis* in GCF between healthy and diseased sites within the same individual.

Tew et al. (1985b) found no obvious differences in the clinical parameters of pocket depth and attachment level between sites with elevated antibody to *P. gingivalis* and/or *A. actinomycetemcomitans*, and those with normal or low levels. They concluded that elevated antibody in GCF may relate to changes in disease activity that are not detectable by normal clinical assessments. More recently, Kinane et al. (1993) found a correlation between *P. gingivalis* antibody titres and gingival inflammation and pocket depth, but not for *A. actinomycetemcomitans* antibodies. They concluded that antibodies are protective because in patients with greater disease and inflammation there are lower levels of antibodies. Suzuki et al. (1984) demonstrated that local production of IgG to *P. gingivalis* was markedly increased in AP compared with RPP patients, suggesting that disease progression was influenced by local antibody production. Challacombe et al. (1986) reported similar findings in patients with high and low periodontal disease indices.

There are conflicting reports about the correlation between serum and GCF titres. Some studies report a correlation suggesting that the primary source of antibodies is from the serum (Naito et al., 1984; Genco et al., 1985; Kinane et al., 1993; Baranowska et al., 1989). Other studies disagree, demonstrating that

GCF levels do not reflect serum levels and suggesting that there is local production (Martin et al., 1986; Smith et al., 1985; Ebersole et al., 1985c). Recently a study by Mooney and Kinane (1997) showed that systemic and local antibody production contributes to the overall GCF antibody profile. This would make sense given the fact that the majority of the cellular infiltrate in the periodontitis lesion are IgG producing plasma cells, and is reflected in the antibody composition of the GCF, which is primarily IgG (Holmberg & Killander 1971, Smith et al., 1985, Kinane & Lindhe 1997).

Lamster et al. (1990) found that *P. intermedia* GCF and serum antibodies correlated but *P. gingivalis* antibodies did not and that this local deficiency in IgG to *P. gingivalis* may lead to local disease. Califano et al. (1997) reported a similar lack of antibody to *B. forsythus* and came to the same conclusion.

Ebersole et al. (1985a) and Ebersole & Cappelli (1994) showed that the frequency and distribution of antibody in GCF is related to colonisation by the target organism. The later study also showed that the pattern of antibody response to *A. actinomycetemcomitans* is characteristic of localised host-parasite interactions. Therefore antibodies may play an important role in the gingival sulcus in relationship to colonisation and clinical presentation.

Mooney & Kinane (1997) also found that periodontitis sites had significantly lower antibody levels than gingivitis sites, which supported the findings of Danielsen et al. (1993) in that more extensive reactions, i.e. high antibody titres,

to organisms may be a prerequisite for successful reduction or elimination of bacteria. OuYang (1993) had previously corroborated these findings by showing that periodontitis patients had lower GCF levels of antibody to P. gingivalis, when related to serum levels, than gingivitis patients.

Failure of a local immune response appears to be related to periodontal disease at these sites. The failure in production may result from a biological failure (Mooney & Kinane 1997), failure of the organism to stimulate an immune reaction (Califano et al., 1997) or degradation of immunoglobulins by the bacteria in the pocket (Kilian, 1981). Local antibody are derived from both local and systemic antibody production.

1.5.3.6 Effect of therapy on the humoral immune response

An early study by Tolo et al., (1982) investigated the effect of therapy on antibody levels. They reported a mixture of increases and decreases in antibody titres to different organisms, but the only clear pattern was a decrease in *P. gingivalis* antibody titre post-therapy. Ebersole et al. (1985b) found a general increase in antibody levels after treatment, which peaked at 2 to 4 months and returned to pre-treatment levels 8 – 12 months later. The increase was most marked for *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*, *E. corrodens* and *C. concisus* antibody titres. In this study if the organism was detected in subgingival plaque then there was an increase in antibody titre after therapy. This suggests that the increase in titre may result from inoculation of the organism into the bloodstream and the subsequent reaction results in the

increased titre. Further studies have not found a clear pattern in the immune response. Aukhil et al. (1988) reported a general reduction in antibody levels after treatment, significantly for *S. sanguis* and *P. gingivalis*. However antibody levels to *Capnocytophaga* species, *B. melaninogenicus* and *T. denticola* were unchanged. A similar reduction to *P. gingivalis*, both locally and systemically, was found by Murray et al. (1989) in treated patients compared to untreated patients. OuYang (1993) reported elevations in the GCF/serum ratio of antibody occurred one month after periodontal therapy. This suggested that local antibody consumption may be reduced after removal of the organism and that the GCF/serum ratio of antibody level might be used as a significant indicator in evaluation of treatment effectiveness. Most recently, Horibe et al. (1995) demonstrated that mean antibody levels to *P. gingivalis* and *P. intermedia* decreased significantly after treatment, and although titres to *P. loeschii*, *F. nucleatum*, *A. actinomycetemcomitans*, *E. corrodens* and *Capnocytophaga* species decreased slightly, these were not significant. Their results suggested that the change in serum IgG titres was related to the suppression of these pathogens in subgingival plaque.

Mouton et al. (1987) were able to split their patients into two groups. One group had low IgA titres and IgG and IgM titres to *P. gingivalis* similar to healthy subjects. This group responded poorly to treatment. The other group had detectable IgA titres and significantly higher IgG titres to *P. gingivalis*. They responded much better to treatment than the other group. The authors found no peak level of antibody after treatment, which suggested the SRP may not

provoke active immunisation. A more recent study by Chen et al. (1991) reported that 33% of their RPP patients were high responder for *P. gingivalis* antibodies, that is they had over twice the median antibody titre than the control subjects. These high responder patients were found to have a significant decrease in titre post-treatment, whereas the low responder patients had a significant increase. Initially the avidity of the antibodies was lower than the controls, but post-therapy was higher. Mooney et al. (1995) also examined the effect of SRP on antibody response to *P. gingivalis* and *A. actinomycetemcomitans* in high responder and low responder periodontitis patients. Overall there were significant increases in *A. actinomycetemcomitans* IgG, IgA and IgM titres, no change in *A. actinomycetemcomitans* antibody avidity, significant increase in *P. gingivalis* IgG titres and significant increase in *P. gingivalis* IgA avidity. When the results were analysed by sero-status, high responder patients for IgG antibodies to *P. gingivalis* showed a significant increase in antibody avidity and a better outcome of treatment. The low responder patients had an increase in titre but no change in avidity, whereas the high responder patients were found to have an increase in avidity but little change in titre. The authors concluded that SRP affects the magnitude and quality of the humoral immune response, that the effect is dependent on initial sero-status and initial sero-status may have a bearing on the treatment outcome. The responses to *P. gingivalis* and *A. actinomycetemcomitans* may reflect their role in the disease or the age of the patients.

In general it seems that treatment may increase or decrease antibody titres depending on the organism and sero-status of the patient. The change in avidity also depends on the sero-status before treatment. These studies have examined *P. gingivalis* and *A. actinomycetemcomitans*. Little is known about the other putative pathogens and an investigation into these is warranted. Also the effect of the presence or absence of the organism on sero-status and treatment outcome requires further investigation. In spite of the fact that *B. forsythus* is a named aetiological agent, there is very little information about the humoral immune response to this organism. In this thesis antibody titres to *P. gingivalis*, *P. intermedia*, *B. forsythus*, *A. actinomycetemcomitans* and *T. denticola* were investigated with regard to 1) the effect of treatment on titre, 2) serostatus, and 3) the effect of the presence or absence of the test organisms.

1.6 Smoking and the periodontium

This section discusses the effect of smoking on the periodontium and the host, with particular regard to disease severity, response to treatment and periodontal microflora.

1.6.1 The effect of smoking on periodontal disease

Tobacco use is directly related to the incidence and prevalence of a variety of medical problems including cancer, low birth weight and pulmonary, cardiovascular and gastrointestinal disease (Bartecchi et al., 1994). In the last

two decades there has been an increasing awareness of the role of tobacco use on the prevalence and severity of periodontal diseases.

A number of studies have shown that smoking is a risk factor in periodontal disease (Haber & Kent, 1992; Locker & Leake, 1993; Grossi et al., 1994, 1995; Oliver et al., 1998; Bergstrom, 1989; Ismail et al., 1990; Beck et al., 1990; Horning et al., 1992). Risk assessment analysis of the data collected determined that tobacco users are 2.5 to 8.4 times more likely to develop periodontal disease than are non-smokers (Beck et al., 1990; Haber & Kent, 1992; Grossi et al., 1994; Bergstrom & Preber, 1994; Stoltenberg et al., 1993; Beck & Slade, 1996; Beck, 1994). In addition to increased incidence, smokers tend to exhibit increased severity of periodontal disease (Beck, 1994; Bergstrom & Preber 1994; Grossi et al., 1994, 1995; Kamma et al., 1999). Smokers have increased pocket depths (Ismail et al., 1983; Solomon et al., 1968; Feldman et al., 1987; Bergstrom, 1989; Goultschin et al., 1990; Haber et al., 1993) and attachment loss (Gonzalez et al., 1996; Machtei et al., 1997) compared to non-smokers. Haber et al. (1993) showed that the mean number of sites with PD greater than or equal to 4mm was higher in smokers than non-smokers and similar results have been found by Bergstrom (1989). Interestingly, an earlier study (Preber & Bergstrom, 1986a) reported that the only difference in PD between smokers and non-smokers was that smokers had significantly deeper upper palatal pocketing.

Both cross-sectional (Bergstrom et al., 1991, Bergstrom & Eliasson, 1987; Feldman et al., 1983; Bergstrom & Floderus-Myrhed, 1983) and longitudinal

(Bolin et al., 1986; Feldman et al., 1987; Grossi et al., 1994, 1995; Bergstrom & Preber, 1994) studies have shown that smokers have greater alveolar bone loss.

The severity of the periodontal destruction may be related to the number of cigarettes and pack years smoked (Martinez-Canut et al., 1995; Goultschin et al., 1990; Grossi et al., 1994, 1995; Haber & Kent, 1992; Jette et al., 1993). Gonzalez et al. (1996) positively correlated the severity of periodontal attachment loss, pocket depth and bone loss with serum cotinine levels. Cotinine is a major metabolite of nicotine and its level is directly related to the level of smoking. Hence it is a quantitative method for measuring tobacco usage.

Smoking has also been associated with increased tooth loss (Bergstrom & Floderus-Myrhed, 1983; Feldman et al., 1987; Ahlqvist et al., 1989; Heckert et al., 1986; Osterberg et al.; 1986), tooth mobility (Feldman et al., 1987) and more furcation involvement in patients (Mullally & Linden, 1996).

There are conflicting reports about the effect of smoking on gingival bleeding. Some studies indicate decreased bleeding in smokers (Bergstrom & Floderus-Myrhed, 1983; Preber & Bergstrom, 1985; Feldman et al., 1983; Bergstrom, 1990; Preber & Bergstrom, 1986a), whilst others report increased bleeding (Macgregor et al., 1985; Arno, 1958). Bergstrom (1989) showed in his study population that gingival index did not notably differ between smokers and non-smokers. Generally it would appear that smokers have less gingival inflammation than non-smokers, and this would follow given that nicotine has

vasoconstrictive properties (Bounaneaux et al., 1988; Michel et al., 1988). The reduced level of gingival inflammation is corroborated by reduced gingival crevicular fluid volumes in smokers (Kinane & Radvar, 1997).

Smokers may have poorer oral hygiene and higher plaque scores than non-smokers (Schei et al., 1959; Sheiham, 1971; Preber et al., 1980; Preber & Bergstrom, 1985; Locker, 1992; Eklund et al., 1994; Holm, 1994), which could be the cause of the increased periodontal destruction in these patients. Two early studies (Preber & Kant, 1973; Sheiham, 1971) indicated that smoking had no effect on the periodontium. However a number of more recent studies have controlled for plaque levels and reported more disease in smokers (Ismail et al., 1983; Preber & Bergstrom, 1990; Bergstrom, 1989; Bergstrom, 1987a; Linden et al., 1994) or have shown similar plaque levels (Preber & Bergstrom, 1986a).

A number of reports indicate that smokers have an increased build up of calculus compared to non-smokers (Pindborg, 1947, 1949; Feldman et al., 1987; Preber & Bergstrom, 1990; Anerud et al., 1991; Christen et al., 1985). However it is unclear what effect this may have.

Tobacco use has also been implicated in refractory periodontitis (Macfarlane et al., 1992; Bergstrom & Blomlof, 1992; Haber, 1994; Magnusson et al., 1996). Macfarlane et al. (1992) reported that 90% of refractory periodontitis patients were smokers. It has also been suggested that smoking is significantly involved in GEOP (Monteiro da Silva et al., 1997; Schenkein et al., 1995; Salvi et al.,

1997; Haber et al., 1993). Haber et al (1993) reported that in 19-40 year olds the prevalence and severity of disease were increased in smokers. Schenkein et al. (1995) found smoking to be more prevalent in GEOP patients. In these patients smoking had a significant effect on attachment loss. Also these patients had more affected teeth and greater mean attachment loss than non-smokers. Salvi et al. (1994) in their review of periodontal risk factors suggested that half of periodontal disease in patients under 33 years of age may be smoking related.

Tobacco use has also been implicated in other oral conditions, such as ANUG (Pindborg, 1947, 1949), leukoplakia (Christen et al., 1979), and oral cancer (Bastiaan et al., 1976). Also subjects who smoke seem to be at a greater risk for further breakdown (Haber, 1994; Kaldahl et al., 1996a; Machtei et al., 1997).

1.6.2 The effect of smoking on periodontal therapy

Given the effect of smoking on the prevalence and severity of periodontal disease, and its multitude of effects on the host, it is not surprising that smoking has an effect on periodontal therapy.

Studies have consistently shown that smokers respond less well to scaling and root planing (Ah et al., 1994; Preber & Bergstrom, 1986b; Preber et al., 1995; Kaldahl et al., 1996b; Kinane & Radvar, 1997; Haffajee et al., 1997a; Machtei et al., 1998; Grossi et al., 1997), with as much as 50% greater improvement in PD reduction in non-smokers (Machtei et al., 1998). After non-surgical therapy there seems to be a greater reduction in gingival index in some smoker

populations (Preber & Bergstrom, 1986b) whereas, in other smoker subjects, a similar reduction is found to that of non-smokers (Kaldahl et al., 1996b).

Similarly tobacco users have a poorer response to surgical periodontal therapy (Ah et al., 1994; Preber & Bergstrom, 1990; Kaldahl et al., 1996b), even after accounting for differences in plaque scores (Kaldahl et al., 1996b). Preber & Bergstrom (1990) reported that during maintenance post-surgery smokers experienced a greater loss of horizontal attachment level, and patients with a higher incidence of breakdown tended to be smokers at the initial examination (Kaldahl et al., 1996a).

Kinane & Radvar (1997) reported that there was a poorer response in smoker subjects to anti-microbial therapy. They found that the response in deeper pockets is more greatly affected by smoking than in shallower pockets. A greater degree of recession was reported among non-smokers, perhaps due to the greater vasoconstriction in smokers and the less tissue swelling leading to less tissue shrinkage. This theory is supported by the findings of Preber & Bergstrom (1986b) who showed that after non-surgical treatment there was significantly less gingival index reduction in smokers, which may be expected if there was less gingival inflammation to start with.

A poorer success rate with guided tissue regeneration (GTR) has been reported in smokers (Rosenburg & Cutler 1994; Tonetti et al., 1995) and the stability of GTR is associated with an absence of cigarette smoking (Cortellini et al., 1996).

Miller (1987) showed that smoking had a major deleterious effect on periodontal grafts, and Bain & Moy (1993) found that implants had a greater risk of failure in tobacco users.

It has been suggested that the poorer response is due to poorer oral hygiene in smoker patients but a number of studies have controlled for plaque levels and shown that the less favourable response was not due to poorer plaque scores (Ah et al., 1994; Tonetti et al., 1995; Ismail et al., 1983).

Cessation of smoking may restore normal periodontal healing responses as similar responses between former smokers and those who have never smoked have been reported (Grossi et al., 1997) and that a past history of smoking was not deleterious to the response to therapy (Kaldahl et al., 1996b).

1.6.3 Smoking and the periodontal microflora

Smokers are colonised by the same microorganisms as non-smokers (Zambon et al., 1996; Preber et al., 1992; Stoltenberg et al., 1993; Kamma et al., 1999), but generally in greater numbers (Kamma et al., 1999; Zambon et al., 1996).

There are conflicting reports whether or not smokers harbour a particular microflora. Preber et al. (1992) examined 145 patients, of whom 83 smoked, sampling one site over 6mm pocket depth per patient and investigated the presence of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* by culture. They found no significant differences in bacterial counts or relative

frequencies between smokers and non-smokers. Interestingly in 10% of these patients *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* were not detected. Stoltenberg et al. (1993) similarly found no differences in the prevalence of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia, E. corrodens*, and *F. nucleatum* between smokers and non-smokers. The authors suggested that smoking was more of a risk factor for periodontitis than the presence of the five microorganisms.

However, increased numbers and prevalence of *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* in smokers were reported by Zambon et al (1996). This group also found increased levels of *Capnocytophaga* species and *E. saburreum* in non-smokers, but no differences in the levels of *C. rectus*, *F. nucleatum* and *P. intermedia.* In general smokers had higher mean levels of subgingival infection with *A. actinomycetemcomitans*, *P. gingivalis*, *B. forsythus*, *C. rectus* and *F. nucleatum.* Smoking was a predictor for *B. forsythus* infection and smokers were at a higher risk of *A. actinomycetemcomitans*, and *P. gingivalis* infection. The relative risk of infection by *B. forsythus*, *A. actinomycetemcomitans*, and *P. gingivalis* increased with the number of pack years. In this study attachment loss rather than pocket depth was examined and the samples taken from a wide range of attachment loss scores rather than just deep pockets. This may account for the differences between this study and the two quoted previously.

In a study of GEOP patients Kamma et al. (1999) found that smokers again harboured greater numbers of bacteria with significantly higher prevalence and numbers of *S. sanguis*, *P. micros*, *C. concisus*, *E. coli*, *B. forsythus*, *C. gracilis*, *C. rectus*, *P. gingivalis*, *S. sputigena*, *C. albicans* and *A. fumigatus*. Non-smokers had higher levels of *S. intermedius*, *A. naeslundii*, *A. israelii* and *E. lentum*. In this study the deepest sites were selected, but similar findings were reported to the study by Zambon et al. (1996).

In response to non-surgical therapy, Grossi et al. (1997) reported a lower reduction in *B. forsythus* and *P. gingivalis* levels when compared to the reduction in former and never smokers. Conversely Preber et al. (1995) found similar reductions in *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* in both smoker and non-smoker groups.

It is unclear why smokers may harbour a different microflora compared with non-smokers. Smoking has been shown to decrease the relative temperature of diseased sub-gingival sites compared with non-smokers (Dinsdale et al., 1997), which may favour colonisation by certain organisms. *In vitro* exposure to smoke reduces the viable number of microorganisms (Bardell et al., 1979, 1981; Ertal et al., 1991), but how this may affect the subgingival microflora is unknown. Periodontal pockets are more anaerobic (Kenney et al., 1975) and the decreased oxygen tension may favour the colonisation and growth of anaerobic bacteria (Loesche et al., 1983). Smoking increases bacterial adherence to epithelial cells (Venditto et al., 1992) and once colonised deep periodontal pockets may offer an

especially favourable environment for the growth of anaerobic periodontopathogens by virtue of reduced oxygen tensions (Mettraux et al., 1984). The reduced oxygen tension may favour particular organisms such as *B. forsythus*, *A. actinomycetemcomitans* or *P. gingivalis*. It is possible that the impaired defence mechanisms i.e. the reduced phagocyte ability of macrophages and neutrophils, may facilitate the colonisation of pockets or allow the establishment of a particular microflora.

1.6.4 Smokeless tobacco

The effect of smokeless tobacco is unclear and yet to be demonstrated. There are a number of reports linking it to disease (Robertson et al., 1990; Hoge et al., 1983; Offenbacher et al., 1985; Christen et al., 1985) but this seems to be mainly recession and white patches at the site of application (Robertson et al., 1990).

This study aimed to examine the effect of smoking on scaling and root planing in AP and GEOP subjects. It also examined the effect of smoking on the microflora as a whole and in these patient groups. In addition, in AP subjects, the antibody titres to a number of periodontal organisms were compared between smokers and non-smokers.

1.7 Microbial diagnostic techniques

1.7.1 Introduction

The many techniques generally available for assessing the extent of the presence of microorganisms in infections have been widely applied in the study of

periodontal disease. Tests are useful for: 1) determining causative agents, 2) assessing disease activity for treatment planning, 3) monitoring the effects of treatment, 4) deciding on recall intervals, 5) diagnosing the various forms of periodontal disease, 6) treatment planning for a new patient, 7) assessing prognosis especially for refractory patients and 8) motivating patients if particular organisms are present and changing oral hygiene habits (Genco et al., 1986; Mombelli 1992, 1994).

Techniques may be classified as: growth in culture, microscopical, immunological, enzymatic, and DNA-technology based.

The usefulness of these tests suffers from a number of problems general to all. Only a fraction of the organisms in the periodontal pocket have been identified (Tanner et al., 1994). There are problems with which sites to select, when to sample these sites and interpretation of results (Tanner & Goodson, 1986). Prospective studies have failed to demonstrate the prognostic value of microbial assays (Macfarlane et al., 1988; Nieminen et al., 1995; Listgarten et al., 1991). Currently there is a lack of a suitable gold standard and also a test that provides a comprehensive picture of the flora. Periodontal disease is a poly-infection and monitoring just a few organisms may be erroneous. It seems that in a clinical situation microbial assays still need validation (Smith, 1994). They are however good tools for research purposes.

1.7.2 Growth in culture

Traditionally, microbial culturing has been the preferred means for examination of the oral microflora. The major advantage of culturing is its ability, in principle, to identify all major components of the oral microbiota. Culturing is also a prerequisite for determination of the *in vitro* antimicrobial susceptibility of a given pathogen.

Unfortunately microbial culturing is time-consuming, expensive, technique-sensitive and requires personnel with considerable knowledge of microbiology, especially for the recovery of the many fastidious organisms in the oral cavity and periodontal pocket. Available microbiological media and incubation atmospheres may not reliably grow *B. forsythus*, *P. gingivalis*, *Treponema* species, and other fastidious bacteria. The large spirochaetes and other organisms observed by direct microscopy have not yet been cultured (Tanner et al., 1994).

Most oral samples can be processed by use of anaerobic processing and incubation conditions. Oral microbial isolation can take place on non-selective or selective media. Non-selective culture media aim at growing all microbes in the same proportions as those that are present in a sample. However no available medium fulfils this criterion with the end result that some species are over-represented and others under-represented. Some form of selective pressure has been demonstrated by non-selective media as different rates for recovery of

black-pigmented anaerobes have been reported from the same sample on different non-selective media (Moore, 1987).

An ideal selective medium contains anti-microbial agents that are capable of suppressing all organisms other than the one for which the medium is designed. Full recovery of the study organism and total suppression of the contaminating flora are often mutually exclusive. Detection limits for organisms in periodontal pockets to be identified by culturing are on average 10^4-10^5 and 10^3 cells for non-selective and selective media respectively (Zambon & Haraszthy, 1995).

Conventional taxonomic methods include colony morphology, microscopic features, oxygen tolerance and comprehensive biochemical characterisation.

Although refined by the use of the spiral plater, selective media, improved anaerobic chambers, and better defined and expedient methods for identification of cultivable species, bacterial culture is still cumbersome, time-consuming and costly (Zambon & Haraszthy, 1995).

The tests not reliant on cell viability are only as accurate as the primary reference method which has been microbiological culture. It is becoming increasingly apparent that culture is not a suitable reference, because of its inability to adequately grow all the bacteria present in the periodontal pocket (Cattabriga & Pedrazzoli, 1996). We are thus left with an inadequate "gold standard", against which to measure new diagnostic methods.

1.7.3 Direct Microscopy

Freshly mounted plaque samples may be examined by dark-field or phase-contrast microscopy, which shows the number and morphological features of the microorganisms present. Direct microscopic examination of plaque samples represents a quick, easy and inexpensive means for screening a microbial sample.

Four major groups of microbial morphotypes are distinguishable: coccoid cells, nonmotile rods, motile rods and spirochaetes. Inflammatory cells and oral protozoa that are present in the sample can be evaluated as well. By examining a sample for size, shape and motility of its bacterial content, it is possible to differentiate between a health-associated and disease-associated sample (Listgarten & Hellden, 1978). Therefore it is possible to measure the success of treatment by comparing samples from before and after.

One major disadvantage of direct microscopy is its inability to identify bacteria at the species level. Also it provides no guidelines for antimicrobial medication and it is possible to confuse similar morphotypes e.g. small rods for cocci (Slots & Taubman, 1992). Given the inevitable variations in sampling, dispersion, slide preparation, morphotype identification and interpretation, there are problems with representativity and reproducibility (Wilson et al., 1985). Gram staining can be used to differentiate between Gram-positive and Gram-negative organisms, but may be difficult to interpret because old or dead Gram-positive

cells lose their ability to retain the stain. Gram staining does not provide information about the species present.

1.7.4 Immunologic assays

Immunologic assays are based on the detection of antigen-antibody reactions between antigens of the target organism and specific antisera raised against it. The reaction can be revealed using a variety of procedures including direct and indirect immunofluorescence assays (IFA) (Zambon et al., 1986), particle concentration fluorescence immunoassay (Jolley et al., 1984), flow cytometry (Kornman et al., 1984), enzyme-linked immunosorbent assay (ELISA) (Simonson et al., 1988), membrane assay (Louie & Larjava, 1994) and latex agglutination (Newman & Nisengard, 1988).

Direct IFA employs both monoclonal and polyclonal antibodies conjugated to a fluorescein marker which binds with the antigen to form a fluorescent immunocomplex detectable under a microscope. Indirect IFA employs a secondary fluorescein-conjugated antibody that reacts with the primary antigen-antibody complex. IFA is a relatively simple technique providing both qualitative and quantitative information about bacteria present in mixed subgingival samples (Zambon et al., 1986).

Particle immunofluorescence immunoassay involves the use of polystyrene beads as a substrate (Jolley et al., 1984) coated with antigens which then react with the specific fluorescein labelled antibodies yielding a fluorescent signal

detectable by a fluorimeter. A modification of this technique using bacteria, termed bacterial concentration fluorescence immunoassay (BCFIA), has been developed to detect putative periodontopathic bacteria in plaque samples (Wolff et al., 1991, 1992).

Cytofluorography (Kornman et al., 1984) involves labelling of bacterial cells with species-specific antibodies and a second fluorescein conjugated antibody. The immunocomplex suspension is scanned by the flow cytometer, but the cost of the required equipment precludes widespread usage.

ELISA involves the detection of the antigen-antibody complex with a colorimetric reaction catalysed by an enzyme linked to the antibody. Although it has been used to detect periodontal pathogens (Simonson et al., 1988), its main use has been to detect and quantitate the humoral immune response towards these organisms (Ebersole & Taubman, 1994).

Latex agglutination immunoassay (LA) is based on the binding of the antibody (or antigen) to latex beads and its subsequent agglutination or clumping on exposure to its specific antigen (or antibody), which can be scored. Rapid LA tests have been developed for *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* (Nisengard et al., 1992).

Membrane immunoassay involves linkage between the antigen and a membrane bound antibody to form an immunocomplex that is later revealed through a

colorimetric reaction. One form has been marketed commercially and detects *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* (Snyder et al., 1994).

Immunologic assays are valuable because rapid, quantitative results can be obtained for organisms in direct oral specimens, which is particularly important for organisms that are difficult to grow. These tests require neither viability of test organisms nor aseptic sample handling, and may additionally provide serotyping information. Immunoassays are based on either polyclonal or monoclonal antibody reagents. Since the accuracy of immunodiagnostic tests depends on the quality of reagents used and the method of specimen processing, the results must be interpreted with care (Slots & Taubman, 1992). The inclusion of appropriate positive and negative controls is crucial.

Polyclonal antibodies are inexpensive to produce, but are often lacking in specificity because of cross-reactivity with different monoclonal species. Monoclonal antibodies can be generated against both major and minor antigens. They can provide high serologic specificity because of reduced risk of microbial taxa sharing the antibody recognising epitope. However the sensitivity may be low if the specific epitopes are inherently few on each cell, are not expressed, are destroyed or are masked (Slots & Taubman, 1992).

1.7.5 Enzymatic methods

In addition to the methods mentioned above, several assays for microbial enzymes are available. In general these tests do not detect specific bacterial

species. Instead these tests indicate the presence of destructive enzymes produced by a group of mainly but not exclusively periodontal pathogens. The microbial enzymes that may be used include collagenase, peptidases, trypsin-like enzymes, neutral proteases and elastase. However a number of these enzymes are also produced by the host especially collagenases, and it may be difficult to differentiate between host-derived and bacterial enzymes.

The periodontal pathogens *P. gingivalis*, *B. forsythus*, *T. denticola*, and also some *Capnocytophaga* species that are not usually found to be periodontopathic, invariably produce a trypsin-like peptidase (Laughon et al., 1982; Loesche et al., 1987; Tanner et al., 1985). This enzyme is able to catalyse the hydrolysis of the synthetic substrate, N-benzoyl-DL-arginine-2, naphthylamide (BANA), yielding a product assayed by colorimetric reaction that is the basis for the Perioscan test (Loesche et al., 1990).

SK-013 is a similar colorimetric test capable of demonstrating the presence in subgingival plaque samples of a bacterial peptidase which catalyses the hydrolysis of a synthetic substrate (N-carbobenoxy-glycil-glycil-arginyl) that is specifically produced by *P. gingivalis*, *B. forsythus*, and *T. denticola* (Seida et al., 1992).

Both these tests cannot distinguish between the 3 periodontopathogens and other species that may also produce these enzymes (Loesche et al., 1990).

1.7.6 DNA-technology based

The tests in this group can be subdivided into two main categories: DNA probes and polymerase chain reaction (PCR).

1.7.6.1 Deoxyribonucleic acid probes

Considerable interest has been directed towards the development of deoxyribonucleic acid (DNA) probes and their use in the diagnosis of infections. DNA probes are able to shorten the time required to identify pathogens, detect organisms directly in clinical specimens and reduce the overall costs associated with processing samples (Tenover, 1988). DNA probes identify organisms by nucleic acid content rather than products. Since every properly classified species has some unique nucleotide sequences that distinguish it from every other species, each microorganism's genetic composition is a fingerprint that can be used for its identification.

A DNA probe is simply a piece of DNA, with a detectable label, that binds to a target nucleic acid molecule under appropriate conditions. Hybridisation is the process whereby two simple strands of nucleic acid come together to form a stable double-stranded molecule.

The hybridisation reaction consists of four components: the probe, the target (contained in the sample), the reporter molecule and the hybridisation method. The sample serves as a source of nucleic acid to be analysed and may be a

clinical specimen. The nucleic acid in the sample is referred to as the target DNA (or RNA) and the label on the probe is referred to as the reporter molecule.

1.7.6.2 Types of DNA probes

There are three major types of DNA probes used in the detection of bacterial species: whole genomic, cloned and oligonucleotide. Whole genomic and cloned probes are usually several hundred to several thousand bases long (long probes), whereas oligonucleotide probes are generally shorter than 100 bases, often 16-30 (short probes).

Whole genomic probes

Whole genomic probes are produced by isolating DNA from an organism and labelling it with a reporter group. The advantages of whole genomic probes are that they are the simplest to make and they generally detect all strains of a phylogenetically coherent species. The major disadvantage is that whole genomic probes often cross-react with closely related species.

Cloned probes

There are two types of cloned probes: random and specific. Cloned probes are produced by cutting into fragments the DNA of the target organism with a restriction endonuclease, anywhere for random probes and either side of the desired DNA sequence for specific probes. These can then be inserted into a vector, usually a small plasmid from *E. coli*, sealed to produce a double-stranded

circular molecule and introduced into *E. coli*. The plasmid containing the DNA insert will replicate in *E. coli* making hundreds of copies.

Both randomly and specifically cloned probes require extensive validation against DNA from the target and also related species. The randomly cloned probe that reacts with strains of the target species but not with other species is selected. The advantage of cloned probes is that they are more specific (less cross-reactive) than whole genomic probes. However the disadvantage is that they require extensive validation especially the randomly cloned probes.

Oligonucleotide probes

Oligonucleotide probes are designed upon sequence information for the target DNA (or RNA). These probes display "exquisite" specificity (Tenover, 1988), and, under stringent conditions, may be capable of detecting a change in a single base pair of a DNA or RNA sequence, which will be enough to prevent binding of the probe to the target DNA. The specificity of short probes, as with long probes, varies much more with the temperature and salt concentration. Thus it is important not to vary these conditions. They are relatively simple to prepare and are very stable over time. Because of their short size, they hybridise to target DNA at very rapid rates (sometimes as little as 30 minutes), in contrast to long probes which often require much longer (4-16 hours) even with rate enhancers such as dextrane sulphate.

Short probes can only be labelled with a single reporter molecule, thus may be 10- to 100-fold less sensitive than long probes. The usual target for short probes has been the 16S rRNA. This region is a good target as all eukaryotic and prokaryotic organisms possess this molecule. There are regions of this molecule that are extremely variable for different species, providing unique targets and there are 10 000 copies of the molecules (ribosomes) in a typical bacterial cell providing a greatly amplified target. (Tenover, 1988). These probes still require extensive validation.

1.7.6.3 Reporter groups for detecting DNA probe hybridisation reactions

There are four major classes of reporter groups widely used for probe detection: radioactive, enzyme, fluorescent and chemiluminescent.

Radioactive labels

Initially radioactive labelling was the most common method used to detect and quantitate hybridisation reactions. The isotopic label is directly incorporated into the probe by nick translation. ^{32}P has been the most commonly used and its incorporation does not radically change the hybridisation characteristics of the probe. After hybridisation the binding of the DNA probe to the target can be detected by autoradiography or scintillation counting. This method has one of the highest sensitivities of all the labelling methods but ^{32}P has a short half life (roughly 14 days) requiring frequent probe preparation. An alternative is ^{125}I with a half life of 60 days, but there are safety concerns with both probes that require careful technique, proper storage and disposal.

Enzyme labels

Horseradish peroxidase and alkaline phosphatase are the two most common enzyme labels. The enzyme may be attached directly to the DNA or to a second detector group after a primary label has been incorporated into the DNA sequence, such as the Biotin-Avidin system. After the addition of a substrate, a colorimetric product is produced.

Fluorescent labels

Instead of a colorimetric reaction, the use of a photoreactive substrate produces a fluorescent reaction that can be measured by a fluorometer.

Chemiluminescence

Chemiluminescence is the production of light by a chemical reaction. Both horseradish peroxidase and alkaline phosphatase may be used with luminol and an enhancer molecule or a substituted dioxetane respectively. The light emitted can be detected by a luminometer (and captured on computer software) or by radiographic film.

1.7.6.4 The hybridisation reaction

1) Stringency

After a double-stranded DNA molecule is denatured to single strands, it is capable of re-associating with either a DNA or RNA strand of complementary sequence. The degree and specificity of binding depends on the temperature,

pH, use of a denaturant such as formamide, and the salt concentration of the reaction buffer. Nucleic acid molecules can tolerate a certain number of mismatched base pairs and still form stable duplexes as long as a significant number of base pairs do match and form bonds. The greater the degree of mismatched bases along the strands of nucleic acid, the more likely the two molecules are to come apart. The degree of mismatch that can be tolerated in a hybridisation reaction and still maintain a double-stranded molecule is referred to as the "stringency" of hybridisation. Under conditions of high stringency, only exact matches of DNA will anneal and stay together. Under conditions of low stringency (e.g. low temperature, high salt concentration, low formamide concentration) two DNA strands that are only 80-90% homologous may bind together and result in a positive hybridisation signal. If the stringency of a reaction is changed, the specificity of the probe will change. The range of conditions that can be tolerated without affecting the specificity of a probe vary depending on the length of the probe and the percentage of guanine and cytosine residues in the probe. The shorter the probe, the more narrow the range of temperature and salt concentration that can be tolerated.

2) Formats

Hybridisation reactions can be performed in four formats: on a solid support, in solution, in situ or by using the Southern blotting procedure after gel electrophoresis. Most DNA probe reports in the literature have used a solid support especially filter hybridisation (Tenover, 1988).

In a filter hybridisation reaction the sample is spotted directly on the filter or concentrated into a small area by placing in vacuum manifold. The sample is lysed and the DNA denatured by addition of NaOH. Once denatured the DNA is attached to the filter by baking and more recently by ultraviolet light. The filter is prehybridised with non-homologous DNA, such as salmon or herring sperm, to prevent non-specific binding of the probe to the filter. After hybridisation the filter is washed at various temperatures determined by the stringency of the reaction. Both nitro-cellulose and synthetic nylon filters have been used.

The second format is to carry out the hybridisation reaction in solution. In this format both target and probe are free to move, maximising the chance that complementary sequences will align and bind. Solution hybridisations go to completion 5- to 10-fold faster than those on solid supports. After the hybridisation step, the nascent duplexes are removed from solution by the addition of hydroxyapatite and quantitated by scintillation counting or spectrophotometry.

The third format is in-situ hybridisation. In most instances in-situ hybridisation is carried out on formalin-fixed paraffin embedded tissues and allows one to examine the tissue by other methods e.g. haemotoxylin and eosin staining.

The last format for hybridisation is the Southern hybridisation gel. In this technique purified DNA is cleaved with restriction endonucleases and the

fragments separated by size using electrophoresis through agarose. The DNA is transferred to a filter, baked and is then ready for hybridisation.

1.7.6.5 Advantages of DNA probes

The use of DNA probes can simplify the identification of bacteria. The technique is faster, less labour intensive and less costly than culture. The direct detection of infectious agents overcomes problems with slow growing or non-culturable organisms. It removes the need for viable cells and esoteric identification protocols. The technique is more comprehensive and uses the same methodology for all species. Additionally samples may be stored and it can detect organisms within tissues.

1.7.6.6 Disadvantages of DNA probes

Initially the use of DNA probes requires extensive preparation and testing. Construction of DNA probes is only feasible when bacterial species have already been isolated and characterised. However it is extremely unlikely that all of the species of periodontopathic bacteria have been identified as only about 60% of oral microorganisms can be cultured (Dewhirst & Paster, 1991). DNA probes are unable to detect yet to be identified species. The technique does not allow for antimicrobial sensitivity, although it is possible to detect the presence of resistance genes by using the appropriate probe (Lacroix & Walker, 1995). It provides semi-quantitative counts but no information about proportions of target bacteria in samples. There may be cross-reactivity of DNA probes between

homologous species, and false positives/negatives if the wrong reaction conditions are used.

1.7.6.7 DNA probes in the detection of periodontopathic organisms

The first probes developed for use in the detection of periodontopathogens were whole genomic probes (French et al., 1986). They detected *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* with 100% accuracy even in low quantities in mixtures of *A. viscosus*. However there was cross-reactivity between the *A. actinomycetemcomitans* probe and a related species, *Haemophilus* (French et al., 1986; Strzempko et al., 1987), and also a low level of cross-reactivity between the *P. intermedia* and other black-pigmented Bacteroides. Roberts et al. (1987) developed genomic probes to *Bacteroides* species but these also suffered from problems with cross-reactivity. These whole genomic probes have a detection limit between 10^3 and 10^6 (Strzempko et al., 1987, Savitt et al., 1988, Lippke et al., 1991).

To overcome the problem of cross-reactivity, cloned DNA (French et al., 1986; Di Rienzo et al., 1991) and oligonucleotide probes (Chuba et al., 1988; Dix et al., 1990; Gersdorf et al., 1993; Moncla et al., 1990) were developed. The oligonucleotide probes were directed towards bacterial 16S rRNA, which removed the problem of cross-reactivity but selection of the appropriate sequence was important (Moncla et al., 1990). The higher specificity of the oligonucleotide probes was counteracted by their generally higher detection limits (10^4-10^5 cells, Gersdorf et al., 1993), although some studies did report

detection limits of around 10^3 cells (Chuba et al., 1988; Dix et al., 1990). More recently the products from polymerase chain reactions have been labelled and used as DNA oligonucleotide probes (Preus & Russell, 1994; Lotufo et al., 1994; Bodinka et al., 1994). These proved to be very specific and, because they were much longer, had lower detection limits.

The first probes were ^{32}P labelled and later biotinylated probes were developed. More recently streptavidin/alkaline phosphatase, and horseradish peroxidase methods were tested (Smith et al., 1989) with detection limits of 10^4-10^5 cells and 10^5-10^6 cells respectively. Recently non-radioactive digoxigenin (DIG) labeled probes have been used (Preus & Russell, 1994; Socransky et al., 1994; Lotufo et al., 1994; Bodinka et al., 1994) by random priming techniques (Feinberg & Vogelstein, 1983) and digoxigenin labeled dUTP.

DNA probes have been developed and used to detect the following organisms in plaque samples, in addition to those mentioned above, *B. forsythus* (Lippke et al., 1991), *F. nucleatum* (Maiden et al., 1991), *E. corrodens* (Lippke et al., 1991), *C. rectus* (Söder et al., 1993), *T. denticola* (Loesche et al., 1992b).

1.7.6.8 Comparison of DNA probes with other techniques

Although some authors feel that there is no sufficiently accurate gold standard for the identification and enumeration of periodontal microorganisms (Loesche et al., 1992a), culture is the most commonly used method for assessing the performance of DNA probes.

In general DNA probes detect much higher frequencies of bacteria than culture (Savitt et al., 1988, Kornman et al., 1992; Ali et al., 1994; Yasui et al., 1993; Gersdorf et al., 1993; Slots & Chen, 1993), although some studies have found similar detection frequencies (Loesche et al., 1992b; Tanner et al., 1998b). The agreement between the two methods ranges from 55% (Slots & Chen, 1993) to 83% (Tanner et al., 1997b), with sensitivity values of 82% to 100% (Tanner et al., 1998b; Gersdorf et al., 1993), and with specificity values of 38% to 100% (Slots & Chen, 1993; Gersdorf et al., 1993). The disagreement between two techniques reflects the lower detection limits of the DNA probes, the accuracy of identification of the organism by DNA probes, and the need for viable cells for culture which is dependent on sampling, transport, processing and growth conditions (Ali et al., 1994; Gersdorf et al., 1993; Slots & Chen 1993). The comparison of the probes against the less sensitive "gold standard" culture results in the varying specificity and sensitivity scores. Oligonucleotide probes generally have lower detection limits than whole genomic probes, 10^3 versus 10^5, and this is generally reflected in the lower sensitivity and specificity scores for these probes when compared to culture. Maiden et al. (1991) reported agreement from 35.6% for *P. gingivalis* to 77.2% for *A. actinomycetemcomitans*, comparing oligonucleotide probes to culture. Sensitivity varied from 0.26 to 0.95 and specificity 0.21 to 0.93. *P. gingivalis* and *F. nucleatum* oligonucleotide probes were much more sensitive than culture, and the figures reflect the lower detection limits for the probes.

Melvin et al. (1994) reported a 59% to 76% agreement comparing DNA probes with ELISA identification. Although DNA probes were better for detecting *T. denticola* and had about 1000-fold lower detection limit, ELISA was better for detecting *P. gingivalis* and *C. rectus*. Again the figures reflect the different detection limits. Loesche et al. (1992a) reported similar accuracies, 88-97%, for DNA probes and ELISA compared to culture, and the detection frequencies for the two methods were generally similar. The same group reported (Loesche et al., 1992b) 90% agreement between DNA probes and the BANA enzyme test. However the BANA test could not distinguish between species making its use questionable.

A number of studies have compared IFA and DNA probes. Listgarten et al. (1995) found IFA much more sensitive, but both techniques were superior to culture. In this study the detection limit for the DNA probes was $5x10^5$ cells. Other studies using lower detection limits for the probes have found DNA probes to be as or more sensitive than IFA (Zappa et al., 1990; Loesche et al., 1992b; Tanner et al., 1998b). In these studies there was no cross-reactivity of the DNA probes or antibodies with other species.

Given the accuracy of DNA probes, its superiority to culture, and its comparable performance to ELISA and IFA, DNA probe technology is an excellent tool to detect periodontal microorganisms.

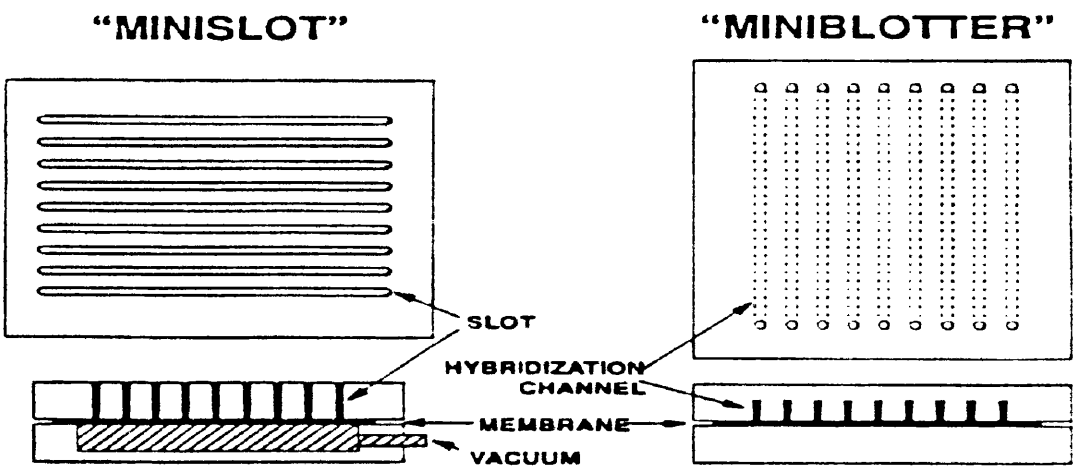
1.7.7 Checkerboard DNA-DNA Hybridisation

1.7.7.1 Development and use of the technique

A major problem with DNA-DNA hybridisation assays has been the limitation in the number of DNA probes that could be simultaneously hybridised with large numbers of DNA samples. Previous methods that have been used only evaluated a large number of samples against a small number of DNA probes or a small number of samples against a large number of DNA probes (Smith et al., 1989). This limited the usefulness of these results especially as a large number of bacteria may be found in a periodontal pocket (Moore & Moore, 1994).

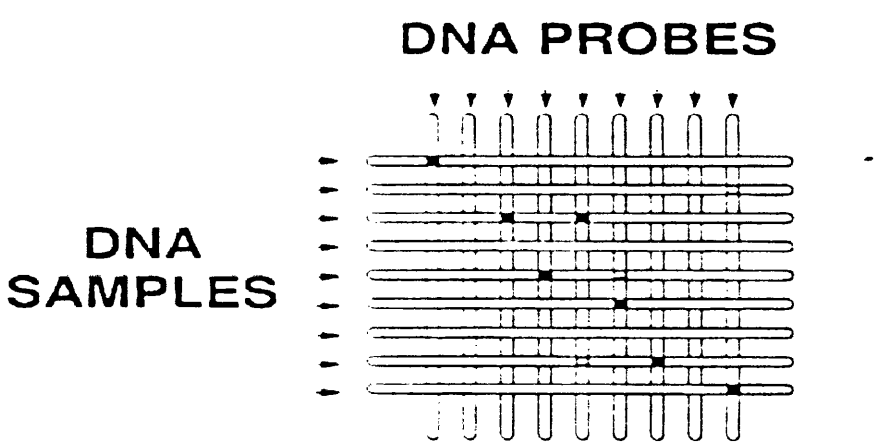
Socransky and co-workers (1994) developed a technique whereby large numbers of samples could be screened against a large number of DNA probes, called Checkerboard DNA-DNA hybridisation. The method used was based on the use of Slotblot devices previously employed for the detection of multiple antigen-antibody reactions on a single solid support membrane (Kazemi et al., 1990; Levin & Branton, 1986). After denaturation, the DNA samples were loaded into lanes using a Minislot apparatus and fixed to the nitro-cellulose membrane by ultraviolet light or baking. By rotating the membrane 90° from its original orientation, the DNA probes were then run across the face of the membrane, allowing each probe to come into contact with part of the bound sample (Figure 1.1). Use of the Minislot apparatus allowed up to 45 samples and 45 probes to be hybridised at any one time. Using randomly DIG-labelled whole chromosomal probes (Feinberg & Vogelstein, 1983), incubation with an anti-digoxigenin antibody conjugated with alkaline phosphatase and a suitable

Figure 1.1 Diagram of the essential features of the Minislot and Miniblotter devices (reproduced from Socransky et al. 1994).



The samples are loaded into lanes on a membrane placed between the halves of the Minislot device. The probes are placed in lanes in the Miniblotter device allowing hybridisation with the membrane-bound samples

Figure 1.2 Diagrammatic representation of "checkerboard" hybridisation format. Only a few channels are shown for simplicity (reproduced from Socransky et al. 1994)



chemiluminescent substrate, the hybridisation reactions could be detected on radiographic film. The characteristic pattern of the hybridisation reactions gives the technique its name (Figure 1.2).

Checkerboard hybridisation offers a number of advantages for DNA-DNA hybridisation. The principal advantage is that large numbers of DNA, RNA, tissue, bacterial or viral samples can be screened with multiple probes at the same time. The technique is relatively economical in that it uses smaller volumes of reagents and probes. A large number of reactions are performed per membrane, so there is less outlay for nylon or nitro-cellulose membrane. Thirdly, because of the large number of reactions possible per membrane, it is quicker and less labour intensive than previous DNA probe techniques.

The detection limit is similar for other techniques using whole genomic probes: lowest limit is about 10^4 cells (Haffajee et al., 1997a). The technique is also suitable for oligonucleotide probes (Socransky et al., 1994).

The Checkerboard technique has been used to identify a large number of disease and health-associated organisms in periodontal patients (Haffajee et al., 1995, 1997a; Tanner et al., 1998a; Papapanou et al., 1997b)

1.7.7.2 Comparison of the Checkerboard technique with other methods

Three studies to date have compared the Checkerboard technique to culture, Maiden et al. (1997), Papapanou et al. (1997a) and Tanner et al. (1998a).

The studies by Maiden et al. (1997) and Tanner et al. (1998a) used the same patients but samples were compared from separate but adjacent sites, rather than the same site. Both studies showed similar results, with culture yielding higher *B. forsythus* and *C. rectus* prevalences, and the Checkerboard technique higher *P. intermedia*, *P. nigrescens*, *F. nucleatum* subsp *vincentii*, *S. oralis*, *S. sanguis* and *V. parvula* prevalences. However, there are problems with comparing samples from different sites, when the microflora between sites may vary considerably (Moore & Moore, 1994).

A better study by Papapanou et al. (1997b) split each of 283 subgingival plaque samples in two; one of which was analysed by culture and the other by the Checkerboard technique for ten periodontal organisms. The Checkerboard technique resulted in higher prevalence figures for *P. gingivalis*, *P. intermedia/P. nigrescens*, *F. nucleatum* and *B. forsythus*. If these species were used as the reference, the Checkerboard technique detection sensitivities ranged from 0.17 to 0.86, specificities 0.17 to 1.0 and diagnostic accuracies from 0.51 to 0.81 depending on the bacterial species. The authors suggested that these differences may be due to difficulty in identifying similar species by biochemical tests, division of non-homologous samples, cross-reactivity of the probes especially in large plaque samples, and incorrect stringency conditions for the whole chromosomal probes. However, there was "reasonable" agreement between the two techniques.

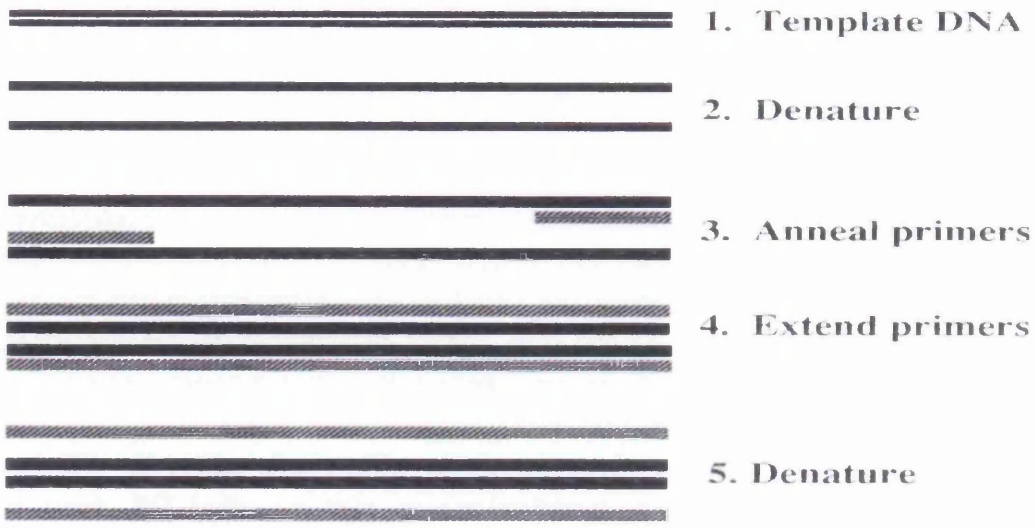
1.7.8 Polymerase chain reaction

1.7.8.1 Basic concepts

The polymerase chain reaction (PCR) is an *in vitro* technique that allows the amplification of a specific DNA region that lies between two regions of known DNA sequence. It allows the amplification of as little as one copy of the specific target sequence to generate large quantities of DNA. PCR is quick, generally taking 4-6 hours, accurate and a reliable technique. PCR amplification of DNA is achieved by using oligonucleotide primers. These are short (usually 15-30 bases in length), single-stranded DNA molecules which are complementary to the ends of a defined sequence of DNA. Two primers are required for PCR amplification. One primer is complementary for a sequence downstream and one primer is complementary for a sequence upstream. Figures 1.3 and 1.4 show the typical PCR cycle. Potentially, after 20 cycles of PCR there will be 2^{20}- fold amplification, assuming 100% efficiency during each cycle. In practice PCR amplification is not unlimited as the amount of enzyme becomes limiting after 25-30 cycles and also due to thermal denaturation of the enzyme during the process.

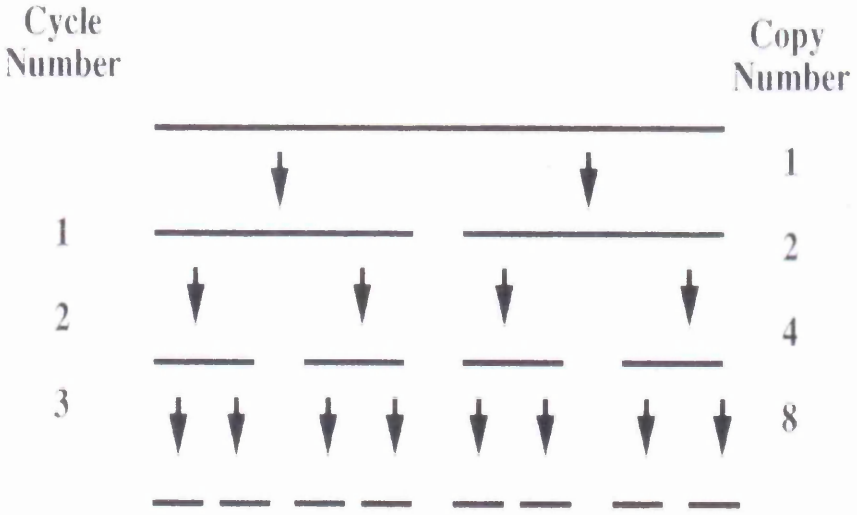
If primers are selected that amplify a specific region of an organism that is unique to that organism, this technique can be used to detect and identify that organism by visualising and sizing the product DNA, usually by gel electrophoresis. The product appears as a discrete band on the gel that can be

Figure 1.3 The polymerase chain reaction (reproduced from Zambon & Haraszthy, 1995).



Double-stranded DNA is denatured into single stranded DNA by heating to 90-95°C. Cooling to 40-60°C enables the DNA primers to hybridise to specific locations on the single-stranded sample DNA. At 70-75°C in the presence of *Taq* polymerase, the primers extend and fill in the DNA between the 2 primers bound to the template of single-stranded DNA. Raising the temperature back to 90-95°C denatures the newly created DNA strand from the DNA template.

Figure 1.4 Repeated cycles of denaturation, annealing and extension produce numerous copies of the DNA segment and geometric amplification of a single DNA sequence (reproduced from Zambon & Haraszthy, 1995).



sized by comparison to a base-pair ladder of known sizes. PCR can be used to amplify any part of the genome, DNA or RNA.

1.7.8.2 Advantages of PCR

PCR is well established as a relatively inexpensive technique, generally taking fewer than six hours. It does not rely on viable cells and samples may be stored frozen for a period of time before analysis.

1.7.8.3 Disadvantages of PCR

Specific PCR primers can only be prepared for already characterised organisms, although there are associated techniques applied to unknown organisms (Harper-Owen et al., 1999). Extensive testing of the primers is required to check specificity. PCR can be used quantitatively, but if so used becomes unreliable after about twenty cycles or so. The number of species that can be detected per reaction is limited. Initially only one species per reaction was detected, but currently up to three periodontal organisms can be detected (Conrads et al., 1999).

1.7.8.4 Detection limit of PCR

Theoretically PCR could detect as little as one cell of a species in a sample, but, more realistically, 10^2 cells is the lowest detection limit for this technique (Riggio et al., 1996)

1.7.8.5 PCR and detection of periodontopathic microorganisms

PCR has been used to detect the presence of a number of the major periodontopathic bacteria using primers for the 16S rRNA sequences (Slots et al., 1995), 23S rRNA sequences (Haraszthy et al., 1992) or specific genes such as the leukotoxin A gene in *A. actinomycetemcomitans* (Goncharoff et al., 1993) or the fimbrillin gene (*fimA*) in *P. gingivalis* (Watanabe & Frommel, 1993). Genes coding for virulence factors are target sequences, such as the collagenase gene in *P. gingivalis* (Bodinka et al., 1994). In this instance it is not present in all strains but it may indicate a more virulent strain (Bodinka et al., 1994).

Table 1.7 shows the pathogens that have been investigated using PCR. It should be noted that PCR only detects the presence or absence of an organism and has not been used as a quantitative technique in the study of periodontal pathogens as yet. Many of the primer sequences are species-specific but cannot distinguish between strains (Watanabe & Frommel, 1996). PCR has also been used to examine the presence of viruses (Parra & Slots, 1996; Contreras & Slots, 1996) and yeasts (Hannula et al., 1997) in periodontal samples.

A variant of the polymerase chain reaction uses DNA segments of arbitrary rather than defined sequences as primers. This method, known as arbitrarily-primed PCR (AP-PCR) can be used for molecular typing of bacteria within the same species (van Steenbergen et al., 1993; Lotufo et al., 1994) and also to track the source of an infection, i.e. the transmission of *A. actinomycetemcomitans* in families (Preus et al., 1994). The arbitrary primers hybridise to the bacterial

Table 1.7 PCR identification of periodontal bacteria. Bacteria that have been identified by PCR, primer target regions and references

Organism	Target	Reference
A. actinomycetemcomitans	Leukotoxin A gene	Goncharoff et al. 1993
		Tonjum et al. 1993
	16S rRNA	Slots et al. 1995
		Matto et al. 1998
		Griffen et al. 1992
		Furcht et al. 1996
P. gingivalis	Fimbrillin gene (fimA)	Watanabe & Frommel 1993
		Riggio et al. 1996
	16S rRNA	Slots et al. 1995
	Collagenase gene (prtC)	Bodinka et al. 1994
P. intermedia	16S rRNA	Conrads et al. 1997
		Riggio et al. 1998
		Slots et al. 1995
T. denticola	tdp A gene	Watanabe & Frommel, 1996
	16S rRNA	Slots et al. 1995
B. forsythus	16S rRNA	Slots et al. 1995
		Meurman et al. 1997
C. rectus	16S rRNA	Slots et al. 1995
E. corrodens	16S rRNA	Slots et al. 1995
		Furcht et al. 1996
P. nigrescens	16S rRNA	Slots et al. 1995
		Riggio et al. 1998
H. pylori	16S rRNA	Asikainen et al. 1994
		Riggio et al. 1999
B. heparinolyticus	16S rRNA	Ashimoto et al. 1995
P. asaccharolytica	16S rRNA	Tran et al. 1997
B. fragilis	16S rRNA	Tran et al. 1997
C. pneumoniae	16S rRNA	Tran et al. 1997

DNA at specific sites depending on the conditions of the assay and amplify corresponding regions. The amplified DNA segments from PCR are resolved on gel electrophoresis and the banding pattern or amplitype is compared between strains.

Antibiotic sensitivity may be examined by determining the presence of resistance genes, such as tet (M) and tet (Q) (Olsvik et al., 1995; Lacroix & Walker, 1995). The PCR product, which is specific for that one organism, may be labelled and used as a probe in DNA-DNA hybridisation (Tonjum et al., 1993; Preus & Russell, 1994).

Traditionally the DNA for one organism is amplified per reaction, but recently multiplex techniques have been introduced allowing the amplification of DNA from more than one organism (Wahlfors et al., 1995; Tran & Rudney, 1996; Garcia et al., 1998; Conrads et al., 1999). Multiplex PCR should allow quicker, more comprehensive and less costly analysis of samples (Conrads et al., 1999). By using primers that amplify sequences from all organisms, followed by sequencing of the product, it is possible to characterise and identify the bacterial DNA from periodontal samples (Riggio, personal communication). This would allow the identification of unculturable species (Harper-Owen et al., 1999).

1.7.8.6 Comparison of PCR with other techniques

Comparisons of PCR and culture have consistently shown that PCR is the more sensitive technique and recorded higher prevalences of any organisms

investigated. (Slots et al., 1995; Riggio et al., 1996; Meurman et al., 1997; Wahlfors et al., 1995; Ashimoto et al., 1996). Ashimoto et al. (1996) reported discrepancies between 28% (*A. actinomycetemcomitans*) and 71% (*B. forsythus*), when comparing the two techniques. The use of selective media resulted in a 71% similarity for *A. actinomycetemcomitans*, but only a 28% match was achieved with *B. forsythus*, a slow growing and difficult organism to culture. In addition to its greater sensitivity, PCR may also detect dead organisms. Culture requires viable organisms, and a reduction may occur due to transportation, processing of samples and culture conditions (Slots et al., 1995; Riggio et al., 1996). PCR is quicker, with the results available in a few hours, cheaper, and less labour intensive, which lead one group to suggest PCR as the "gold standard" for identifying periodontal pathogens (Riggio et al., 1996).

A comparison of PCR and DNA probes showed that they produced similar results but PCR and culture did not (Ashimoto et al., 1995). Ashimoto et al. (1996) achieved an 84% match for *B. forsythus*, and 70% for *P. gingivalis*. There were similar PCR positive/DNA probe negative and PCR negative/DNA probe positive discrepancies for *B. forsythus*, but many more samples were DNA probe positive for *P. gingivalis* suggesting some cross reactivity by the DNA probe. Conrads et al. (1997) reported that PCR was slightly more sensitive for detecting *P. intermedia* and *P. nigrescens*, though oligonucleotide DNA probes allowed semi-quantification.

Table 1.8 summarises the advantages and disadvantages reported for the various assays.

In this study PCR and "Checkerboard" technique were used to investigate the microflora in AP and GEOP smoker and non-smoker subjects both before and after treatment. A comparison of the two techniques was also performed.

1.8 Aims of the study

The aims of this thesis are to:

1) a) Investigate the effect of SRP on AP and GEOP clinical and microbiological parameters,

   b) Compare clinical and microbiological parameters in AP and GEOP subjects before and after SRP,

   c) Investigate the inter-relationship between clinical parameters and the microflora in AP and GEOP subjects,

2) Compare PCR and Checkerboard using all plaque samples collected,

3) a) Examine the effect of SRP on AP antibody titres,

   b) Examine the effect of the flora on AP antibody titre and avidity,

   c) Examine systemic and local AP antibody correlations,

   d) Examine the effect of AP patient antibody serostatus on clinical and microbiological parameters, and treatment outcome,

4) a) Compare clinical and microbiological parameters in smokers and non-smokers before and after treatment in all patients, as well as AP and GEOP subjects,

Table 1.8 Microbial diagnostic assays and their use in the study of the periodontal microflora (adapted from Cattabriga & Pedrazzoli, 1996)

Type of Assay	Method	Detection Limit	Chairside	Antibiotic Susceptibility	Time required	Specific	Quantitative	Detects dead cells	Advantages	Disadvantages
Direct Mircoscopy	Phase Contrast Darkfield	?	yes	no	minutes	no	yes	yes	Results are immediately available	Requires immediate examination of samples
Culture	Selective media	10^4-10^5	no	yes	1-3 weeks	yes	yes	no	Broadest spectrum	Multiple sources of error
	Non-selective	10^3	no							Cannot detect spirochaetes
Immunologic	Immuno-fluorescence	10^3-10^4	no	no	minutes to hours	yes	yes	yes	Analysis of samples can be delayed	Cross-reactivity with polyclonal antibodies
	Particle fluorescence	10^3-10^4	no				yes			Cannot detect unidentified species
	Cytofluoro-graphy	minimal	no				yes			
	ELISA	10^3-10^5	no				no			
	Membrane assay	?	yes				no			
	Latex Agglutination	10^6	no				no			
	Slot immunoblot	$2x10^4$	no				yes			
DNA-based technology	Whole genomic	10^3-10^6	no	no	30 minutes to 20 hours	yes	semi	yes	Analysis of samples can be delayed	Radioactive probes need special handling
	Cloned/Oligo-nucleotide probes	$<10^4$-10^7	yes	yes			semi			Cannot detect unidentified species

	PCR	10^2	no	yes			no			
	Colony lift + DNA Probes	see culture	no	no			yes			
	Checkerboard	10^3-10^6	no	yes			semi			
Enzymatic	BANA	10^5-10^6	yes	no	15 minutes	no	no	?	Can detect	Supragingival species may give a positive reaction
	SK-013	$6x10^6$	yes						Pg/Bf & Td	Other species may produce the enzymes

b) Compare antibody titres, avidity and serostatus in AP smokers and non-smokers.

1.9 Layout of thesis

After the next chapter, Methodology, the results and discussion sections of this thesis have been divided in four further chapters: 3) Comparison of AP and GEOP patients, 4) Comparison of PCR and Checkerboard, 5) The humoral immune response and periodontal therapy, and 6) Effect of smoking on periodontal therapy. In each of these chapters the relevant results are presented together with a discussion of these results. This arrangement is intended to clarify the results and discussion sections in this thesis for the reader, rather just grouping everything together into two chapters, which was confusing. These chapters are followed by a brief final chapter (7) summing up the findings in this thesis.

CHAPTER 2

METHODOLOGY

This chapter describes the clinical and laboratory methods used in this thesis, and is split into two parts. The first part describes the selection of patients and sites, study outline, clinical indices and sampling techniques used. The second part describes the laboratory techniques used to prepare and analyse the clinical samples. Lastly the methods of statistical analysis are detailed.

2.1 Clinical methodology

2.1.1 Subject selection

Prior to the commencement of these studies, ethical approval was obtained from the Glasgow Dental Hospital Ethics Committee. Subjects participating in these studies were informed of the protocol and consent was obtained. All patients taking part in the investigation were free to withdraw from the study at any time.

Fifty seven patients with untreated periodontal disease were recruited from new referrals to Glasgow Dental Hospital and School between January 1996 and July 1998. Each patient had at least two non-adjacent sites per quadrant with pocket depths of 5 millimetres or over, with no history of systemic disease or antibiotic therapy in the three month period prior to recruitment. Thirty three patients were aged 35 and over (mean age 47 years), and were designated adult periodontitis (AP) patients. Twenty four patients aged between 24 and 35 (mean age 33) at the time of diagnosis were designated generalised early-onset periodontitis (GEOP), according to the criteria of Hart et al., (1991). This states that there should be a generalised pattern of severe periodontal destruction with clinical attachment loss of at least 5mm on 8 or more teeth, 3 of which were not first

molars and at least one of which was a permanent molar. The subject should be less than 35 years of age.

2.1.2 Site selection

Site selection was carried out at the screening visit, after full mouth periodontal pocket charting was performed using a PCP-12 periodontal probe (Ash, Densply, UK). In each patient, four non-adjacent sites with pocket depths equal to 5 mm or over were selected for sampling purposes. These sites, where possible, were in different quadrants and in no cases were sites with furcation involvement included. In order to facilitate sample collection and increase the accuracy of the measurements, buccal and anterior sites were preferred to lingual and posterior sites. Non-adjacent sites were used so as to reduce the possibility of contamination and ensure independence of the gingival crevicular fluid and plaque samples.

2.1.3 Clinical design

The patients in this study were seen at the following visits: 1) screening visit, 2) baseline measurements, 3) four sessions for quadrant root planing under local anaesthetic, 4) reassessment, and 5) post-treatment sampling visit (Figure 2.1).

At the screening visit, all study subjects signed an informed consent form. The medical history was checked and a full periodontal examination with pocket charting carried out. The patients were asked to reattend at the first available appointment for baseline measurements.

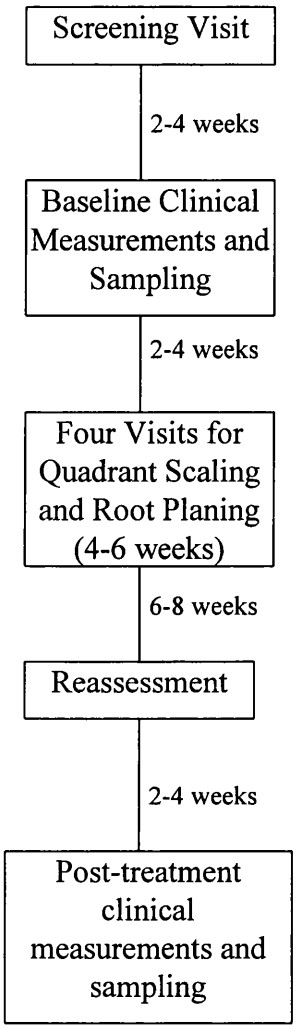


Figure 2.1 Study Outline: Details of patient visits and treatment undertaken at each visit with time intervals between stages and duration of SRP

At the baseline visit the following were sampled or recorded from the study sites in the order listed:

1) Modified Ginigval Index (MGI) (Lobene et al., 1986) and Plaque Index (PLI) (Silness & Löe, 1964) from all sites.

2) Gingival crevicular fluid (GCF) samples were taken from all sites, the volume recorded using the Periotron 6000 and stored frozen at -20°C in separate sterile 1.5ml micro-centrifuge tubes until analysis.

3) The first set of pocket depth (PD) measurements using an electronic pressure sensitive probe from all sites.

4) Bleeding on probing (BOP) and suppuration (Supp) from all sites.

5) After changing the patient's position the second set of pocket depth measurements were recorded from all sites.

6) After changing the probe tip, the first set of attachment level measurements were recorded from all sites.

7) Again after changing the patient's position the second set of attachment level measurements were taken from all sites.

8) Following probing a sub-gingival plaque sample was taken from each site using a different sterile scaling instrument, and transferred to 500µl of TE Buffer in a sterile 1.5ml micro-centrifuge tube for subsequent PCR analysis.

9) Lastly, 21ml of venous blood was drawn from the Anterior Cubital vein of either arm.

Following baseline measurements each subject was seen for four visits where oral hygiene instruction was given according to individual needs and each

quadrant in the patient's mouth scaled and root planed (SRP) under local anaesthetic.

After a period of eight weeks to allow for healing, the subjects were re-examined and pocket charted to determine the success of the therapy. At the next visit the clinical measurements and samples were collected in the same order as before.

2.1.4 Clinical measurements

Two examiners carried out the clinical measurements and treatment (IBD for all the AP patients, and PJH for all the GEOP patients). The following parameters were recorded at each site in each patient, and a total of twice per site.

2.1.4.1 Modified Gingival Index

The Modified Gingival Index (MGI) (Lobene et al., 1986) was used to assess gingival inflammation and is described below:

0	Absence of inflammation
1	Mild inflammation; slight change in colour, little change in texture but not the entire marginal or papillary gingival unit.
2	Mild inflammation; criteria as above but involving the entire marginal or papillary gingival unit.
3	Moderate inflammation; glazing, redness, oedema and/or hypertrophy of the marginal gingival unit.

4 Severe inflammation; marked redness, oedema and/or hypertrophy of the marginal or papillary gingival unit, spontaneous bleeding, congestion or ulceration.

The MGI was preferred to the more commonly used Gingival index (Löe & Silness, 1963) because it provides greater sensitivity to the earliest changes of gingivitis as the lower part of the scale is expanded resulting in a scoring system of 0-4. Since bleeding on probing is not a criterion in the MGI, it is a completely non-invasive procedure. A non-invasive method was essential in this study since GCF samples were taken after MGI scoring, and any procedures that might irritate the gingival tissue, and thereby alter the GCF constituent profile, had to be avoided.

2.1.4.2 Plaque Index

The Plaque index (PLI) of Silness & Löe (1964) was used for recording plaque accumulation. The scoring system is as follows:

0 No plaque in the gingival area

1 A film of plaque adhering to the free gingival margin and adjacent surfaces of the tooth. The plaque may only be noticed by running a probe across the tooth surface.

2 A moderate accumulation of soft tissue deposits within the gingival crevice, on the gingival margin and/or adjacent tooth surface.

3 An abundance of soft matter within the gingival crevice and/or on the gingival margin and adjacent tooth surface.

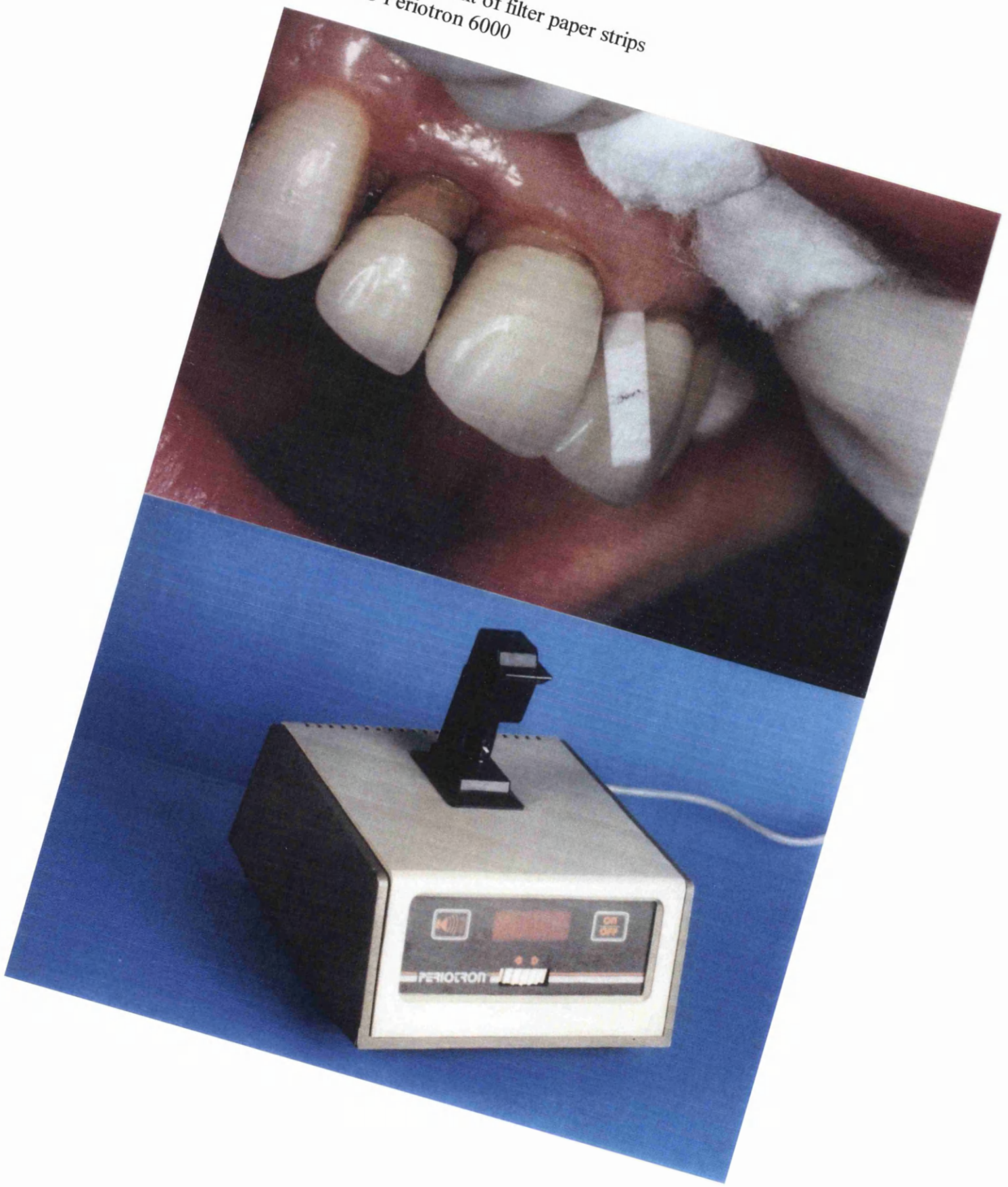
2.1.4.3 Gingival crevicular fluid collection

Sample collection was performed using filter paper strips and the fluid volume on the strip was measured immediately after sampling with a Periotron 6000 (IPE Interstate, Amytiville, NY).

In the clinic, GCF was sampled after MGI and PLI scores were taken but before any other clinical recordings which could cause irritation of the tissue and serum contamination of the sample.

The site to be sampled was isolated with cotton wool rolls and supra-gingival plaque was carefully removed. The region was dried with a gentle stream of air and GCF was collected with a Whatman grade 4 paper strip (Whatman International Ltd., Maidstone, Kent, UK) (2x13 mm) (Griffiths et al., 1988) inserted into the crevice until mild resistance was felt and left in place for thirty seconds (Figure 2.2). Strips visually contaminated with saliva or blood were discarded. The sampling method is reliable and causes no significant disturbance of the gingival blood vessels (Gustafsson et al., 1992). The fluid volume on the strip was measured immediately after sampling with a Periotron 6000 (IPE Interstate, Amytiville, NY) (Figure 2.3). The jaws of the Periotron were wiped with absolute ethanol and then dried between readings. The Periotron 6000 was reset to zero between measurements (Chapple et al., 1995).

Figure 2.2 Placement of filter paper strips
Figure 2.3 Periotron 6000

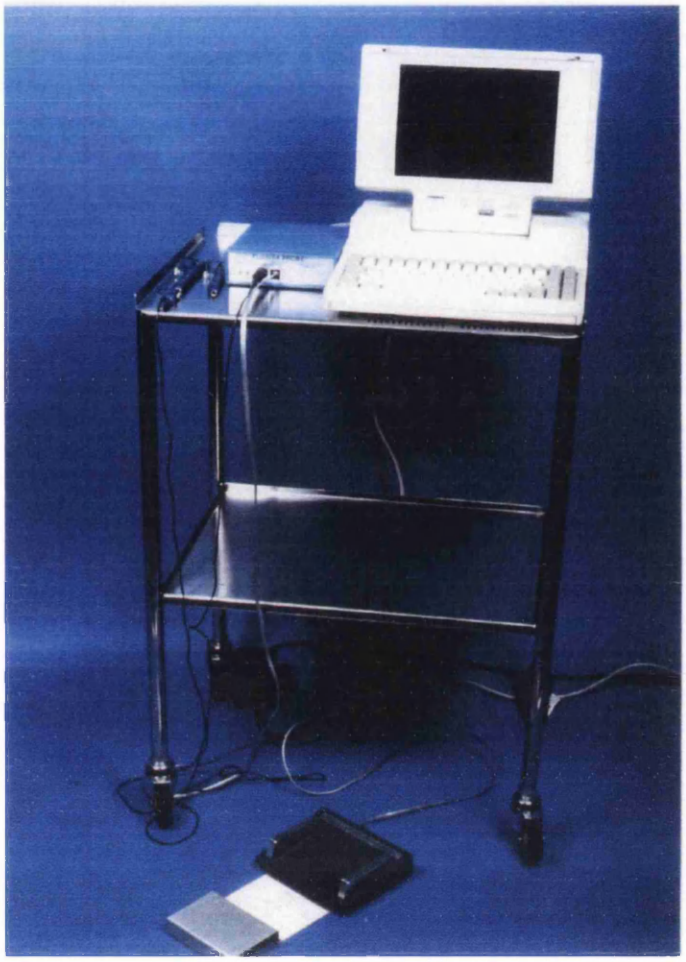


The strips were then placed into individual sterile micro-centrifuge tubes and stored at -20°C until elution. Subsequently the strips were eluted into 1ml of incubation buffer at room temperature using a rotary mixer for one hour. The strips were then discarded and the elutant aliquoted into sterile 0.5ml micro-centrifuge tubes and stored at -20°C. The aliquots were subsequently analysed for IgG titres to *P. gingivalis*, *P. intermedia*, *B. forsythus*, *A. actinomycetemcomitans*, and *T. denticola.*

2.1.4.4 Probing depth and attachment level assessments

In this study pocket depth (PD) and attachment level (AL) were recorded to the nearest 0.2 mm using the Florida probe (Florida Probe Corporation, Florida, USA) (Gibbs et al., 1988). The Florida probe is an electronic pressure sensitive probe with a constant force of 20 grams. The system consists of pocket depth and attachment level handpieces, a foot switch, a computer interface and a desktop computer (Figure 2.4). When the foot switch is pressed, the measurements of PD and AL are made electronically, using the pocket depth or attachment level handpieces. Measurements were recorded by an assistant from the computer screen, so that the operator was blind to the measurement. The probe tip has a diameter of 0.4 mm, with no visible graduation along its length and it reciprocates through a sleeve. The edge of the sleeve is the reference from which measurements are recorded.

Figure 2.4 Florida probe monitor, computer interface and foot switch.



PD was assessed by using the point at which the probe sleeve was brought into contact with the gingival margin. Care was taken to ensure that the probe tip was held parallel to the long axis to the tooth (Figure 2.5).

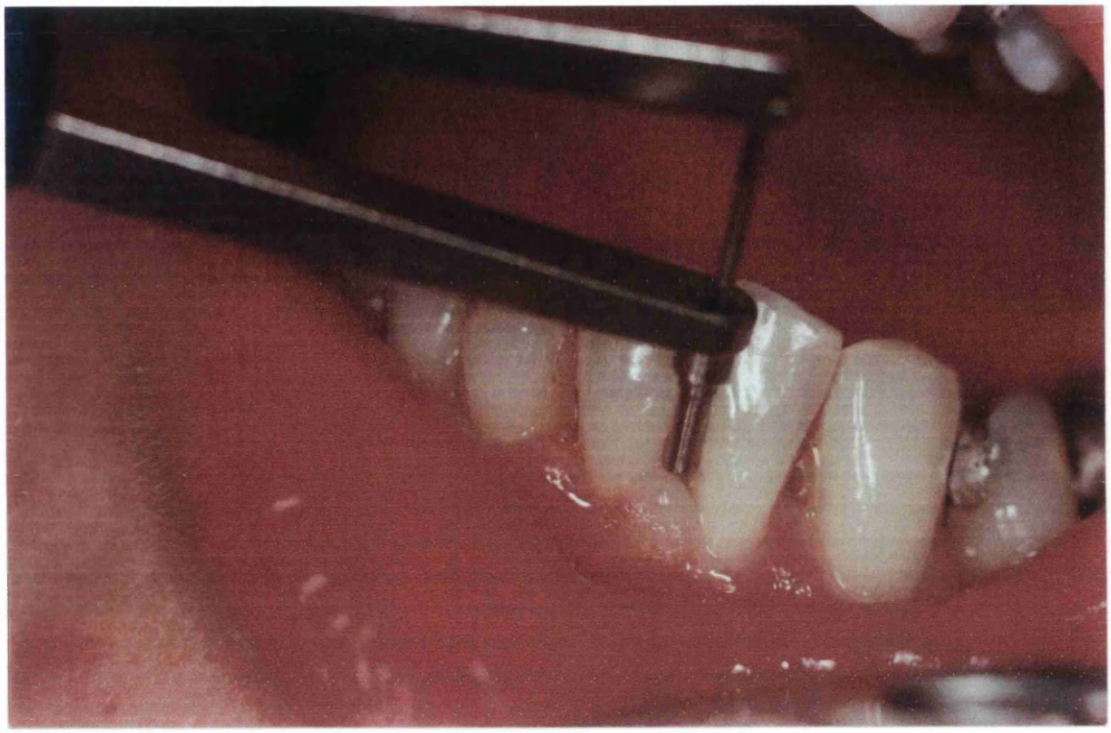
A fixed reference point is required for attachment level measurements and, as identification of the cemento-enamel junction (CEJ) is often complicated by its sub-gingival location or the presence of restorations, in this study the occlusal plane was used. Near the end of the shank of the attachment level handpiece is a disc which can be placed on the occlusal surfaces of the associated teeth and provides a fixed reference point. The attachment level was recorded relative to the point at which the disc was seated on the teeth. Once the disc was seated on the tooth, care was taken to ensure that the probe tip was parallel to the long axis of the tooth.

PD and AL measurements were taken in duplicate to improve accuracy. If the first two measurements were more than 1 mm in disagreement then two more were taken and the middle two measurements recorded. After the first set of measurements was obtained, the patient's position was changed before the next set. After each measurement the probe tip was cleaned with isopropyl alcohol.

2.1.4.5 Bleeding on probing

Bleeding on probing was visually assessed within 30s of the first set of pocket depth measurements by the Florida probe. It was scored as present or absent.

Figure 2.5 Florida pocket depth probe in use



2.1.4.6 Suppuration

Suppuration was visually assessed after BOP by applying gentle pressure from the end of a periodontal probe in an apical-coronal direction. It was recorded as present or absent.

2.1.4.7 Subgingival plaque sampling

A subgingival plaque sample was taken from each site after pocket depth and attachment level measurements were performed in order to avoid distortion of the pocket, and so alter the pocket depth. Each plaque sample was taken using a sterile scaling instrument, usually a periodontal hoe, using a single stroke. The sample was immediately placed in 500µl of TE Buffer (10mM Tris HCl pH 7.6, 1mM EDTA pH 8.0) in a 1.5ml micro-centrifuge tube and stored at -20°C until processing.

2.1.4.8 Serum collection

Twenty one millilitres of venous blood were collected from the anterior cubital region using butterfly needles and three 7ml red-capped Vacutainer tubes (Becton Dickinson Vacutainer Systems Europe, Meylan, France). The blood was allowed to clot overnight and then the serum removed and aliquoted for ELISA analysis.

2.2 Experimental methodology

2.2.1 Experimental materials

2.2.1.1 Whatman grade 4 paper strips

Whatman grade 4 paper strips (Whatman International Ltd., Maidstone, Kent) were cut manually to a standard size, 13 mm in length and 2 mm wide, using a steel ruler and a scalpel. A line was drawn on each strip at 8 mm, indicating the length of paper strip to be inserted between the Periotron jaws and the part of the strip to be used for GCF sampling. Rubber gloves were worn during their preparation in order to avoid contamination of the strips by substances from the operator's hands. The strips were then placed in glass universal bottles for autoclaving and storage.

2.2.1.2 ELISA buffers and reagents

The buffers used were as follows:

1) Coating buffer (CB): 1.59g Na_2CO_3, 2.93g $NaHCO_3$ was dissolved in 800ml distilled H_2O. The pH was adjusted to 9.6 at just under 1 litre, by adding 1M HCl up to 1 litre in a volumetric flask. It was stored in a sterilised bottle at 4°C for a maximum of 1 week.

2) Incubation buffer (IB): 8g NaCl, 0.2g KH_2PO_4, 1.44g Na_2HPO_4. $2H_2O$, 0.2g KCl, 0.5g Tween 20 (SIGMA chemical company Ltd., Poole, Dorset) dissolved in 800 millilitres of distilled H_2O, and made up to 1 litre with the addition of 1g of lyophilised bovine serum albumin (BSA) (SIGMA, St. Louis, USA). This was layered on the surface until dissolved, then mixed, and stored at 4°C for a maximum of 1 week (pH 7.4).

3) Wash buffer (PBST): This was prepared at 10 times the concentration of incubation buffer (nil BSA) and stored at room temperature. It was diluted 1/10 immediately before use.

2.2.1.3 Checkerboard buffers and reagents

The buffers and reagents used were as follows:

1) PO_4 buffer (4 litres x 4): 11.36g 20 mm Na_2HPO_4, 1.49g 1 mm EDTA, and 1% SDS were dissolved in approximately 3 litres distilled H_2O and then the volume made up to 4 litres with distilled H_2O. After autoclaving it was stored at room temperature for a maximum of 3 to 4 days.

2) Maleic acid (4 litres): 46.43g maleic acid, 701.3g NaCl were dissolved in 2.5 litres of distilled H_2O. The volume was made up to 4 litres with distilled H_2O and the pH adjusted to 8.0 at room temperature using about 32g NaOH pellets. To this 12ml Tween 20 was added and the solution autoclaved and stored at room temperature for a maximum of a week.

3) Blocking solution (500ml): 25ml stock block (solution 12) was added to 475ml maleic acid.

4) Antibody solution (two membranes): 6µl antidigoxigenin (Boehringer Mannheim GmbH, Mannheim, Germany) was added to 100ml blocking solution.

5) Formamide (800ml): 40g AG 501-x8 resin/800ml of formamide beads were deionised by stirring for 30 minutes and filtering. After aliquoting into 5ml samples, the tubes were capped with red caps and stored at -20°C until use.

6) Denhardt's solution (50x) (500ml): 1% Ficoll and 1% polyvinylpyrrolidone were dissolved in distilled H_2O. 1% bovine serum albumin fraction V was added

and the solution aliquoted into 5ml samples. After capping with blue lids the solution was stored at -20°C until use.

7) Denatured sheared herring sperm DNA (10mg/ml): 250mg herring sperm was dissolved in 25ml distilled H_2O and allowed to mix overnight at 4°C. The solution was brought to room temperature and forced through a 50ml syringe and needle to shear the DNA. This was repeated four times. The DNA was transferred to a glass tube and boiled for 10 minutes. After cooling on ice the shearing and boiling process was repeated. The DNA was stored in 1.5ml micro-centrifuge tubes at -20°C until use.

8) 20xSSC (2 litres): 350.64g NaCl and 176.5g Na Citrate were dissolved in 2 litres of distilled H_2O.

9) 2xSSC (500ml): 50ml 20xSSC was added to 450 ml distilled H_2O, autoclaved and used within a week.

10) Buffer 1 (1 litre): 11.6g maleic acid, 8.7g NaCl and 7.5g NaOH were added to 1 litre distilled H_2O, autoclaved and stored at room temperature.

11) Buffer 3 (2 litres): 31.4g Tris HCl and 11.6g NaCl were dissolved in 1 litre of distilled H_2O. The pH was adjusted to 9.5 using about 7g NaOH. In another flask, 20.2g $MgCl_2.6H_2O$ was dissolved in 1 litre distilled H_2O. The solutions were autoclaved separately and mixed together afterwards.

12) Stock block: 10% block (casein) was added to buffer 1. The solution was placed in the microwave to dissolve the casein, autoclaved and stored at 4°C until use.

13) Pre-hybridisation PO_4 & SSC (500ml): To about 400ml distilled H_2O, 88g NaCl, Na Citrate and 6.9g NaH_2PO_4 were added. The solution was topped up to

500ml and the pH was adjusted to 6.5. The solution was autoclaved, and stored at 4°C until use.

14) Hybridisation PO_4 & SSC (300ml): 30.7g NaCl, 16.1g Na Citrate, and 2.01g NaH_2PO_4 were dissolved in 300ml distilled H_2O. The pH was adjusted to 6.5 and the solution autoclaved and stored at 4°C until use.

15) Hybridisation solution:

For 20ml: 9ml formamide (2 tubes minus 1ml), 0.4ml Denhardt's, 2ml stock block, 8.2ml hybridisation PO_4 & SSC, 2g Dextrane sulphate and 0.4ml herring sperm. Once the herring sperm was dissolved in the hybridisation PO_4 & SSC, the other reagents were added and thoroughly mixed together.

For 1ml: 450µl formamide, 20µl Denhardt's, 100µl stock block, 410µl hybridisation PO_4 & SSC. 0.1g Dextrane sulphate and 20µl herring sperm. Mixing was as above.

2.2.1.4 Preparation of PCR primers

The primers used for the PCR analyses are shown in (table 2.1) with the size of the amplification product, target and references. The *P. gingivalis* primers targeted the *fimA*, fimbrillin gene, which all *P. gingivalis* strains possess and is specific for *P. gingivalis*. The sequences for the primers were (from 5' to 3') ATAATGGAGAACAGCAGGGAA and TCTTGCCAACCAGTTCCATTGC and the expected product size 131 base pairs (bp). The primer sequences for *P. intermedia* targeted the 16S rRNA and were specific for *P. intermedia.* These sequences were developed by Riggio et al. (1998) and were reported not to cross

Table 2.1 Sequences, expected product size, target and references for PCR primers

Primer Pairs (5'-3')	Amplicon length (bp)	Target	Reference
P.gingivalis		Fimbrillin gene	Watanabe &
ATAATGGAGAACAGCAGGGAA			Frommel (1993)
TCTTGCCAACCAGTTCCATTGC	131		
P.intermedia		16S RNA	Riggio et al., (1998)
CCTAATACCCGATGTTGTCCACA			
AAGGAGTCAACATCTCTGTATCC	855		
A.actinomycetemcomitans		Leukotoxin gene	Goncharoff et al.,
GGAATTCCTAGGTATTGCGAAACAAT			(1993)
GGAATTCCTGAAATTAAGCTGG	262		
B.forsythus		16S RNA	Slots et al., (1995)
GCGTATGTAACCTGCCCGCA			
TGCTTCAGTGTCAGTTATACCT	641		
T.denticola		16S RNA	Slots et al., (1995)
TAATACCGAATGTGCTCATTTACAT			
TCAAAGAAGCATTCCCTCTTCTTCTTA	316		

react with the closely related *P. nigrescens*. The primer sequences were (from 5' to 3') CCTAATACCCGATGTTGTCCACA and AAGGAGTCAACATCTCTGTATCC and the expected product size 855bp. The primer sequences for *A. actinomycetemcomitans* targeted the leukotoxin gene which all *A. actinomycetemcomitans* strains possess and had been shown not to cross react with related species (Goncharoff et al., 1993). The sequences were (from 5' to 3') GGAATTCCTAGGTATTGCGAAACAAT and GGAATTCCTGAAATTAAGCTGG and the expected PCR product size was 262bp. The sequences for *B. forsythus* and *T. denticola* were developed by Slots et al., (1995) and were specific for *B. forsythus* and *T. denticola* only. Both targeted the 16S rRNA region. The *B. forsythus* primers were (from 5' to 3') GCGTATGTAACCTGCCCGCA and TGCTTCAGTGTCAGTTATACCT, and the expected product 641bp. The *T. denticola* primers were (from 5' to 3') TAATACCGAATGTGCTCATTTACAT and TCAAAGAAGCATTCCCTCTTCTTCTTA and the expected product 316bp. All primers were made by Cruachem Ltd. (Glasgow, Scotland). They were re-suspended with sterile, distilled, de-ionised water and tested on whole genomic DNA for accuracy and specificity before use on plaque samples.

2.2.1.5 Preparation of Checkerboard probes

Digoxigenin-labeled, whole genomic probes were prepared by using the High Prime kit (Boehringer Mannheim, Mannheim, Germany) and cultured organisms (Papapanou et al., 1997a). Genomic DNA was extracted from *A. actinomycetemcomitans* FDC Y4, *P. gingivalis* FDC 381, *P. intermedia* ATCC

25611, *B. forsythus* ATCC 43037 and *T. denticola.* The Checkerboard was performed in a dedicated laboratory in the Oral Microbiology department, Faculty of Odontology, University of Gothenburg, Sweden and all probes had been tested for accuracy and specificity previously.

2.2.2 Experimental techniques

2.2.2.1 Calibration of the Periotron 6000 and determination of GCF volume

In order to transform the Periotron digital readings for each paper strip into volumes, and also to verify the accuracy of the instrument, a calibration curve was constructed. First a blank paper strip was placed between the jaws of the machine and the instrument zeroed. Known volumes of a fifty/fifty PBS/sera mixture were pipetted onto the paper strips using a Hamilton micro-syringe at a range of volumes from 0.05-1 μl and the Periotron readings recorded. Each measurement was performed three times and the digital display reset to zero after each sample (Chapple et al., 1995). The mean value for each volume was used to construct a calibration curve, the slope and intercept were used to determine the volumes of GCF collected. The curve was analysed in two separate lines (fitted by regression method) as shown in Fig 2.6. The upper portion included volumes of 0.4 to 1.2μl, and the lower portion volumes 0.05 to 0.4μl. The calibration was performed each day samples were taken, in order to reduce daily variability due to differences in factors such as temperature and humidity.

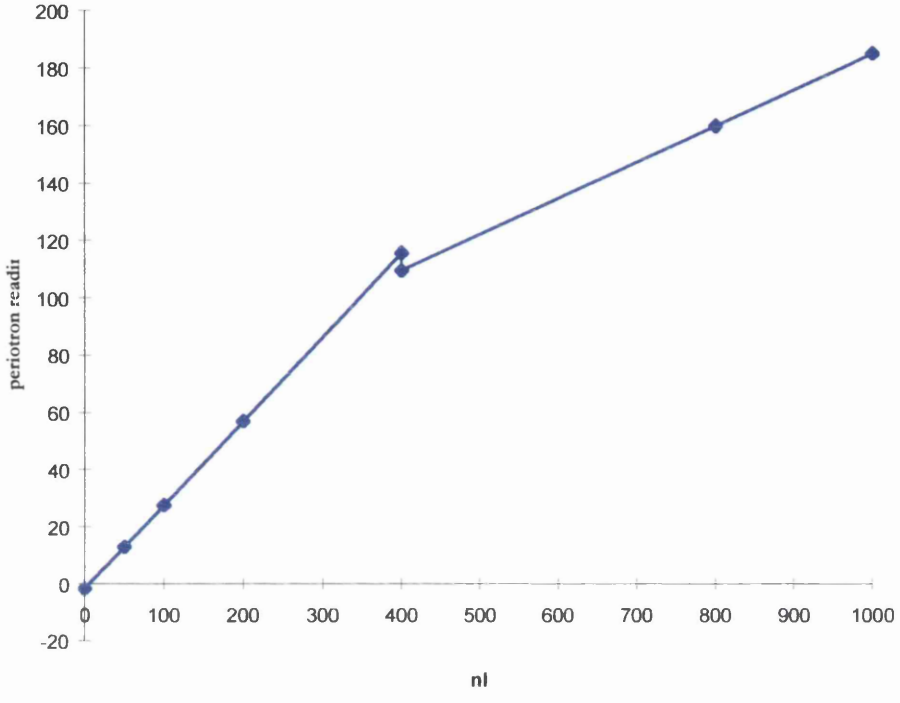


Figure 2.6 GCF calibration curve. There are two lines for the calculation of GCF volume, meeting at 400nl. The first line is for 0-400nl and has the equation, in the form $y = mx + c$, $y = 3.4x + 6.41$, and the second line for 400-1000nl, $y = 7.93x - 469.18$, where y is the Periotron reading, m the gradient, x the volume in nl, and c the intercept.

2.2.2.2 Ginigval crevicular fluid elution

GCF samples were eluted into 1ml of incubation buffer for 1 hour at room temperature using a rotary mixer. The strips were then discarded and the elutes aliquoted into 125µl samples and stored at -20°C until analysis.

2.2.2.3 Preparation of plaque samples

The plaque samples were stored at -20°C until analysis. Once thawed the plaque samples were vortex mixed for 30 seconds before being divided in two, as PCR and Checkerboard require different methods of sample preparation.

For PCR, 26µl of 10 times lysis buffer (100mM Tris-HCl pH 8.0, 10mM ethylenediamine tetra-acetic acid, 10% Triton X-100) was added to 250µl of the sample which was then boiled for 5 minutes. 10µl of this lysate was used in each PCR reaction. The samples were stored frozen in numbered, but unnamed microcentrifuge tubes so that the analyses could be performed blind.

For Checkerboard analysis the samples were prepared as follows. 167µl of 0.5M NaOH was added to the 250µl plaque sample aliquot and boiled for 10 minutes to denature the DNA. 0.86ml of 7.5M ammonium acetate was added to each tube to neutralise the reaction. A 15 x 15cm nylon membrane (Boehringer Mannheim GmbH, Mannheim, Germany) was mounted on 10 15 x 15cm Whatmann grade 3MM filter paper squares (Whatman International, Maidstone, Kent) in the bottom half of a Minislot apparatus (Immunetics, Cambridge, MA, USA) and the top screwed tightly in place. Each plaque sample was pipetted

into a separate lane on the top of the apparatus with 20 samples per membrane. The aliquots were allowed to soak in for 10-15 minutes, until the lanes were dry, and then aspirated through the membrane by vacuum. At the bottom of each membrane samples containing whole genomic DNA from 10^5 and 10^6 mixed bacterial cells were placed to act as controls. The controls consisted of a mixture of the five test organisms. The DNA deposited on the membrane surface was fixed by exposure to ultraviolet light for 1 minute each side and then by baking for twenty minutes at 120°C. The membranes were stored at 4°C until hybridisation.

2.2.2.4 Preparation of serum samples

Blood samples were allowed to clot overnight at 4°C, centrifuged at 2000rpm for 10 minutes and the sera removed, aliquoted into 125µl samples and stored frozen at -20°C until analysis.

2.2.3 Polymerase chain reaction

2.2.3.1 Polymerase chain reaction cycle

PCR amplification was carried out in a reaction volume of 100µl consisting of 10µl sample lysate and 90µl of reaction mixture containing 1xPCR buffer (10mM Tris-HCl pH8.8, 1.5mM $MgCl_2$, 50mM KCl, 0.1%Triton X-100), 2 units of Dynazyme DNA polymerase (Flowgen, Lichfield, England, UK), 0.2 mM dNTPs (dATP, dCTP, dGTP, dTTP) and 50 pmol of each primer. The primers were separated from the other components of the reaction mixture by a layer of wax ("hot start" PCR) preventing the reaction from starting until the wax had

melted upon commencement of PCR cycling. PCR cycling was carried out in an OmniGene thermal cycler (Hybaid, Teddington, England, UK). The cycling conditions for *A. actinomycetemcomitans* and *P. intermedia* comprised an initial denaturation for 5 minutes at 95°C, 35 amplification cycles of denaturation at 95°C for 1 minute, annealing of primers at 55°C for 1 minute and primer extension at 72°C for 1.5 minutes, followed by a final extension step at 72°C for 10 minutes. The cycling conditions for the other organisms were the same except that in the amplification cycles the primer extension step lasted for 1 minute. The reaction products were either stored at -20°C or analysed immediately. Negative and positive controls were included in each batch of samples being analysed by PCR. The negative control was a 90µl reaction mixture with the sample replaced by 10µl of sterile water. The positive control contained 100ng of genomic DNA from the relevant organism in 90µl of reaction mixture, with sterile water added to bring the volume to 100µl. The genomic DNA for *T. denticola* was kindly supplied by Dr. C. Wyss, Zurich, Switzerland.

2.2.3.2 Analysis of PCR products

20µl of each reaction product was fractionated on a 2% agarose gel containing ethidium bromide (0.5µg/ml), using a 100bp DNA ladder (Pharmacia Biotech, St. Albans, UK) as a size marker, and visualised and photographed using an ImageMaster video documentation system (Pharmacia Biotech).

2.2.4 Checkerboard DNA-DNA hybridisation

After halving the membranes containing the plaque samples, so that two could be tested at the same time, they were soaked for 5-10 minutes in 2xSSC, and pre-hybridised for 2 hours at 42°C with 50ml pre-hybridisation solution (6 tubes Formamide, 1 tube Denhardt's, 5ml stock block, 12.5ml pre-hybridisation PO_4 and SSC, and 2.5ml denatured herring sperm) in a plastic bag on a shaker in a water bath. During pre-hybridisation, 10ml of hybridisation solution was prepared (4.5ml Formamide, 0.2ml Denhardt's, 1ml stock block, 4.1ml hybridisation PO_4 and SSC, 1g Dextrane sulphate, and 0.2ml herring sperm). Varying amounts of each probe were added to 160µl of hybridisation solution, so that the final probe concentration was 20ng/ml, (Table 2.2) (8µl of *P. gingivalis* probe was added, 6µl *P. intermedia* and *A. actinomycetemcomitans* probes, 22µl of *B. forsythus* probe, and 15µl of *T. denticola* probe). The probe was boiled for 5 minutes to denature it and then stored on ice until use. The membranes were removed from their bags and the pre-hybridisation solution kept. The bottom half of a Miniblotter device was wrapped in cling-film and the membrane placed face up on it. The top half was screwed tightly in place, and the excess prehybridisation fluid aspirated with a vacuum. The probes were carefully pipetted into their lanes avoiding air bubbles, and the empty lanes filled using prehybridisation solution. The whole apparatus was wrapped in clingfilm and placed in a shaking water bath set at 47°C. The probes were allowed to hybridise overnight at a temperature of 42°C. A thermometer was placed on top of the apparatus so that the temperature could be checked.

Table 2.2 Checkerboard hybridisation set up: Lanes for the probes, probe volumes and hybridisation solution volumes

Species	Lane	Probe Vol.	Hyb Sol Vol
P. gingivalis	2	8	152
P. intermedia	5	6	154
B. forsythus	8	22	138
A. actino	11	6	154
T. denticola	14	18	142
P. gingivalis	28	8	152
P. intermedia	31	6	154
B. forsythus	34	22	138
A. actino	37	6	154
T. denticola	40	18	114

The following day, PO_4 buffer was heated to 70°C and the apparatus removed from the water bath whilst the buffer was warming. The probes were aspirated with a vacuum, and the membranes clipped to a frame. Once the buffer had heated up it was poured into a plastic tank and the frame fixed within. The membranes were washed for twenty minutes, followed by a wash for a further twenty minutes. Following a rinse with maleic acid, the membranes were soaked in maleic acid for 5 minutes by placing in plastic boxes on a rotator. The membranes were then placed in blocking solution, again in plastic boxes, for 60 minutes to block non-specific binding sites, and then sealed in a plastic bag with the antibody solution for 30 minutes again on the rotator. Following soaking with maleic acid, the membranes were washed four times for ten minutes with maleic acid in plastic boxes, and then immersed in Buffer 3 for five minutes on the rotator in plastic boxes. Alkaline phosphate substrate solution was made up by adding 50µl CSPD to 4.95ml Buffer 3 for each membrane. Without allowing the membranes to dry out, they were placed on Whatman paper 3MM to remove excess liquid, and then placed within light tight metal trays. CSPD solution was pipetted over the membranes with the excess re-pipetted over the membranes three to four times to ensure that the surface of the membrane was completely covered. The trays were then incubated in the dark for five minutes at room temperature. To remove excess solution the membranes were again placed on Whatman paper 3MM, but not allowed to dry out. The membranes were placed in plastic bags, sealed without air bubbles and allowed to incubate in the dark at 37°C for 20 minutes. They were then placed in the Lumi-Imager image capture system (Boehringer Mannheim GmbH, Mannheim, Germany) for 20 minutes,

which detected and recorded the chemiluminescent reactions on the membrane. The captured image was displayed on the screen of a linked desk-top computer (Hewlett Packard), and printed or saved to disc. Finished membranes were allowed to dry out overnight and then kept at 4°C.

Samples were scored positive and absent for each organism by comparison with the standards on each membrane both by eye and by the software on the desktop computer. No attempt was made to semi-quantitate the results.

2.2.5 ELISA

Specific antibody titres were measured using enzyme-linked immunosorbent assay (ELISA) by the method of Ebersole et al., (1980).

2.2.5.1 Preparation of microorganisms and coating of plates

A. actinomycetemcomitans, *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola* were prepared for coating the ELISA plates as follows. *A. actinomycetemcomitans* strain Y4 was grown on blood agar plates and harvested after 24 hours. *P. gingivalis* NCTC 11834, *P. intermedia* ATCC 25611, and *B. forsythus* ATCC 43037were grown on fastidious anaerobe agar and harvested after seven days. The organisms were harvested with swabs and dispersed into PBS containing 0.1mM disodium EDTA (PBSE). They were then washed once in PBSE and fixed overnight in 10% formal saline. *T. denticola* ATCC 35405 cells were grown, fixed and kindly donated by Dr C. Wyss, Zurich, Switzerland. After washing twice with PBSE, the fixed organisms were re-suspended in

coating buffer (CB). The organisms were further washed once with coating buffer, and were used to coat the plates at the following OD_{600}; *A. actinomycetemcomitans* 0.02, *P. gingivalis* 0.05, *P. intermedia* 0.05, *B. forsythus* 0.02, and *T. denticola* 0.001, as determined as optimal by previous workers (Mooney et al., 1994).

2.2.5.2 Analysis of sera samples

The plates were pre-washed three times with coating buffer and coated overnight with the whole cells at 4^0C using 100µl per well. Immulon 1B plates (Dynex Technologies, VA, USA) were used because of their low protein-binding characteristics and low non-specific background. Control wells for each aspect of the ELISA process were arranged in the wells around the outside of the plates.

The ELISA plates were removed from the refrigerator and washed four times, then four more, four times again and lastly once more with wash buffer (4x4x4x1). The non-specific binding sites were blocked with 100µl per well of incubation buffer (IB) containing 5% skimmed milk powder (Marvel, Premier Beverages, Stafford, UK) for 1 hour at 37°C. The plates were then washed twice and once again (2x1) before addition of the sera.

Human sera collected from the study patients were serially diluted from 1/100 to 1/25600 using IB and control sera diluted to 1/1000. 50µl of sera were added to each well and incubated at 37°C for one and a half hours. Each serum was tested in duplicate and the sera samples from the same patient at different time points

were tested on the same plate. Following incubation with the sera the plates were washed 4x4x4x1. Afterward the plates were incubated at 37°C for 60 minutes with Biotin-Goat-Anti-Human IgG (Sigma, St. Louis, MI, USA) at 1/2000 dilution in IB. The plates were then washed 4x4x4x1, and the plates either incubated for 60 minutes at 37°C or overnight at 4°C with 1/2000 dilution of extravidin peroxidase. After washing 4x4x4x1, the reactions were visualised using 100µl per well of TMB Peroxidase substrate (Kirkegaard & Perry Laboratories, Maryland, USA) and stopped after 5-10 minutes, depending on the speed of the reaction, using 50µl per well of 0.12M HCl.

The reactions were read using a Dynex Technologies MRX II plate reader at 450nm with a reference at 630nm and the results printed out. The duplicate results were averaged and the final titre expressed as ELISA units using the method of Gmür et al., (1986). The results were calculated with a regression line and derived equation from serial dilutions of a reference serum (Mooney et al., 1993).

2.2.5.3 Analysis of GCF samples

The same ELISA methodology that was used for analysis of the sera samples was used for analysis of the GCF samples. 50µl of GCF aliquot was used per well instead of serum and it was not serially diluted. The samples were also analysed in duplicate. All the GCF samples from the same patient were tested together on the same plate. The results were expressed as EU/30s.

2.2.5.4 Avidity analysis

The dissociation assay to determine antibody avidity was performed in a similar manner to the ELISA for the serum analysis described above. After incubation with serum, the wells were treated with increasing concentrations of ammonium thiocyanate, 0.2M to 8M. Ten wells were used per patient serum dilution, in duplicate, and the thiocyanate dilutions compared to a buffer blank with 100% binding. The plates were incubated for 60 minutes at 37°C, washed 4x4x4x1, and the ELISA continued as before. The concentration of thiocyanate as a molarity required to dissociate 50% of the bound antibody was calculated by linear regression. This concentration was termed the 50% inhibitory dose and provides a measure of the relative avidity (Macdonald et al., 1988; Pullen et al., 1986). All the samples from the same patient were tested together on the same plate.

2.2.5.5 Control sera

Sera from eight clinically healthy subjects, aged between 21 and 51, mean 32 years, were analysed by ELISA as above. The data produced was used to determine serostatus of the AP patients using the method of Mooney et al. (1995). AP patients with a serum titre greater than twice the median control sera titre were designated high responder and those with titres less than twice the control median low responder.

2.3 Statistical analysis of data

The clinical, microbiological, and antibody titre and avidity data for each site and patient were statistically analysed using Minitab statistical package (Minitab, release 12, Minitab Inc., State College, PA) and SPSS statistical software (SPSS, version 5, SPSS Inc., Chicago, IL, USA). For the patient based analysis the clinical data were averaged from the four sites and the mean GCF antibody titre of the middle two values used. The patient was recorded as positive for an organism if one or more sites were positive for that organism. BOP, Supp and prevalence of the microorganisms are presented as percentages in this thesis, but the raw data not the percentage was used in the analysis . All patient/site data was used in the analyses. No patients were excluded from the analyses on the grounds of poor response to therapy. Decreases were scored as positive and increases as negative

The differences before and after treatment were compared in AP, GEOP, smoker and non-smoker groups. The Wilcoxon test was used to compare MGI and PLI, a paired t-test for PD, AL and GCF volume, and McNemar's test for BOP, Supp, and the presence or absence of each organism before and after treatment.

The data was analysed for differences between AP and GEOP at baseline, after treatment, and to compare the changes in parameters with treatment, on a site and patient basis. Smoking subjects were compared to non-smokers in three groups; all subjects, AP subjects and GEOP subjects, and compared before and after treatment, and for any differences in the response to treatment. The Mann-

Whitney test was used to compare MGI and PLI, a two sample t-test for PD, AL and GCF volume, and Chi squared for BOP, Supp and the presence or absence of each organism, except when expected counts were less than five where Fisher's exact test was used.

In both AP and GEOP subjects, differences in baseline, after treatment and change with treatment scores were analysed for BOP positive and negative sites, Supp positive and negative sites, PD loser and gain sites and AL loser or gain sites. For the change in PD or AL, ANOVA was used for PD, AL and GCF volume, Kruskal-Wallis test for MGI and PLI, and Chi squared for BOP, Supp and the presence or absence of each organism, with Fisher's exact test used as above.

Serum antibody titres in AP patients were compared before and after treatment using Wilcoxon's test. In smoker and non-smoker groups baseline, after treatment and change with treatment antibody titres were analysed using Mann-Whitney test. GCF titres were analysed similarly. Avidity scores were compared before and after treatment using a paired t-test, and a two sample t-test in smokers and non-smokers. Where changes in treatment were analysed, ANOVA was used. Subjects were separated into high responders and low responders groups on the basis of antibody titres from control patients. The two groups were statistically analysed using the same tests used to compare smokers and non-smokers. The effect of serostatus on the presence of organisms was analysed using the Chi squared test. Spearman's rank correlation efficient was used to assess the relationship of sera and GCF titres with each other and PD.

Multiple regression analysis with backward elimination was performed to assess the effect of the clinical parameters, smoking, disease category and microbiological scores on pocket depth, the effect of these on the change in pocket depth, and the change in clinical and microbiological scores on the change in PD.

PCR and Checkerboard were compared statistically using McNemars's test and by percentage and number of sites which agreed with each other. PCR was used as the "gold standard" to determine the sensitivity and specificity of the Checkerboard.

CHAPTER 3

COMPARISON OF AP AND GEOP PATIENTS

This chapter deals with the comparison of AP and GEOP patients before and after SRP. In addition, the changes in clinical and microbiological parameters in response to SRP are examined. The inter-relationships between clinical parameters and the microflora are also investigated and discussed. Clustering and absence of bacteria are also examined. It should be noted that the prevalence of organisms in the microflora was determined solely by PCR in this chapter.

3.1 Comparison of AP and GEOP patients: Results

3.1.1 Demographic details

The demographic details of the patients recruited are shown in table 3.1. Thirty three AP subjects were recruited of whom 28 completed hygiene phase therapy. The average age of these patients was 47 (±7) years and the average time between sampling was 17.8 (±6.6) weeks. A total of 27 GEOP patients were recruited, 24 of whom completed therapy. Their average age was 33 (±3) years and all were diagnosed as GEOP before the age of thirty five. The average time between sampling was 16.2 (±5.2) weeks. Eight patients were dropped from the study due to systemic illness or antibiotic therapy not related to periodontal therapy or condition. The statistical analysis in this study used only the 28 AP patients and 24 GEOP patients who completed SRP.

3.1.2 Effect of SRP on clinical and microbiological parameters

Adult Periodontitis

Table 3.2 shows AP clinical parameters before and after SRP. Scaling and root planing resulted in significant reductions in PD after treatment (5.9 (±1.0) mm at

Table 3.1 Demographic details of the AP and GEOP patient groups

Disease	No. Pts baseline	No. Pts post-SRP	Mean Age (±SD)	Male	Female	Smokers	Mean Time (wks) between samples
AP	33	28	47 (±7)	13	20	10	17.8 (±6.6)
GEOP	27	24	33 (±3)	10	17	12	16.2 (±5.2)

Table 3.2 Effect of SRP on AP and GEOP mean clinical parameters. SD in brackets beside each number

Clin. Param.	AP Pre-SRP	AP Post-SRP	p value	GEOP Pre-SRP	GEOP Post-SRP	p value
MGI	2.5 (±0.6)	1.2 (±0.9)	<0.0001	1.9 (±1.0)	0.8 (±0.8)	<0.0001
PLI	1.5 (±0.7)	1 (±1.0)	0.0001	1.1 (±1.0)	0.7 (±0.9)	0.0006
BOP (%)	86	47	<0.0001	74	34	<0.0001
Supp (%)	29	8	<0.0001	34	4	<0.0001
PD (mm)	5.9 (±1.0)	4.4 (±1.5)	<0.001	6.8 (±1.0)	4.8 (±1.2)	<0.001
AL (mm)	13.4 (±2.2)	13 (±2.3)	<0.001	13.8 (±1.5)	12.7 (±1.7)	<0.001
GCFVol (nl/30s)	403.0 (±199.5)	421 (±365)	0.5	298 (±246)	338.5 (±299)	0.27

Table 3.3 Percentage of positive AP and GEOP patients for each organism before and after SRP

Micro. Param. (%)	AP Pre-SRP	AP Post-SRP	p value	GEOP Pre-SRP	GEOP Post-SRP	p value
P. gingivalis	54.4	54	1	62.5	50	0.45
P. intermedia	72.9	54	0.23	79.2	29.2	0.002
B. forsythus	63.6	54	0.79	91.7	62.5	0.016
A. actinomycetem	3	0	1	20.8	8.3	0.25
T. denticola	54.4	32	0.09	45.8	20.8	0.11

baseline compared to 4.4 (±1.5) mm post-treatment, $p<0.001$) and AL (13.4 (±2.2) mm compared to 13 (±2.3) mm, $p<0.001$). MGI, PLI, percentage of sites with BOP and Supp were reduced significantly also. There was an increase in GCF volume of on average 25 nl/30s, but this was not significant.

There were no significant differences in the microflora on a patient basis (table 3.3), although the reduction in percentage of patients positive for *T. denticola* approached significance. *P. intermedia* and *B. forsythus* decreased after SRP by 5-10%, and *T. denticola* by 25%. *A. actinomycetemcomitans* was eliminated and *P. gingivalis* prevalence did not change.

On a site basis (table 3.4) there were significant reductions in *P. intermedia*, *B. forsythus* and *T. denticola* of about 20%. *P. gingivalis* decreased by about 10% and again *A. actinomycetemcomitans* was eliminated.

GEOP

Following SRP there were significant decreases in all clinical variables except GCF volume. PD decreased from 6.8 (±1.0) mm pre-treatment to 4.8 (±1.2) mm post-treatment, $p<0.001$, and AL from 13.8 (±1.5) mm to 12.7 (±1.7) mm, $p<0.001$. BOP reduced by on average 40% and suppuration by 30%. GCF volume increased by roughly 40 nl/30s, but not significantly (Table 3.2).

There were significantly lower percentages for *P. intermedia* and *B. forsythus* in GEOP patients after therapy (table 3.3). *P. gingivalis*,

Table 3.4 Percentage of positive sites for each organism before and after SRP in AP and GEOP subjects

Micro. Param. (%)	AP Pre-SRP	AP Post-SRP	p value	GEOP Pre-SRP	GEOP Post-SRP	p value
P. gingivalis	43.9	33.9	0.1	54.2	26	0.0003
P. intermedia	56.8	33	0.03	51	12.5	<0.0001
B. forsythus	57.6	36.6	0.005	83.3	38.5	<0.0001
A. actinomycetem	1.5	0	0.5	12.5	4.2	0.04
T. denticola	37.1	18.8	0.0001	34.5	5.2	<0.0001

A. actinomycetemcomitans and *T. denticola* were also lower post-treatment. *A. actinomycetemcomitans* was not eliminated.

All the tested for bacteria were detected at significantly lower percentages after treatment on a site basis, with percentage decreases ranging from just under 10% to almost 50% (table 3.4).

3.1.3 Comparison of AP and GEOP patients at baseline

Table 3.5 shows the average clinical measurements of the selected sites between the two groups. Averaged clinical parameters on a patient basis were identical to those on a site basis. The MGI scores were 2.5 (±0.6) for the AP patients compared to 1.9 (±0.8) for GEOP patients, and were significantly different (p=0.006). The percentage of AP sites with BOP was significantly greater than GEOP sites. PI and Supp scores were not significantly different between the two patient groups. Average pocket depth measurements were significantly different (p=0.0002) with 5.9 (±1.0) mm for AP patients and 6.8 (±0.6) mm for GEOP patients. Gingival crevicular fluid (GCF) volumes were significantly different (p=0.015) with the average AP volume of 403 (±199.5) nl/30s and average GEOP volume 298.2 (±114.5) nl/30s.

The percentage of positive patients for each organism are shown in Table 3.6. *P. gingivalis* was detected in 54% of AP patients compared to 63% of EOP patients. *P. intermedia* was detected in 71% of AP patients and 79% of EOP patients. *B. forsythus* was found in 64% of AP patients and 92% of EOP

Table 3.5 Comparison of AP and GEOP mean clinical parameters at baseline, post-SRP, and change in response to SRP. Number of sites in brackets beside title of each box and SD in brackets beside each number

Clin. Param.	AP Pre (112)	GEOP Pre (96)	p val.	AP Post (112)	GEOP Post (96)	p val.	AP Chan (112)	GEOP Chan(96)	p val
MGI	2.5 (±0.6)	1.9 (±0.8)	0.006	1.2 (±0.9)	0.8 (±0.8)	<0.001	1.2 (±1.0)	1.1 (±1.0)	0.7
PLI	1.5 (±0.7)	1.1 (±0.7)	0.11	1.0 (±0.9)	0.7 (±0.9)	0.001	0.5 (±1.1)	0.4 (±1.2)	0.86
BOP (%)	86	74	0.049	47	34	0.06	38	40	0.98
Supp (%)	27	34	0.95	8	4	0.25	21	30	0.32
PD (mm)	5.9 (±1.0)	6.8 (±0.6)	0.0002	4.4 (±1.5)	4.8 (±1.3)	0.05	1.5 (±1.4)	2.0 (±1.3)	0.01
AL (mm)	13.4 (±2.2)	13.8 (±1.5)	0.13	13.0 (±2.3)	12.7 (±1.7)	0.23	0.5 (±1.2)	1.2 (±1.1)	0.0002
GCFVol (nl/30s)	403.0 (±199.5)	298.2 (±114.5)	0.015	420.7 (±365.1)	338.5 (±289.5)	0.08	-23.7 (±382)	-40.3 (±357.4)	0.75

Table 3.6 Comparison of percentage of AP and GEOP patients positive for each organism at baseline, post-SRP, and change in response to SRP. Number of patients in brackets

Micro. Param. (%)	AP Pre (28)	GEOP Pre (24)	p value	AP Post (28)	GEOP Post (24)	p val.	AP Chan (28)	GEOP Chan(24)	p val
P. gingivalis	54.4	62.5	0.37	54	50	0.8	0.4	12.5	0.6
P. intermedia	72.9	79.2	0.52	54	29	0.08	18	50	0.16
B. forsythus	63.6	91.7	0.01	54	63	0.5	7	29	0.05
A. actinomycetem	3	20.8	0.04	0	8	0.12	4	12.5	0.23
T. denticola	54.4	45.8	0.52	32	21	0.36	25	25	0.93

patients. 4% of AP patients were positive for *A. actinomycetemcomitans* compared to 21% of EOP patients. *T. denticola* was detected in 54% of AP patients and 46% of EOP patients. There were significant differences between the groups in the detection of *B. forsythus* (p=0.05) and *A. actinomycetemcomitans* (p=0.04).

The comparison of the percentage of positive sites is shown in Table 3.7. *P. gingivalis* was detected in 44% of AP sites and 54% of EOP sites. 57% of AP sites were positive for *P. intermedia* and 51% of EOP sites. *B. forsythus* was found in 58% of AP sites and 83% of EOP sites. *A. actinomycetemcomitans* was detected in 2% of AP sites and 12.5% of EOP sites. 37.5% of AP sites and 35% of EOP were positive for *T. denticola*. There were significant differences again between the two groups for the detection of *B. forsythus* (p=0.01) and *A. actinomycetemcomitans* (p=0.001).

3.1.4 Comparison of AP and GEOP patients after SRP

A comparison of AP and GEOP clinical parameters is shown in table 3.5. The values of the clinical parameters were identical on a site and when averaged for patient basis. Both MGI and PLI scores were significantly higher in AP patients. AL, GCF volume, BOP and Supp were also higher but not significantly. Pocket depth was, however, statistically significantly higher in GEOP subjects.

On a patient basis, table 3.6 shows higher frequencies of detection for *P. gingivalis*, *P. intermedia* and *T. denticola* in AP and, in GEOP subjects,

Table 3.7 Comparison of percentage of AP and GEOP sites positive for each organism at baseline, post-SRP, and change in response to SRP. Number of sites in brackets.

Micro. Param. (%)	AP Pre (112)	GEOP Pre (96)	p value	AP Post (112)	GEOP Post (96)	p val.	AP Chan (112)	GEOP Chan(96)	p val
P. gingivalis	43.9	54.2	0.44	34	26	0.22	10	28	0.01
P. intermedia	56.8	51.0	0.62	33	12.5	0.001	22	39	0.12
B. forsythus	57.6	83.3	<0.001	37	39	0.8	19	45	0.01
A. actinomycetem	1.5	12.5	0.002	0	4	0.03	2	8	0.001
T. denticola	37.1	35.4	0.76	19	5	0.003	22	30	0.48

higher prevalences for the other two test organisms. These differences were not statistically significant, although the greater percentage of AP with detectable *P. intermedia* approached significance.

Table 3.7 displays the comparison of the percentage of positive sites in AP and GEOP subjects after SRP. *P. intermedia* and *T. denticola* were significantly higher in AP subjects and *A. actinomycetemcomitans* significantly increased in GEOP sites. *P. gingivalis* was higher in AP and *B. forsythus* higher in GEOP, but these differences were not statistically significant.

3.1.5 The change in clinical and microbiological parameters in response to SRP

The change in clinical measures for AP and GEOP in response to SRP is shown in table 3.5. There were similar changes in both groups for MGI, PLI and BOP. Supp scores improved more in GEOP than AP patients and in GEOP subjects there was a slightly greater increase in mean GCF volume, but these were not statistically significantly different. GEOP patients had significantly greater reduction in PD 2 (±1.3) mm compared to 1.5 (±1.4) mm, (p=0.0002). A significantly greater reduction was noted in GEOP subjects compared to AP subjects 1.2 (±1.1) mm compared to 0.5 (±1.2) mm.

In GEOP patients (table 3.6) there was a greater decrease in the percentage of patients positive for *P. gingivalis*, *P. intermedia*, *B. forsythus* and *A.*

actinomycetemcomitans, but only the reduction in *B. forsythus* was significant. The decrease in *T. denticola* was the same for both groups.

Table 3.7 shows the greater reductions in the percentage of positive sites in GEOP compared to AP patients for all organisms, but only *P. gingivalis* and *B. forsythus* were significantly different.

3.1.6 Relationship between Pocket Depth and BOP, Supp and the microflora

Adult Periodontitis

The relationship between PD, BOP, Supp and the prevalence of the bacteria on a site basis at baseline is shown in table 3.8. 83.9% of sites were between 4-7mm and 16.1% over 7mm. Sites with initial pocket depths over 7mm had a statistically significantly greater percentage of positive sites for Supp, *P. gingivalis*, and *P. intermedia*. As the pocket depth increased the number of sites with none of the test bacteria decreased.

The response to treatment is shown in table 3.9. The deeper sites had a lower reduction for BOP, Supp, *P. gingivalis*, *P. intermedia*, and *T. denticola*. However only the difference in Supp scores was statistically significant. *T. denticola* prevalence did not alter in the deep sites compared to a decrease of 26.6% in the shallower sites. *P. gingivalis* increased by 5.6% against a reduction of 12.8% in shallower sites. The change in *P. intermedia* was the same for both groups. The mean change in PD for the shallower sites was 1.4 (±1.2) mm

Table 3.8 Relationship between initial pocket depth, BOP, Supp and microflora (percentage) in AP and GEOP patients

Pocket Depth	No. Sites	BOP	Supp	Pg	Pi	Bf	Aa	Td	None
AP									
4-6.99mm	94	84	22	39.4	51.1	52.1	2.1	39.4	24.5
7mm+	18	94	61	66.7	77.8	72.2	0	50	16.7
p value		0.25	0.001	0.032	0.037	0.17	na	0.4	0.47
GEOP									
4-6.99mm	52	71.2	25	46.2	57.7	83.7	13.5	38.5	5.8
7mm+	44	77.3	45.5	63.6	43.2	84.1	11.4	31.8	0
p value		0.49	0.036	0.087	0.16	0.86	0.76	0.5	0.11

Table 3.9 Relationship between initial pocket depth and change in mean clinical and microbiological parameters following SRP in AP and GEOP patients

Pocket Depth	No. Sites	BOP	Supp	Pg	Pi	Bf	Aa	Td	PD (mm) (±SD)	AL (mm) (±SD)
AP										
4-6.99mm	94	40.4	17	12.8	22.2	20.2	2.1	26.6	1.4(±1.2)	0.5(±1.2)
7mm+	18	27.8	38.9	-5.6	22.2	11.1	0	0	2.4(±1.7)	0.8(±1.6)
p value		0.44	0.01	0.43	0.86	0.52	na	0.16	<0.0001	<0.001
GEOP										
4-6.99mm	52	38.5	21.2	21.2	46.2	50	7.8	32.7	1.5(±1.1)	1.3(±1.1)
7mm+	44	40	40.9	36.4	29.6	38.6	9.1	27.3	2.6(±1.3)	1.0(±1.1)
p value		0.62	0.04	0.009	0.24	0.01	0.76	0.67	<0.0001	0.23

compared to 2.4 (±1.7) mm for the deeper sites which was significant at $p<0.0001$. Mean change in AL was also significantly greater for the deeper site category.

Post-treatment 43.9% of sites were 0-4mm, 50% between 4-7mm and 7.1% over 7mm (table 3.10). There were significantly higher numbers of positive sites for BOP, Supp, *P. gingivalis*, *P. intermedia*, *B. forsythus*, *T. denticola*, and a reduction in sites with no bacteria as the pocket depth increased.

GEOP

Table 3.8 shows the relationship between pocket depth, BOP, Supp and the prevalence of the bacteria on a site basis at baseline. Before treatment 54.2% of sites were between 4 and 7mm, and 45.8% over 7mm.

Percentage of sites with BOP increased slightly in deeper pockets but not significantly. Suppuration was statistically significantly higher in deeper sites. The prevalence of *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans* and *T. denticola* decreased at deeper sites and the prevalence of *B. forsythus* was similar. 5.3% of sites had none of the bacteria investigated, but all sites over 7mm had detectable flora.

In response to SRP pocket depth improved significantly more at the deeper sites 2.6 (±1.3) mm compared to 1.5 (±1.1) mm, $p<0.0001$ although gain in attachment was lower 1.0 (±1.1) mm to 1.3 (±1.1) mm, $p=0.23$ (table 3.9). The

Table 3.10 Relationship between post-SRP pocket depth, BOP, Supp and microflora (percentage) in AP and GEOP patients

Pocket Depth	No. Sites	BOP	Supp	PG	PI	BF	AA	TD	None
AP									
0-3.99mm	48	31.3	0	20.8	20.8	18.8	0	6.3	60.4
4-6.99mm	56	55.4	10.7	37.5	39.3	48.2	0	26.8	28.6
7mm+	8	87.5	37.5	87.5	62.5	62.5	0	37.5	0
p value		0.003	0.001	0.001	0.025	0.02	na	0.01	<0.001
GEOP									
0-3.99mm	23	34.8	0	34.8	0	43.5	0	0	47.8
4-6.99mm	69	31.9	5.8	24.6	13	36.2	5.8	5.8	46.4
7mm+	4	75	0	0	75	50	0	25	0
p value		0.2	0.2	0.3	<0.001	0.74	0.2	0.035	0.18

decrease in BOP was similar for both pocket depth categories although the change in suppuration was significantly greater at the deeper sites. There were greater reductions in *P. gingivalis* and *A. actinomycetemcomitans* at deeper sites but otherwise lower decreases for the other organisms. The differences in the reductions of *P. gingivalis* and *B. forsythus* were statistically significant.

After treatment 24% of sites were between 0-4mm, 71.9% between 4-7mm and 4.1% over 7mm (table 3.10). There were no significant differences in the number of sites positive for suppuration, *B. forsythus* or *A. actinomycetemcomitans*. BOP increased markedly in sites over 7 mm as did the prevalences of *P. intermedia*, *B. forsythus* and *T. denticola*. The higher percentages of *P. intermedia* and *T. denticola* at deeper sites were statistically significant. The number of sites with no detectable bacteria decreased sharply over 7mm, though these results should be interpreted with caution given the low number of sites over 7mm after treatment.

3.1.7 Relationship between bleeding on probing and clinical and microbiological parameters

Adult Periodontitis

A comparison of clinical and microbiological parameters at AP BOP positive and negative sites at baseline is shown in table 3.11. Significantly more BOP negative sites were associated with smoking (62% compared to 23%, p=0.001). At baseline BOP sites had significantly more suppuration, and attachment loss. PD and GCF volume were higher, but not significantly compared to non-

Table 3.11 Comparison of AP mean clinical and microbial parameters at sites with (+) and without (-) bleeding on probing at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	BOP+ Pre (96)	BOP- Pre (16)	p val.	BOP+ Chan (96)	BOP- Chan (16)	p val.	BOP+ Post (59)	BOP- Post (53)	p val
MGI	2.4 (±0.7)	2.1 (±0.7)	0.11	1.2 (±1.0)	1.2 (±1.0)	0.83	1.3 (±0.9)	1.1 (0.9)	0.23
PLI	1.5 (±0.9)	1.5 (±1)	0.91	0.4 (±1.1)	0.7 (±1.2)	0.83	1.2 (±0.9)	0.9 (±0.9)	0.16
BOP (%)	100	0	na	53	-50	<0.001	100	0	na
Supp (%)	32	6	0.03	24	0	0.056	13	3	0.06
PD (mm)	6 (±1.3)	5.4 (±1.5)	0.16	1.6 (±1.3)	1.0 (±1.5)	0.14	5.0 (±1.5)	3.9 (±1.4)	0.0001
AL (mm)	13.7 (±2.2)	12.6 (±1.9)	0.04	0.6 (±1.3)	0.2 (±0.9)	0.15	13.65 (±2.6)	12.4 (±1.9)	0.005
GCFVol (nl/30s)	409.7 (±342.4)	320.9 (±269.3)	0.25	-14.1 (±390.3)	-81.4 (±333.1)	0.47	447 (±380)	397 (±352)	0.48
Smoker (%)	23	62	0.001				34	24	0.23
Micro. Param. (%)									
P. gingivalis	43.8	43.8	1	7	25	0.27	41.5	27.1	0.11
P. intermedia	58.3	37.5	0.12	26	0	0.17	41.5	25.4	0.07
B. forsythus	55	56	0.94	19.8	12.5	0.22	41.5	32.2	0.31
A. actinomycetem	2	0	0.56	2	0	0.56	0	0	1
T. denticola	41	43.8	0.8	22	25	0.47	24.5	13.6	0.14

bleeding sites. There were no significant differences in the microflora at baseline. *P. intermedia* was slightly increased at bleeding sites and *A. actinomycetemcomitans* was not detected at non-bleeding sites.

Bleeding sites had greater though not significant reductions in Supp, PD and AL in response to SRP (table 3.11). Non-BOP sites had a five-fold greater increase in GCF volume. Bleeding sites reduced by just over half whereas half of the non-bleeding sites became positive after treatment. In response to therapy, *P. intermedia* and *B. forsythus* decreased more at bleeding sites, and *P. gingivalis* and *T. denticola* at non-bleeding sites. *P. intermedia* and *A. actinomycetemcomitans* prevalence did not change in non-bleeding sites. However, there were no statistically significant differences.

Table 3.11 also shows the comparison of clinical and microbiological parameters at sites with and without BOP post-treatment. Sites which bled after treatment had significantly higher suppuration, pocket depths and attachment loss. Sites with bleeding post-SRP had higher percentages of sites with detectable test organisms than sites without bleeding, but these were not statistically significant. *A. actinomycetemcomitans* was not detected post-treatment

GEOP

Significantly fewer BOP positive sites were found in smokers (Table 3.12). There were no significant differences in clinical and microbial parameters between BOP positive and negative sites at baseline.

Table 3.12 Comparison of GEOP mean clinical and microbial parameters at sites with (+) and without (-) bleeding on probing at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	BOP+ Pre (71)	BOP- Pre (25)	p value	BOP+ Chan (71)	BOP- Chan (25)	p value	BOP+ Post (33)	BOP- Post (63)	p value
MGI	2 (±1.1)	1.7 (±0.85)	0.18	1.2 (±1.0)	0.9 (±0.9)	0.13	1.1 (±1.0)	0.6 (±0.7)	0.037
PLI	1.2 (±1.1)	1.0 (±1.0)	0.35	0.5 (±1.2)	0.2 (±1.2)	0.26	0.8 (±0.9)	0.6 (±0.8)	0.14
BOP (%)	100	0	na	63 (±49)	-28 (±46)	<0.001	100	0	na
Supp (%)	31 (±47)	44 (±51)	0.24	27 (±48)	40 (±50)	0.22	9.1	15.9	0.08
PD (mm)	6.8 (±1.0)	6.7 (±0.9)	0.42	2.1 (±1.4)	1.8 (±1.1)	0.4	4.8 (±1.6)	4.8 (±1.0)	0.93
AL (mm)	13.9 (±1.5)	13.6 (±1.5)	0.34	1.2 (±1.2)	1.1 (±0.9)	0.93	12.6 (±1.7)	12.7 (±1.7)	0.64
GCFVol (nl/30s)	279 (±219)	352 (±310)	0.28	-56 (±354.9)	4.3 (±368.1)	0.48	340.4 (±326.9)	337.4 (±287.1)	0.96
Smoker (%)	41 (±50)	76 (±44)	0.0025				45.5	52.4	0.52
Micro. Param. (%)									
P. gingivalis	52	60	0.5	18	56	0.052	30.0	23.8	0.49
P. intermedia	51	52	0.9	39	36	0.75	15.2	11.1	0.57
B. forsythus	85	80	0.6	38	64	0.15	45.5	34.9	0.31
A. actinomycetem	13	12	0.93	10	4	0.38	6.1	3.2	0.5
T. denticola	37	32	0.68	30	32	0.28	3	6.4	0.48

Response to therapy produced a 63% reduction in BOP at positive sites and a 28% increase in negative sites (table 3.12). Otherwise there were no other statistically significant reductions in clinical parameters. The greater reduction in *P. gingivalis* at BOP negative sites compared to BOP positive sites approached significance. There was a reduction in the microflora at all sites.

Sites with BOP after treatment had significantly higher gingival inflammation, but were otherwise similar to non-bleeding sites (table 3.12). The flora was not significantly different also.

3.1.8 Relationship between suppuration and clinical and microbiological parameters

Adult Periodontitis

Suppurative sites at baseline were significantly associated with increased MGI, BOP, PD and AL (table 3.13). *P. gingivalis* and *T. denticola* were found at significantly higher frequencies in suppurative sites at baseline.

Both groups however had similar changes in response to SRP in all clinical parameters except GCF volume which decreased in suppurative sites and increased at non-suppurative sites (table 3.13). Suppurative sites reduced by 81% and increased by 4% in negative sites. Suppurative sites had a significantly greater change in *T. denticola*, and also greater reduction in *P. gingivalis*, *P. intermedia* and *B. forsythus*.

Table 3.13 Comparison of AP mean clinical and microbial parameters at sites with (+) and without (-) suppuration at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	Supp+ Pre (32)	Supp- Pre (80)	p value	Supp+ Chan (32)	Supp- Chan (80)	p value	Supp+ Post (9)	Supp- Post (103)	p value
MGI	2.75 (±0.5)	2.2 (±0.7)	<0.001	1.4 (±0.8)	1.1 (±1.1)	0.1	1.9 (±1.0)	1.2 (±0.9)	0.03
PLI	1.6 (±1.0)	1.4 (±0.9)	0.31	0.6 (±0.8)	0.4 (±1.2)	0.57	1.1 (±0.3)	1.0 (±0.9)	0.54
BOP (%)	97	81	0.03	40	38	0.51	78	45	0.056
Supp (%)	100	0	na	81	-4	<0.001	100	0	na
PD (mm)	6.7 (±1.6)	5.6 (±1.0)	0.002	1.6 (±1.5)	1.5 (±1.3)	0.64	6.3 (±1.3)	4.2 (±1.5)	0.001
AL (mm)	14.4 (±2.4)	13.2 (±2.0)	0.02	0.6 (±1.1)	0.5 (±1.3)	0.57	16.1 (±2.1)	12.7 (±2.1)	0.001
GCFVol (nl/30s)	438.2 (±346)	380.6 (±329)	0.42	14.7 (±428)	-39 (±364)	0.53	499 (±410)	414 (±362)	0.56
Smoker (%)	28	29	0.95				44	27	0.27
Micro. Param. (%)									
P. gingivalis	65.6	35	0.003	16	8	0.23	88.9	29.1	0.0003
P. intermedia	69	50	0.07	34	18	0.32	33.3	33.0	1
B. forsythus	66	51	0.17	25	16	0.73	77.8	33.0	0.008
A. actinomycetem	0	2	0.57	0	2	0.56	0	0	1
T. denticola	62.5	32.5	0.004	34	18	0.04	33.3	17.5	0.24

Post-therapy suppurative sites had significantly increased gingival inflammation, bleeding on probing, pocket depths, and attachment loss (table 3.13). More suppurating sites were from smokers than non-smokers. Significantly higher frequencies of *P. gingivalis* and *B. forsythus* were detected at suppurating sites, with increased *T. denticola.*

GEOP

Suppurating sites had significantly greater PD and AL at baseline (table 3.14). An average PD of 7.1 (±0.9) mm was recorded at suppurating sites compared to 6.6 (±1.0) mm at non-suppurating sites. The percentage prevalences of the test organisms were slightly, but not significantly, higher at suppurating sites.

A statistically significant reduction in BOP was noted at non-suppurating sites compared to suppurating sites (46% compared to 27%, 0.01) in response to SRP (table 3.14). Two percent of non-suppurating sites become suppurative and there was a decrease of 91% in the number of suppurative sites. The reduction in non-suppurative sites for *A. actinomycetemcomitans* was significantly greater than suppurative sites, though all sites experienced a reduction in the test organisms after therapy (table 3.14).

Sites with suppuration after treatment had no significant differences in clinical parameters, though suppurative sites had a tendency for deeper pockets and more BOP (table 3.14). *T. denticola* and *A. actinomycetemcomitans* were not detected

Table 3.14 Comparison of GEOP mean clinical and microbial parameters at sites with (+) and without (-) suppuration at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	Supp+ Pre(33)	Supp- Pre (63)	p value	Supp+ Chan(33)	Supp- Chan (63)	p value	Supp+ Post (4)	Supp- Post (92)	p value
MGI	2.0 (±1.1)	1.9 (±1.0)	0.7	0.9 (±0.9)	1.2 (±1.0)	0.09	0.5 (±1)	0.8 (±0.8)	0.37
PLI	1.2 (±1.0)	1.1 (±1.1)	0.7	0.3 (±1.4)	0.5 (±1.1)	0.7	0.75 (±0.5)	0.7 (±0.9)	0.5
BOP (%)	67 (±48)	78 (±42)	0.2	27 (±76)	46 (±53)	0.01	75	32.6	0.08
Supp (%)	100	0	na	91 (±29)	-2 (±13)	<0.0001	100	0	na
PD (mm)	7.1 (±0.9)	6.6 (±1.0)	0.03	2.1 (±1.3)	1.9 (±1.3)	0.4	5.2 (±0.3)	4.8 (±1.3)	0.07
AL (mm)	14.4 (±1.4)	13.5 (±1.5)	0.005	1.1 (±0.8)	1.2 (±1.3)	0.8	12.5 (±0.3)	12.7 (±1.7)	0.45
GCFVol (nl/30s)	281.4 (±208.2)	307 (±265.1)	0.6	-47.5 (±284.5)	-36.4 (±392.5)	0.9	166.3 (±147.6)	346 (±302.8)	0.089
Smoker (%)	52	49	0.94				75	48.9	0.31
Micro. Param. (%)									
P. gingivalis	64	49	0.2	39	22	0.1	25	26.1	0.96
P. intermedia	61	46	0.2	46	35	0.09	50	10.9	0.001
B. forsythus	87	61	0.15	48	43	0.3	50	38	0.63
A. actinomycetem	18	10	0.2	6	9	0.04	0	4.4	0.67
T. denticola	36	35	0.9	27	32	0.5	0	5.4	0.63

in suppurative sites post-treatment and *P. intermedia* was found at a significantly higher prevalence at these sites compared to non-suppurative sites (table 3.14).

3.1.9 Comparison of sites which gained and lost pocket depth

Adult periodontitis

Thirteen sites increased in pocket depth and 96 decreased after therapy. Before treatment, there were no significant differences between these sites although loser sites had a tendency for lower bacterial prevalences and slightly more sites from smokers (table 3.15). In response to therapy, there was significantly less resolution of gingival inflammation, bleeding on probing and suppuration at loser sites. In addition pocket depth and attachment increased significantly at loser sites. GCF volume decreased slightly compared to an increase at gain sites. Gain sites had significantly greater reductions in bacterial prevalence for *P. gingivalis*, and *T. denticola* and loser sites recorded a slight increase in *P. gingivalis*. Loser sites had no change in percentage of positive sites for *B. forsythus*, *A. actinomycetemcomitans* and *T. denticola.* Post treatment, in addition to significantly deeper pockets, loser sites had significantly higher MGI, suppuration and attachment scores, and slightly higher bleeding. Sites which improved generally had lower bacterial prevalences, although there were no significant differences.

GEOP

Only three sites increased in pocket depth and this was not sufficient for meaningful analysis.

Table 3.15 Comparison of AP mean clinical and microbial parameters at sites which lost and gained pocket depth at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	Loser Pre (13)	Gain Pre (96)	p value	Loser Chan (13)	Gain Chan (96)	p value	Loser Post (13)	Gain Post (96)	p value
MGI	2.3 (±0.9)	2.4 (±0.7)	0.8	0.54 (±1.0)	1.3 (±1.0)	0.05	1.8 (±0.8)	1.1 (±0.9)	0.024
PLI	1.5 (±1.0)	1.5 (±0.9)	0.26	0.46 (±1.1)	0.43 (±1.1)	0.22	1.0 (±0.9)	1.0 (±0.9)	0.78
BOP (%)	77	88	0.16	7	42.7	0.01	69.2	44.8	0.23
Supp (%)	31	28	0.79	7.7	22.9	0.002	23.1	5.2	0.015
PD (mm)	5.8 (±1.1)	6 (±1.3)	0.16	-0.7 (±0.6)	1.9 (±1.1)	na	6.5 (±1.1)	4.1 (±1.4)	<0.001
AL (mm)	14.3 (±2.1)	13.4 (±2.2)	0.42	-0.6 (±1.1)	0.7 (±1.1)	0.002	14.8 (±2.7)	12.7 (±2.1)	0.006
GCFVol (nl/30s)	380.4 (±323.4)	397.5 (±330.8)	0.94	64 (±375)	-45.3 (±380.3)	0.23	316.2 (±307.5)	442.7 (±373.1)	0.24
Smoker (%)	38.5	26	0.08						
Micro. Param. (%)									
P. gingivalis	38.5	44.8	0.67	-7.7	13.5	0.003	46.2	31.3	0.28
P. intermedia	38.5	57.3	0.2	7.7	25	0.4	30.8	32	0.91
B. forsythus	53.8	56.3	0.87	0	22.9	0.38	53.8	33.3	0.15
A. actinomycetem	0	2.1	na	0	2.1	na	0	0	na
T. denticola	30.8	41.7	0.45	0	24	0.05	30.8	17.7	0.26

3.1.10 Comparison of sites which gained and lost attachment

Adult periodontitis

Twenty nine sites lost attachment compared to 80 which gained attachment. Not all sites which lost attachment were the same as those sites which lost pocket depth. At baseline sites which were to lose attachment, there was significantly less bleeding and a higher number were from smokers (table 3.16). These sites had significantly lower *P. gingivalis* prevalence and, in general, lower bacterial prevalences. Response to treatment resulted in significantly lower changes in bleeding on probing, suppuration and pocket depth in loser sites. There were lower reductions in bacterial prevalence and an increase in *P. gingivalis* at loser sites. The differences in the reductions for *B. forsythus* and *T. denticola* approached significance. Post-therapy loser sites had significantly greater gingival inflammation, suppuration and pocket depth, as well as attachment loss. Loser sites had a significantly higher detection frequency for *P. gingivalis* and, again in general, bacterial prevalences were higher.

GEOP

Eleven sites lost attachment and 82 gained attachment. Table 3.17 shows the clinical and microbial comparison of GEOP sites which gained and lost attachment at baseline. Clinically, sites which were to lose attachment had significantly lower attachment loss. There were no other significant differences at baseline, although the difference in suppuration approached significance. Before treatment, loser sites had lower prevalences for *P. gingivalis* and *T.*

Table 3.16 Comparison of AP mean clinical and microbial parameters at sites which lost and gained attachment at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	Loser Pre (29)	Gain Pre (80)	p value	Loser Chan (29)	Gain Chan (80)	p value	Loser Post (29)	Gain Post (80)	p value
MGI	2.5 (±0.7)	2.3 (±0.7)	0.24	1.1 (±1.1)	1.2 (±1.0)	0.87	1.4 (±0.9)	1.1 (±0.9)	0.04
PLI	1.7 (±1.1)	1.4 (±0.8)	0.32	0.6 (±1.3)	0.4 (±1.0)	0.5	1.1 (±0.9)	1.0 (±0.9)	0.77
BOP (%)	72	90	0.025	10.3	48.8	0.001	62	41.3	0.13
Supp (%)	24	31	0.18	2	27.5	0.007	20.7	3.8	0.004
PD (mm)	6.0 (±1.2)	5.9 (±1.4)	0.8	0.7 (±1.3)	1.8 (±1.3)	0.0001	5.3 (±1.7)	4.1 (±1.4)	0.001
AL (mm)	13.5 (±2.3)	13.7 (±2.1)	0.1	-0.9 (±0.8)	1.1 (±0.9)	na	14.4 (±2.3)	12.6 (±2.0)	0.0001
GCFVol (nl/30s)	428.5 (±295.5)	379.3 (±342.9)	0.54	-68.8 (±433.5)	-9.7 (±370.6)	0.75	497.3 (±394.2)	389.0 (±349.2)	0.35
Smoker (%)	41	24	0.07						
Micro. Param. (%)									
P. gingivalis	24.1	48.8	0.01	-17	20	0.0002	41.4	28.8	0.02
P. intermedia	44.8	57.5	0.14	17.2	23.8	0.27	27.6	33.8	0.76
B. forsythus	48.3	57.5	0.64	3.4	25	0.052	44.8	32.5	0.27
A. actinomycetem	0	2.5	na	0	2.5	na	0	0	na
T. denticola	34.5	42.5	0.5	6.9	27.5	0.072	27.6	15	0.1

Table 3.17 Comparison of GEOP mean clinical and microbial parameters at sites which lost and gained attachment at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	Loser Pre (11)	Gain Pre (82)	p value	Loser Chan (11)	Gain Chan (82)	p value	Loser Post (11)	Gain Post (82)	p value
MGI	1.8 (±1.1)	1.9 (±1.0)	0.82	1.3 (±0.9)	1.1 (±1.0)	0.77	0.5 (±0.5)	0.8 (±0.9)	0.42
PLI	1.1 (±1.1)	1.1 (±1.0)	0.98	0.7 (±0.9)	0.4 (±1.2)	0.71	0.4 (±0.5)	0.7 (±0.9)	0.55
BOP (%)	81.8	73.2	0.5	39	36.4	0.1	45.5	34.2	0.34
Supp (%)	9.1	36.6	0.096	9.1	31.7	0.031	0	4.9	0.4
PD (mm)	6.9 (±1.2)	6.8 (±1.0)	0.77	1.3 (±1.5)	2.1 (±1.3)	0.15	4.7 (±1.1)	4.9 (±2.3)	0.11
AL (mm)	12.8 (±1.5)	14.0 (±1.3)	0.003	-0.6 (±0.6)	1.4 (±0.9)	na	13.3 (±1.7)	12.6 (±1.7)	0.32
GCFVol (nl/30s)	423 (±302.8)	277 (±229.2)	0.12	115 (±339)	-69.4 (±357)	0.15	307.9 (±196.5)	346.5 (±315.5)	0.76
Smoker (%)	54.5	48.8	0.79						
Micro. Param. (%)									
P. gingivalis	36.4	57.3	0.32	27.3	28	0.7	9.1	29.3	0.076
P. intermedia	54.5	48.8	0.21	36.4	37.8	0.83	18.2	11.0	0.19
B. forsythus	90.9	82.9	0.3	45.5	46.3	0.96	45.5	36.6	0.51
A. actinomycetem	0	13.4	0.09	0	9.8	0.13	0	3.7	0.41
T. denticola	27.3	36.6	0.83	18.2	31.7	0.2	9.1	4.9	0.47

denticola, higher *P. intermedia* and *B. forsythus*, and *A. actinomycetemcomitans* was absent. These differences were not statistically significant however. Table 3.17 also shows the change in clinical parameters in response to scaling and root planing at loser and gain sites. A significantly lower reduction in suppuration at loser sites was recorded compared to gain sites. These sites also had a lower, non-significant, reduction in pocket depth, and a decrease in GCF volume compared to an increase at gain sites. Both loser and gain sites had similar reductions for *P. gingivalis*, *P. intermedia* and *B. forsythus*. Gain sites had greater, but not significant, decreases in *A. actinomycetemcomitans* and *T. denticola* prevalences. Post-therapy, there were no significant differences between the sites. Loser sites had slightly higher bleeding and attachment loss but lower scores for the other parameters. Following SRP, loser sites showed higher detection frequencies for *P. intermedia*, *B. forsythus* and *T. denticola* and lower *P. gingivalis*. However these were not significant.

3.1.11 Relationship between the presence and absence of each organism and clinical and microbiological parameters

AP sites

The presence of *P. gingivalis* in baseline sites was significantly related with suppuration and deeper pockets (table 3.18). Microbiologically, *P. gingivalis* positive sites had significantly greater frequencies of detection for *P. intermedia*, *B. forsythus*, and *T. denticola* at baseline. Although *P. gingivalis* positive sites had initially deeper pocketing, the reduction in clinical measurements was similar for positive and negative sites. The only significant difference was the

Table 3.18 Comparison of AP mean clinical and microbial parameters in sites with (+) and without (-) *P. gingivalis* at baseline, change in response to SRP and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	PG+ Pre (49)	PG- Pre (63)	p value	PG+ Chan (49)	PG- Chan (63)	p value	PG+ Post (38)	PG- Post (74)	p value
MGI	2.41 (±0.67)	2.35 (±0.74)	0.77	1.0 (±1.0)	1.3 (±1.0)	0.35	1.5 (±1.0)	1.1 (±0.8)	0.028
PLI	1.5 (±0.9)	1.44 (±0.9)	0.56	0.5 (±0.9)	0.4 (±1.2)	0.87	1.0 (±0.7)	1.1 (±0.9)	0.84
BOP (%)	86	86	1	31	44	0.45	57.9	41.9	0.11
Supp (%)	43	17	0.003	35	9.5	0.01	21	1.4	<0.001
PD (mm)	6.3 (±1.5)	5.6 (±1.1)	0.01	1.6 (±1.3)	1.5 (±1.4)	0.7	5.2 (±1.7)	4 (±1.3)	0.0005
AL (mm)	13.5 (±2.1)	13.6 (±2.2)	0.96	0.6 (±1.0)	0.5 (±1.4)	0.53	13.6 (±3.0)	12.7 (±1.7)	0.083
GCFVol (nl/30s)	396 (±315)	398 (±34)	0.97	-47 (±364)	-5 (±398)	0.56	478.7 (±391)	391 (±350)	0.25
Smoker (%)	33	25	0.4				34.2	25.7	0.34
Micro. Param. (%)									
P. gingivalis	100	0	NA	51	-22	<0.001	100	0	na
P. intermedia	77.6	38.1	<0.001	37	11	0.04	55.3	21.6	<0.001
B. forsythus	81	35	<0.001	41	2	0.003	60.5	24.3	<0.001
A. actinomycetem	4.1	0	0.11	4.1	0	0.11	0	0	na
T. denticola	67.4	20.6	<0.001	41	8	0.001	36.8	9.5	<0.001

significantly greater reduction in suppuration at positive sites. Twenty two percent of negative sites became positive for *P. gingivalis* after treatment. The percentage reductions in *P. intermedia*, *B. forsythus*, and *T. denticola* were significantly greater at *P. gingivalis* positive sites. Post-treatment *P. gingivalis* positive sites were associated with greater gingival inflammation, suppuration and pocketing. In addition, these positive sites had significantly higher percentages for *P. intermedia*, *B. forsythus* and *T. denticola* compared to negative sites.

Table 3.19 shows the differences in clinical measurements between *P. intermedia* positive and negative sites. *P. intermedia* positive sites had significantly greater GCF volume scores at baseline. PD, BOP and Supp were slightly greater but not significantly. At baseline *P. gingivalis* and *B. forsythus* were detected at significantly higher frequencies in positive sites compared to negative sites. There were no significant differences in the response to treatment between positive and negative sites. Positive sites had slightly greater reductions in pocket depth and the microflora. Negative sites gained *P. intermedia*, whereas *P. intermedia* was markedly reduced at positive sites. Following therapy, *P. intermedia* positive sites had significantly greater pocketing and suppuration. The presence of *P. gingivalis* and *B. forsythus* was significantly greater at positive sites than negative sites.

B. forsythus positive sites showed significantly lower MGI and PLI scores (table 3.20). PD was significantly deeper at *B. forsythus* positive sites. Significantly

Table 3.19 Comparison of AP mean clinical and microbial parameters at sites with (+) and without (-) *P. intermedia* at baseline, change in response to SRP and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	PI+ Pre (62)	PI- Pre (50)	p value	PI+ Chan (62)	PI- Chan (50)	p value	PI+ Post (37)	PI- Post (75)	p value
MGI	2.4 (±0.7)	2.3 (±0.7)	0.41	1.1 (1.1)	1.2 (1.0)	0.65	1.4 (±0.9)	1.1 (±0.9)	0.15
PLI	1.6 (±1.0)	1.3 (±0.9)	0.06	0.5 (1.1)	0.4 (1.2)	0.8	1.1 (±1.0)	1.0 (±0.8)	0.48
BOP (%)	90	80	0.12	37	40	0.29	59.5	41.3	0.07
Supp (%)	35	20	0.07	27	12	0.22	8.1	8	0.98
PD (mm)	6.1 (±1.4)	5.7 (±1.2)	0.52	1.6 (1.3)	1.4 (1.5)	0.5	4.9 (±1.6)	4.2 (±1.5)	0.025
AL (mm)	13.7 (±2.4)	13.4 (±1.9)	0.4	0.6 (1.1)	0.5 (1.3)	0.6	13.5 (±2.5)	12.8 (±2.2)	0.15
GCFVol (nl/30s)	455 (±357)	325 (±289)	0.036	-5 (392)	-46 (371)	0.6	437.6 (±383.9)	412.4 (±357.8)	0.74
Smoker (%)	32	24	0.34				37.8	24	0.13
Micro. Param. (%)									
P. gingivalis	62	22	<0.001	15	4	0.6	56.8	22.7	<0.001
P. intermedia	100	0	NA	55	-18	<0.001	100	0	na
B. forsythus	67.7	40	0.003	24	12	0.17	59.5	25.3	<0.001
A. actinomycetem	1.6	2	0.88	1.6	2	0.88	0	0	na
T. denticola	48.4	32	0.08	29	14	0.26	24.3	16	0.29

Table 3.20 Comparison of AP mean clinical and microbial parameters at sites with (+) and without (-) *B. forsythus* at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	BF+ Pre (62)	BF- Pre (50)	p value	BF+ Chan (62)	BF- Chan (50)	p value	BF+ Post (41)	BF- Post (71)	p value
MGI	2.2 (±0.7)	2.5 (±0.6)	0.027	1.0 (±1.0)	1.4 (±1.1)	0.53	1.6 (±0.7)	1.0 (±0.9)	<0.001
PLI	1.3 (±0.9)	1.7 (±0.9)	0.028	0.2 (±0.9)	0.8 (±1.2)	0.001	1.2 (±0.8)	0.9 (±0.9)	0.04
BOP (%)	85	86	0.94	32	46	0.43	53.7	43.7	0.31
Supp (%)	35	22	0.17	31	8	0.024	17.1	2.8	0.008
PD (mm)	6.2 (±1.4)	5.6 (±1.1)	0.02	1.5 (±1.4)	1.6 (±1.4)	0.59	5.2 (±1.4)	4.0 (±1.5)	0.0001
AL (mm)	13 (±2.1)	14.2 (±2.1)	0.006	0.5 (±1.2)	0.5 (±1.3)	1	13.6 (±2.4)	12.7 (±2.2)	0.049
GCFVol (nl/30s)	404 (±313)	389 (±360)	0.08	-31 (±375)	-15 (±394)	0.83	455.6 (±369.5)	400.6 (±363.6)	0.45
Smoker (%)	21	38	0.05				51.2	15.5	<0.001
Micro. Param. (%)									
P. gingivalis	64.5	18	<0.001	24	-8	0.01	56.1	21.1	<0.001
P. intermedia	67.7	40	0.003	31	12	0.09	53.7	21.1	<0.001
B. forsythus	100	0	NA	58	-30	<0.001	100	0	na
A. actinomycetem	3.2	0	0.8	3.2	0	0.8	0	0	na
T. denticola	62.9	14	<0.001	34	8	0.008	48.8	1.4	<0.001

more negative sites were associated with smokers. Before treatment *P. gingivalis*, *P. intermedia*, and *T. denticola* were significantly higher at positive sites. There was a significantly better improvement in plaque scores at *B. forsythus* negative sites, but a significantly better reduction in Supp at positive sites in response to SRP. Thirty percent of negative sites became positive for *B. forsythus*. Significantly greater reductions in *P. gingivalis* and *T. denticola* occurred at *B. forsythus* positive sites, and a slight increase in *P. gingivalis* was reported at negative sites. Sites positive after treatment had significantly higher gingival inflammation, plaque scores, suppuration, pocket depth, attachment loss, and percentage of sites from smoker subjects. At positive sites significantly higher prevalences of *P. gingivalis*, *P. intermedia*, and *T. denticola* were recorded.

There were only two *A. actinomycetemcomitans* positive sites, which was too small a number for a meaningful statistical analysis. Table 3.21 shows the clinical and microbiological parameters.

Table 3.22 shows a significantly increased number of suppurating sites and smokers in *T. denticola* positive sites compared to negative sites at baseline. At baseline, positive sites had significantly increased frequencies for *P. gingivalis* and *B. forsythus*. SRP produced a significantly greater decrease in suppurative sites in those positive for *T. denticola* compared to negative sites. Eleven percent of negative sites became positive for *T. denticola*. Significant changes in the prevalences of *P. gingivalis* and *B. forsythus* were noted at positive sites

Table 3.21 Comparison of AP mean clinical and microbial at sites with (+) and without (-) *A. actinomycetemcomitans* at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	AA+ Pre (2)	AA- Pre (110)	AA+ Chan (2)	AA- Chan (110)	AA- Post (112)
MGI	2.0 (±0)	2.4 (±0.7)	1 (±1.4)	1.2 (±1)	1.2 (±0.9)
PLI	2.5 (±0.7)	1.5 (±0.9)	0.5 (±0.7)	0.5 (±1.1)	1 (±0.9)
BOP (%)	100	85	50	38	47.3
Supp (%)	0	29	0	21	8
PD (mm)	5.7 (±0.6)	5.9 (±1.3)	2.9 (±0.6)	1.5 (±1.4)	4.4 (±1.5)
AL (mm)	12.2 (±0.5)	13.6 (±2.2)	1.3 (±0.3)	0.5 (±1.2)	13 (±2.3)
GCFVol (nl/30s)	282 (±33.7)	399 (±336)	-681 (±185)	-12 (±374)	420.7 (±365.1)
Smoker (%)	0	29			
Micro. Param. (%)					
P. gingivalis	100	42.7	50	9	33.9
P. intermedia	50	55.5	50	22	33
B. forsythus	100	54.5	100	17	36.6
A. actinomycetem	100	0	100	0	0
T. denticola	100	40	100	21	18.8

Table 3.22 Comparison of AP mean clinical and microbial parameters at sites with (+) and without (-) *T. denticola* at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	TD+ Pre (46)	TD- Pre (66)	p value	TD+ Chan (46)	TD- Chan (66)	p value	TD+ Post (21)	TD- Post (91)	p value
MGI	2.4 (±0.7)	2.4 (±0.7)	0.94	0.9 (±1.1)	1.3 (±0.9)	0.1	1.8 (±0.6)	1.1 (±0.9)	0.001
PLI	1.6 (±1.0)	1.4 (±0.9)	0.31	0.5 (±1.0)	0.5 (±1.2)	0.9	1.4 (±0.8)	0.9 (±0.9)	0.03
BOP (%)	85	86	0.81	33	42	0.44	61.9	44	0.14
Supp (%)	43	18	0.004	35	11	0.016	14.3	6.6	0.24
PD (mm)	6.1 (±1.4)	5.8 (±1.2)	0.18	1.35 (±1.3)	1.6 (±1.4)	0.3	5.6 (±1.5)	4.1 (±1.4)	0.0003
AL (mm)	13.4 (±2.1)	13.6 (±2.3)	0.67	0.4 (±1.0)	0.6 (±1.3)	0.3	14 (±2.7)	12.8 (±2.2)	0.07
GCFVol (nl/30s)	432 (±325)	373 (±339)	0.36	-13 (±374)	-31 (±390)	0.8	441.7 (±333.6)	415.9 (373.5)	0.76
Smoker (%)	39	21	0.04				38.1	26.4	0.28
Micro. Param. (%)									
P. gingivalis	71.7	24.2	<0.001	33	-6	0.002	66.7	26.4	<0.001
P. intermedia	65.2	48.5	0.08	22	23	0.98	42.9	30.8	0.29
B. forsythus	84.8	34.9	<0.001	37	6	0.033	95.2	23.1	<0.001
A. actinomycetem	4.4	0	0.9	4.4	0	0.9	0	0	na
T. denticola	100	0	NA	70	-11	<0.001	100	0	na

compared to negative sites. Post-treatment significantly greater gingival inflammation scores, plaque scores, and pocket depths were associated with *T. denticola* positive sites. BOP and suppuration and AL were also higher. *P. gingivalis* and *B. forsythus* were detected at significantly higher percentages at positive sites, and *P. intermedia* was also increased post-treatment.

GEOP sites

Table 3.23 shows the clinical measurements at *P. gingivalis* positive and negative sites. There were no significant differences at baseline or after treatment. Microbiologically there were no significant differences at baseline. The only significant difference comparing the change in clinical parameters in response to SRP was that *P. gingivalis* positive sites gained significantly more attachment. Just over a quarter of negative sites gained detectable *P. gingivalis* in response to therapy and there was a significantly greater reduction in *B. forsythus* at these sites. After treatment the proportion of *P. gingivalis* negative sites that were in smokers was significantly higher. Post-therapy significantly higher levels of *B. forsythus* were detected at positive sites.

At baseline, detection of *P. intermedia* at sites was associated with significantly deeper pockets, as shown in table 3.24. *B. forsythus* was found at significantly higher levels in negative sites before treatment. A significantly greater reduction in MGI was recorded for positive sites in response to treatment. Eleven percent of *P. intermedia* negative sites became positive. The reduction in *A. actinomycetemcomitans* was significantly greater at negative sites than

Table 3.23 Comparison of GEOP mean clinical and microbial parameters at sites with (+) and without (-) *P. gingivalis* at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	PG+ Pre (52)	PG- Pre (44)	p value	PG+ Chan (52)	PG- Chan (44)	p value	PG+ Post (25)	PG- Post (71)	p value
MGI	2.1 (±2.1)	1.75 (±1.0)	0.17	1.2 (±1.0)	1.0 (±1.0)	0.28	0.8 (±0.9)	0.8 (±0.8)	0.62
PLI	1.1 (±1.1)	1.1 (±1.0)	0.9	0.4 (±1.1)	0.5 (±1.2)	0.82	0.6 (±0.8)	0.7 (±0.9)	0.96
BOP (%)	71 (±46)	77 (±42)	0.5	35	45	0.62	40	32.4	0.49
Supp (%)	40 (±50)	27 (±45)	0.18	37	23	0.11	4	4.2	0.96
PD (mm)	6.9 (±1.0)	6.7 (±0.9)	0.26	2.2 (±1.3)	1.8 (±1.2)	0.098	4.7 (±1.2)	4.8 (±1.2)	0.6
AL (mm)	14 (±1.3)	13.6 (±1.7)	0.23	1.4 (±1.1)	0.9 (±1.1)	0.03	12.5 (±2.2)	12.7 (±1.5)	0.65
GCFVol (nl/30s)	289 (±226)	305.7 (±266.7)	0.75	-25 (±365)	-58 (±352)	0.66	289 (±245.5)	355.9 (±316.3)	0.28
Smoker (%)	42	50	0.1				24	59.2	0.003
Micro. Param. (%)									
P. gingivalis	100	0	Na	75	-27	<0.001	100	0	na
P. intermedia	50	52.2	0.82	42	34	0.75	8	14.1	0.43
B. forsythus	80.7	86.4	0.46	42	48	0.01	68	28.2	<0.001
A. actinomycetem	13.5	11.4	0.76	10	7	0.31	0	5.6	0.23
T. denticola	28.9	43.2	0.14	25	36	0.57	8	4.2	0.47

Table 3.24 Comparison of GEOP mean clinical and microbial parameters at sites with (+) and without (-) *P. intermedia* at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	PI+ Pre (49)	PI- Pre (47)	p value	PI+ Chan (49)	PI- Chan (47)	p value	PI+ Post (12)	PI- Post (84)	p value
MGI	2.1 (±1.0)	1.7 (±1.0)	0.12	1.3 (±1.0)	0.9 (±1.0)	0.043	0.4 (±0.7)	0.8 (±0.8)	0.08
PLI	1.1 (±1.0)	1.1 (±1.1)	0.97	0.6 (±1.2)	0.3 (±1.1)	0.11	0.6 (±0.7)	0.7 (±0.9)	0.96
BOP (%)	73 (±45)	74 (±44)	0.91	43	36	0.86	41.7	33.3	0.57
Supp (%)	41 (±50)	28 (±45)	0.18	35	26	0.11	16.7	2.4	0.02
PD (mm)	7.0 (±1.0)	6.6 (±1.0)	0.035	1.8 (±1.3)	2.2 (±1.3)	0.14	6.1 (±1.2)	4.6 (±1.1)	0.002
AL (mm)	13.6 (±1.5)	14 (±1.5)	0.22	1.0 (±1.0)	1.4 (±1.2)	0.089	13.8 (±1.3)	12.5 (±1.7)	0.009
GCFVol (nl/30s)	290 (±216)	304.3(±273)	0.78	-59 (±314)	-21 (±401)	0.6	254.9 (±252.4)	350.4 (±305.3)	0.25
Smoker (%)	57	43	0.15				100	42.9	<0.001
Micro. Param. (%)									
P. gingivalis	53.1	55.3	0.82	39	17	0.15	16.7	27.4	0.43
P. intermedia	100	0	na	86	-11	<0.001	100	0	na
B. forsythus	73.5	93.6	0.008	47	42	0.92	41.7	38.1	0.8
A. actinomycetem	10.2	14.9	0.49	6	11	0.04	8.3	3.6	0.44
T. denticola	30.6	40.4	0.32	27	34	0.45	8.3	4.8	0.6

positive sites. Significantly higher pocket depth and attachment loss were recorded at *P. intermedia* positive sites after treatment. All positive sites were from smokers. There were no significant differences in the prevalences of the flora.

At the outset *B. forsythus* positive sites had significantly lower MGI scores (table 3.25). Before treatment *T. denticola* was found significantly more prevalent at positive sites and *P. intermedia* significantly increased at negative sites. In response to SRP the change in suppuration was significantly lower at *B. forsythus* positive sites. A quarter of negative sites had detectable levels of *B. forsythus* after treatment. *A. actinomycetemcomitans* and *T. denticola* were not found before or after treatment in negative sites. Consequently the reduction for these two organisms was significantly greater at positive sites. *P. intermedia* reduced significantly more at negative sites. *B. forsythus* positive sites post-SRP were not significantly different from negative sites, although BOP, suppuration and PD were slightly higher. However, a significantly higher number of sites were positive for *P. gingivalis* and *B. forsythus*, with *A. actinomycetemcomitans* and *P. intermedia* more frequent also.

A. actinomycetemcomitans positive sites had significantly lower MGI scores and GCF volume at baseline, shown in table 3.26. *T. denticola* was found with higher frequency at positive sites and this was significant. In response to SRP the change in MGI was significantly greater at negative sites and PLI at positive sites. Positive sites had a much greater reduction in *T. denticola* and negative

Table 3.25 Comparison of GEOP mean clinical and microbial parameters at sites with (+) and without (-) *B. forsythus* at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	BF+ Pre (80)	BF- Pre (16)	p value	BF+ Chan (80)	BF- Chan (16)	p value	BF+ Post (12)	BF- Post (84)	p value
MGI	1.8 (±1.0)	2.4 (±0.7)	0.025	1.1 (±0.9)	1.5 (±1.0)	0.07	0.8 (±0.9)	0.8 (±0.8)	0.96
PLI	1.1 (±1.1)	1.2 (±0.9)	0.62	0.5 (±1.2)	0.1 (±1.4)	0.2	0.6 (±0.8)	0.7 (±0.9)	0.87
BOP (%)	75 (±44)	69 (±48)	0.61	41	31	0.68	40.5	30.5	0.31
Supp (%)	31 (±47)	50 (±52)	0.15	29	38	0.01	5.4	3.4	0.63
PD (mm)	6.8 (±1.0)	6.7 (±0.8)	0.73	2.0 (±1.4)	2.0 (±1.1)	1	5 (±1.4)	4.7 (±1.1)	0.21
AL (mm)	13.8 (±1.5)	13.9 (±1.3)	0.92	1.2 (±1.2)	1.1 (±0.9)	0.65	12.5 (±2)	12.8 (±1.5)	0.49
GCFVol (nl/30s)	296 (±256)	301 (±180)	0.93	-49 (±376)	-42 (±253)	0.98	302.1 (±246.6)	361.3 (±328.6)	0.32
Smoker (%)	54	31	0.1				43.2	54.2	0.29
Micro. Param. (%)									
P. gingivalis	52.5	62.5	0.46	26	38	0.16	46	13.6	<0.001
P. intermedia	45	81.2	0.008	34	63	0.021	13.5	11.9	0.81
B. forsythus	100	0	Na	59	-25	<0.001	100	0	na
A. actinomycetem	15	0	0.098	10	0	0.1	8.1	1.7	0.13
T. denticola	42.5	0	0.001	36	0	0.001	13.5	0	0.004

Table 3.26 Comparison of GEOP mean clinical and microbial parameters at sites with (+) and without (-) *A. actinomycetemcomitans* at baseline, change in response to SRP and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	AA+ Pre (12)	AA- Pre (84)	p value	AA+ Chan (12)	AA- Chan (84)	p value	AA+ Post (4)	AA- Post (92)	p value
MGI	1.25 (±0.9)	2.0 (±1.0)	0.013	0.5 (±0.7)	1.2 (±1.0)	0.01	1.8 (±1.0)	0.8 (±0.8)	0.03
PLI	1.4 (±0.9)	1.1 (±1.1)	0.21	1.25 (±0.9)	0.3 (±1.2)	0.004	0.5 (±0.6)	0.7 (±0.9)	0.87
BOP (%)	75 (±45)	74 (±44)	0.9	33	40	0.14	50	33.7	0.5
Supp (%)	59 (±52)	32 (±47)	0.23	42	29	0.37	0	4.4	0.67
PD (mm)	7.0 (±1.0)	6.8 (±1.0)	0.49	1.6 (±1.3)	2.0 (±1.3)	0.29	5.7 (±0.7)	4.8 (±1.2)	0.085
AL (mm)	14.6 (±1.5)	13.7 (±1.5)	0.13	1.0 (±0.9)	1.2 (±1.1)	0.7	13.2 (±1.3)	12.6 (±1.7)	0.48
GCFVol (nl/30s)	188 (±99)	321.5 (±255)	0.003	-154 (±367)	-24 (±355)	0.27	466 (±436)	332.9 (±294.5)	0.59
Smoker (%)	50	50	1				100	47.8	0.04
Micro. Param. (%)									
P. gingivalis	58.3	53.4	0.76	25	29	0.69	0	27.2	0.23
P. intermedia	41.7	52.4	0.49	17	42	0.051	25	12	0.44
B. forsythus	100	81	0.098	58	43	0.34	75	37	0.13
A. actinomycetem	100	0	NA	83	-2.4	<0.001	100	0	na
T. denticola	66.7	31	0.016	67	25	0.01	25	4.4	0.07

sites a greater reduction in *P. intermedia.* Both of these were significantly different. Post-therapy all *A. actinomycetemcomitans* positive sites were from smokers and had significantly higher gingival inflammation. BOP, PD and AL were higher but not significantly. There were higher frequencies of *P. intermedia*, *B. forsythus* and *T. denticola* at positive sites and lower frequencies of *P. gingivalis*, but these were not significant. Only four sites were positive after therapy so these results should be treated with caution.

The clinical parameters for *T. denticola* positive and negative sites are shown in table 3.27. There were no significant differences before treatment. *B. forsythus* was found at all positive sites and this was statistically significant. *A. actinomycetemcomitans* was also found with significantly increased frequency at positive sites. In response to treatment the reduction in plaque levels at positive sites was significantly lower. The reductions in *B. forsythus* and *A. actinomycetemcomitans* were significantly greater at positive sites. There was a 95% decrease in the detection of *T. denticola* at positive sites and a 5% increase at negative sites. The only significant difference between negative and positive sites after therapy was the poorer plaque levels at *T. denticola* positive sites. *B. forsythus* was still detected at all positive sites and *A. actinomycetemcomitans* increased compared to negative sites, almost to significance.

Table 3.27 Comparison of GEOP mean clinical and microbial parameters at sites with (+) and without (-) *T. denticola* at baseline, change in response to SRP, and post-SRP. Number of sites in brackets beside title of each column and SD in brackets beside numbers.

Clin. Param.	TD+ Pre (34)	TD- Pre (62)	p value	TD+ Chan (34)	TD- Chan (62)	p value	TD+ Post (5)	TD- Post (91)	p value
MGI	1.85 (±0.9)	1.9 (±1.1)	0.64	0.9 (±0.9)	1.3 (±1.0)	0.06	1.2 (±1.1)	0.8 (±0.8)	0.31
PLI	1.35 (±1.1)	1.0 (±1.0)	0.12	0.7 (±1.3)	0.3 (±1.1)	0.03	1.4 (±0.9)	0.6 (±0.9)	0.03
BOP (%)	77 (±43)	73 (±45)	0.68	44	37	0.11	20	35.2	0.49
Supp (%)	35 (±48)	34 (±48)	0.89	32	29	0.45	0	4.4	0.73
PD (mm)	6.8 (±0.7)	6.8 (±1.1)	0.88	2.2 (±1.3)	1.9 (±1.3)	0.39	5.8 (±1.4)	4.7 (±1.2)	0.17
AL (mm)	14 (±1.3)	13.7 (±1.6)	0.29	1.2 (±1.3)	1.1 (±1.0)	0.61	12.7 (±1.8)	12.7 (±1.7)	0.99
GCFVol (nl/30s)	268 (±236)	313 (±249)	0.38	-41 (±390)	-40 (±341)	0.99	533 (±453)	327.8 (±288.8)	0.37
Smoker (%)	38	56	0.09				60	49.4	0.65
Micro. Param. (%)									
P. gingivalis	44.1	59.7	0.14	27	29	0.4	40	25.3	0.47
P. intermedia	44.1	54.8	0.32	29	44	0.14	20	12.9	0.6
B. forsythus	100	74.2	0.001	68	32	0.016	100	35.2	0.004
A. actinomycetem	23.5	6.5	0.016	18	3	0.01	20	3.3	0.07
T. denticola	100	0	na	94	-5	<0.001	100	0	na

3.1.12 Relationship between the change in site microflora and change in site pocket depth

Sites that gained any of the test organisms showed a trend for slightly lower reductions in pocket depth. Sites in which *P. gingivalis* and *B. forsythus* reduced had significantly greater pocket depth reductions than those that gained these organisms (table 3.28).

3.1.13 Clustering of bacteria

Figure 3.1 displays the frequency of detection of each organism and combination of organisms for AP and GEOP samples at baseline. In AP samples there were high frequencies for combinations of *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola*. In GEOP samples *B. forsythus* was detected at all *T. denticola* and *A. actinomycetemcomitans* positive sites. Also there were high percentages at sites for combinations of *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola*, but not as high as AP samples. 23.5% of AP and 3.1% of GEOP samples did not have detectable bacteria.

Table 3.29 shows the effect of the presence and absence of combinations of organisms on individual organisms at AP sites. The effect of the presence of each organism on the other bacteria has already been reported in tables 3.18 to 3.22. The analysis of the organisms showed that when any of these bacteria, *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola*, were found, whether singly or in combinations with each other, they had a significant positive effect on the frequency of detection of the other bacteria in this group. *A.*

Table 3.28 Relationship between loss or gain in site bacteria and change in site pocket depth. SD shown in brackets.

Ch Orgs	*P. gingivalis*	*P. intermedia*	*B. forsythus*	*A. actinomyce*	*T. denticola*
Gain (mm)	0.56 (±1.2)	1.5 (±1.9)	0.72 (±1.0)	na	1.0 (±1.8)
Loss (mm)	1.3 (±1.4)	1.8 (±1.2)	1.4 (±1.3)	2.9 (±0.6)	1.2 (±1.2)
p value	0.008	0.42	0.03	na	0.14

Figure 3.1 Bacterial combinations in AP and GEOP subjects

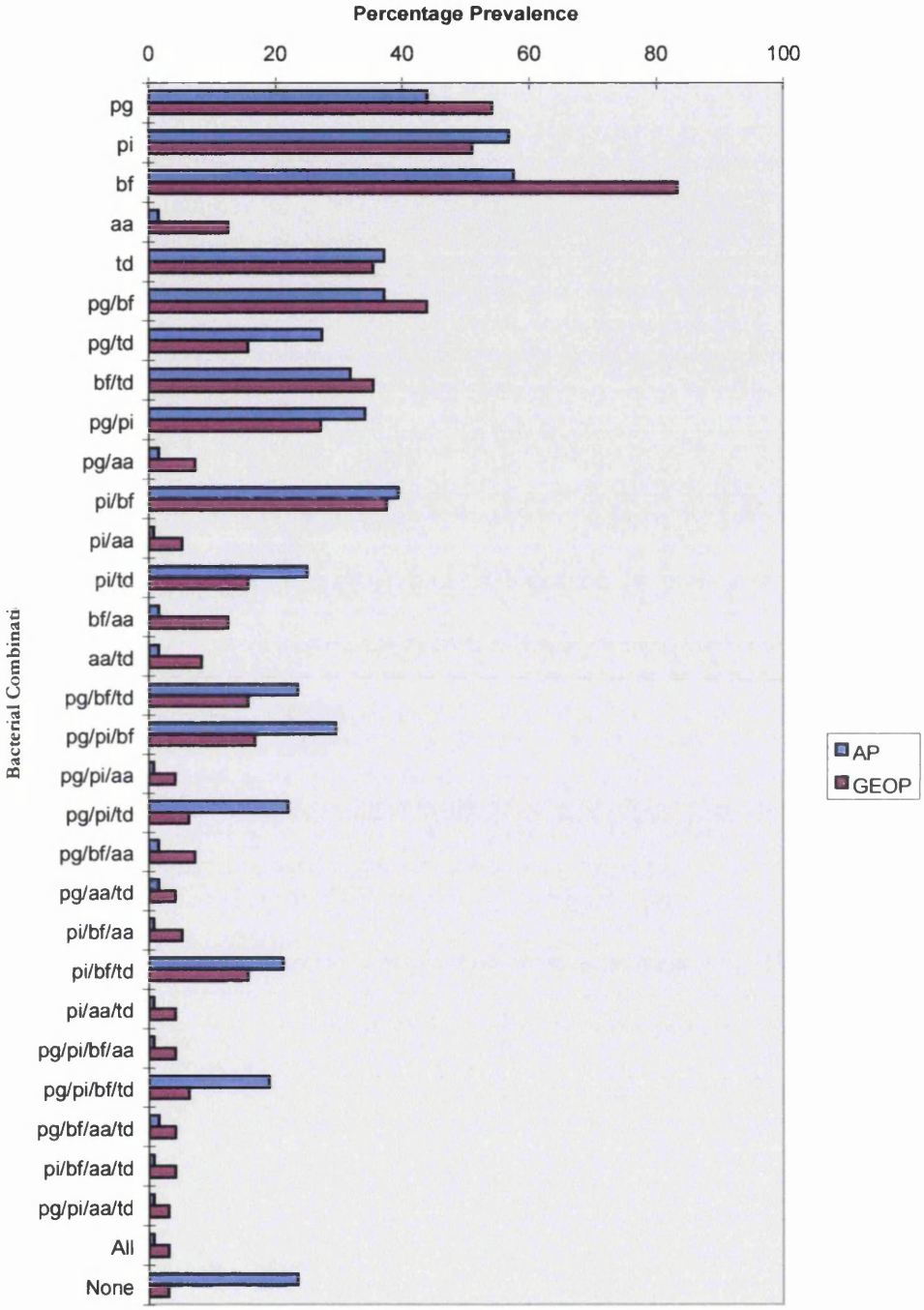


Table 3.29 Relationship between bacterial combinations and the percentage presence or absence of the other organisms in AP baseline samples. Number of sites shown in brackets beside column title.

Organism	pgbf+ (49)	pgbf- (83)	pgtd+ (36)	pgtd- (96)	bftd+ (42)	bftd- (90)	pgpi+ (45)	pgpi- (87)	pibf+ (52)	pibf- (80)	pitd+ (33)	pitd- (99)
P. ging	na	na	na	na	73.8	30 +	na	na	75	23.8 +	87.9	23.9 +
P. inter	79.6	43.4 +	80.6	47.9 *	66.7	52.2	na	na	na	na	na	na
B. forsyth	na	na	86.1	46.9 *	na	na	86.7	42.5 +	na	na	84.9	48.5 +
A. actino	4.1	0	5.6	0	4.8	0	2.2	1.2	1.9	1.3	3	1
T. denti	63.3	21.7 +	na	na	na	na	64.4	22.9 +	53.8	26.3 *	na	na

Organism	pgpibf+ (39)	pgpibf- (93)	pgpitd+ (29)	pgpitd- (103)	pibftd+ (28)	pibftd- (104)	pgbftd+ (31)	pgbftd (101)
P. ging	na	na	na	na	89.3	31.7 +	na	na
P. inter	na	na	na	na	na	na	80.7	49.5 *
B. forsyth	na	na	86.2	49.5 +	na	na	na	na
A. actino	2.6	1.1	3.5	1	3.6	1	6.5	0
T. denti	64.1	25.8 +	na	na	na	na	na	na

+ $p<0.001$ * $p=0.001$

actinomycetemcomitans frequencies were too low for meaningful statistical analysis but the organism was always found at sites with the combination *P. gingivalis*, *B. forsythus* and *T. denticola.*

Table 3.30 shows the effect of the presence and absence of combinations of organisms on the prevalence of individual bacteria in GEOP patients. The prevalence of the individual bacteria at positive and negative sites for each organism has already been reported in tables 3.23 to 3.27. *P. intermedia* was detected significantly more frequently at *P. gingivalis/B. forsythus* positive sites and *B. forsythus* at *P. intermedia/P. gingivalis* positive sites. *A. actinomycetemcomitans* was found significantly more often at *B. forsythus/T. denticola* positive sites. Although the number of positive sites was small *T. denticola* was significantly more frequently detected at *P. intermedia/A .actinomycetemcomitans*, *B. forsythus/A. actinomycetemcomitans* and *P. intermedia/B. forsythus/A. actinomycetemcomitans* positive sites.

3.1.14 Regression analysis

Multiple linear regression with backward elimination was used to determine the relationship between PD and other clinical and microbial parameters in all patients as well as AP and GEOP subjects. In addition the relationships between change in PD and change in the other clinical and microbial parameters, and baseline clinical and microbial parameters were examined.

Table 3.30 Relationship between bacterial combinations and the percentage presence or absence of the other organisms in GEOP baseline samples. Number of sites shown in brackets beside column title. Significance shown by symbol as indicated below.

Organism	pgbf+ (42)	pgbf- (54)	pgtd+ (15)	pgtd- (81)	bftd+ (34)	bftd- (62)	pgpi+ (26)	pgpi- (70)	pibf+ (36)	pibf- (60)	pitd+ (15)	pitd- (81)
P. ging	na	na	na	na	44.1	59.7	na	na	44.4	60.0	40.0	56.8
P. inter	38.1	61.1 ^	40.0	53.1	44.1	54.8	na	na	na	na	na	na
B. forsyth	na	na	100	80.3	na	na	61.5	91.4 +	na	na	100	80.3
A. actino	16.7	9.3	26.7	9.9	23.5	6.5 ^	15.4	11.4	13.4	11.7	26.7	9.9
T. denti	35.7	35.2	na	na	na	na	23.1	40.0	41.7	31.7	100	23.5

Organism	pgaa+ (7)	pgaa- (89)	piaa+ (5)	piaa- (91)	bfaa+ (84)	bfaa- (12)	aatd+ (8)	aatd- (88)
P. ging	na	na	80.0	52.3	58.3	53.6	50	54.6
P. inter	57.1	50.6	na	na	41.7	52.4	50.0	51.1
B. forsyth	100	82.2	100	82.4	na	na	100	81.8
A. actino	na	na	na	na	na	na	na	na
T. denti	57.1	33.7	80.0	33.0 ^	66.7	31.0 ^	na	na

~p=0.05 ^p<0.05 * p=0.001 +p<0.001

Table 3.30 (con'd)

Organism	pgpibf+ (16)	pgpibf- (80)	pgpitd+ (6)	pgpitd- (90)	pibftd+ (15)	pibftd- (81)	pgbftd+ (15)	pgbftd (81)	pgpiaa+ (4)	pgpiaa- (92)
P. ging	na	na	na	na	40.0	56.8	na	na	na	na
P. inter	na	na	na	na	na	na	40.0	53.1	na	na
B. forsyth	na	na	100	82.2	na	na	na	na	100	82.6
A. actino	25.0	10.0	50.0	10.0	26.7	9.9	26.7	9.8	na	na
T. denti	37.5	35	na	na	na	na	na	na	75.0	33.7

Organism	pgbfaa+ (7)	pgbfaa- (89)	pgaatd+ (4)	pgaatd- (92)	pibfaa+ (5)	pibfaa- (91)	piaatd+ (4)	piaatd- (92)
P. ging	na	na	na	na	80.0	52.3	75.0	53.3
P. inter	57.1	50.6	75.0	50.0	na	na	na	na
B. forsyth	na	na	100	82.6	na	na	100	82.6
A. actino	na	na	na	na	na	na	na	na
T. denti	57.1	33.7	na	na	80.0	33.0 ^	na	na

In all subjects baseline pocket depth was significantly and positively related to MGI, presence of suppuration, AL, presence of *B. forsythus*, GEOP and negatively to smoking ($p<0.001$, R^2 46.4%). Change in PD in response to SRP was significantly and positively related to change in AL, change in MGI and GEOP, and negatively to smoking ($p<0.001$, R^2 37.7%). Change in PD in relation to baseline measurements was significantly and positively related to PD and negatively to AL, GCF volume, presence of *B. forsythus* and smoking ($p<0.001$, R^2 26.9%).

In AP subjects baseline pocket depth was significantly and positively related to presence of suppuration, AL, and presence of *B. forsythus* ($p<0.001$, R^2 47.2%). Change in PD in response to SRP was significantly and positively related to change in AL, and change in MGI, and negatively to smoking ($p<0.001$, R^2 35.4%). Change in PD in relation to baseline measurements was significantly and positively related to PD and negatively to AL, presence of *B. forsythus* and smoking ($p<0.001$, R^2 24.0%).

In GEOP subjects baseline pocket depth was significantly and positively related to MGI, PLI, AL, presence of *P. intermedia* ($p<0.001$, R^2 29.6%). Change in PD in response to SRP was significantly and positively related to change in AL and negatively to smoking ($p<0.001$, R^2 36.3%). Change in PD in relation to baseline measurements was significantly and positively related to PD and MGI, and negatively to AL and smoking ($p<0.001$, R^2 43.8%).

3.2 Comparison of AP and GEOP patients: Discussion

3.2.1 Comparison of AP and GEOP patients at baseline

Although the same selection criteria were used for each patient group, and based on PD, there were a number of significant differences in the clinical measurements. AP patients appeared to exhibit significantly more gingival inflammation than GEOP patients. GEOP patients were found to have significantly lower BOP scores than AP patients, and similarly significantly lower GCF volumes. As the BOP score may relate to probing pressure and technique, it is possible that operator variation is responsible for the differences between AP and GEOP subjects. GCF measurements tend to be more objective and provide a continuous variable which relates to degree of gingival inflammation. The GCF samples were taken using identical paper strips, for the same duration, measured in the same machine and calibrated on the same curve. These differences in inflammation are therefore more likely to be due to the higher numbers of smokers in the GEOP group or that GEOP patients have less gingival inflammation.

In AP subjects the patient-based analysis showed that the most common organism was *P. intermedia* (72.7%), followed by *B. forsythus* (63.6%), *P. gingivalis* and *T. denticola* (both 54.4%) and lastly *A. actinomycetemcomitans* (3%). The order was slightly different in the site-based analysis. *B. forsythus* was the most common (57.6%), then *P. intermedia* (56.8%), *P. gingivalis* (43.9%), *T. denticola* (37.1%), and *A. actinomycetemcomitans* (1.5%). The high level of detection of these organisms supports the hypothesis that AP is a

polymicrobial infection, but the low level of *A. actinomycetemcomitans* may suggest that the other organisms may be more relevant in this disease.

B. forsythus was the most commonly detected organism in GEOP patients (91.7%), followed by *P. intermedia* (79.2%), *P. gingivalis* (62.5%), *T. denticola* (45.8%) and *A. actinomycetemcomitans* (20.8%). The site-based analysis gave a slightly different order. Again *B. forsythus* was the most prevalent (83.3%), but followed by *P. gingivalis* (54.2%), *P. intermedia* (51.0%), *T. denticola* (35.4%) and *A. actinomycetemcomitans* (12.5%). The high prevalence of *B. forsythus* in both patients and sites compared to the other organisms suggests that it may be more closely associated with GEOP.

Both the patient and site analysis of the microflora between the two groups revealed significant differences between the prevalences of *B. forsythus* and *A. actinomycetemcomitans* in AP and GEOP subjects. GEOP patients have a significantly higher prevalence of both of these organisms, which may be due to the deeper pockets noted in our GEOP patients or perhaps their greater involvement in the GEOP disease process. Studies by Dzink et al. (1985) and Christersson (1992) have previously reported that *B. forsythus* is associated with deeper pockets. *P. gingivalis* and *P. intermedia* are also found at a higher prevalence in GEOP than AP patients, but this was not statistically significantly different, and may also reflect the deeper pocketing. The prevalence of *T. denticola* was similar between the two groups.

Other studies that have used PCR to detect the presence of periodontopathogens have reported different detection rates than found in this study. Riggio et al. (1996) reported 24% of 43 AP patients positive for both *P. gingivalis* and *A. actinomycetemcomitans*. In this study we found *P. gingivalis* in 54.5% and *A. actinomycetemcomitans* in 3% of AP patients. In a subsequent study, Riggio et al. (1998) found 39% of sites and 52% of AP patients PCR-positive for *P. intermedia*. Mooney et al. (1995) reported that 29% of AP patients were positive for *P. gingivalis* and 47% for *A. actinomycetemcomitans*. Similarly varying prevalences have been reported by other authors (Christersson, 1992; Haffajee et al., 1988a; Dzink et al., 1985; Ashimoto et al., 1996; Moore et al., 1982) indicating that there is considerable variation in the detection of pathogenic flora and also methodolgical variation between different studies. The high levels of positive sites and patients for the organisms investigated in this study supports the polymicrobial nature of periodontal disease.

The level of *T. denticola* detection was similar between both disease groups (37.1% of AP sites and 35.4% of GEOP sites), but much higher than previously reported (Moore et al., 1982; Riviere et al., 1995). Riviere et al. (1995) detected high levels of spirochaetes in AP patients, but only 16% of sites were positive for *T. denticola*. Our higher prevalences may reflect a more accurate and sensitive diagnostic technique. The difficulty in culturing *T. denticola* may have masked its role in periodontal disease, and further studies using non-culture techniques could indicate that *T. denticola* may have an important role in both AP and GEOP.

Kamma et al. (1994) examined a number of periodontopathogens from GEOP patients by culture. It was reported that in pockets over 6mm in depth, *P. gingivalis* was detected in 91.7%, *B. forsythus* in 53.4%, and *A. actinomycetemcomitans* in 10.9% of sites. In that study the prevalence of *P. gingivalis* was higher, with *B. forsythus* and *A. actinomycetemcomitans* lower when compared with our study. *P. gingivalis* was the more prevalent microorganism in the study of Kamma et al. (1994), whereas *B. forsythus* was the more prevalent microorganism in our present study. The fact that two pathogens can be detected at different levels in two similar groups of patients again supports the variable and polymicrobial nature of periodontal disease. Christersson (1992) found a correlation between *B. forsythus* and *P. gingivalis* and deep pockets, but none for *P. intermedia* and *A. actinomycetemcomitans*.

3.2.2 Effect of SRP on clinical parameters

SRP significantly reduced all clinical variables except GCF volume, which increased slightly in both patient groups. In AP patients pocket depth reduced by a mean of 1.5 (±1.4) mm and GEOP patients by on average 2.0 (±1.3) mm. The initial average pocket depth for AP subjects was 5.9 (±1.3) mm and the reduction in these patients is slightly greater than that previously reported of 0.7mm to 1.3 mm (Becker et al., 1988; Hammerle et al., 1991; Hill et al., 1981; Kaldahl et al., 1988; Knowles et al., 1979; Pihlstrom et al., 1981; Ramfjord et al., 1987). The greater reduction in GEOP patients probably reflects the deeper pockets and perhaps differences between operators. The decrease in pocket depth is within

the range of previous studies of 1.2mm to 2.9mm (Becker et al., 1988; Hammerle et al., 1991; Hill et al., 1981; Kaldahl et al., 1988; Morrison et al., 1980; Pihlstrom et al., 1981; Ramfjord et al., 1987). The attachment gains for both patient groups is similar to that of previously published work. In both GEOP and AP patients SRP was effective in reducing pocket depth and there were few pockets that did not benefit from non-surgical therapy (Badersten et al., 1984a).

It has been demonstrated that the extent of connective tissue inflammation is different between bleeding and non-bleeding sites (Greenstein et al., 1981; Harper & Robinson 1987; Passo et al., 1988). Despite the reservation of some authors concerning low reproducibility for bleeding on probing scores (Janssen et al., 1986), evidence suggests that the bleeding on probing decreases after a successful treatment (Caton et al., 1982; Proye et al., 1982) and therefore could be used as a part of a diagnostic test to evaluate treatment outcome.

BOP was significantly reduced for both patient groups and, although not as large as some studies have reported, still agrees with previous reports (Badersten et al., 1984a; Magnusson et al., 1984; Nordland et al., 1987; Cercek et al., 1983). This may reflect differences in pocket depths, and difficulties in resolving inflammation in deeper pockets, the different study populations, the skills of the operator or the plaque control levels of the patients, which if poor may allow early re-colonisation and subsequent re-inflammation. However, in accordance with previous studies SRP is effective in reducing BOP.

The number of suppurating sites decreased significantly after therapy in both groups and indicates that SRP is effective in reducing suppuration. The formation of pus in some periodontal pockets suggests that in these sites the host defences are overwhelmed by the microbial challenge and studies by Badersten et al. (1985c, 1990) and Claffey et al. (1990) have shown that the presence of suppuration increased the positive predictive value for further breakdown. Following therapy there is a significant reduction in bacterial load, which will allow the host defences to re-establish control and promote healing. It is probably the effect of the removal of the bacterial load that reduces suppuration.

The reduction in gingival index reflects the decrease in inflammation as a result of the removal of the bacterial challenge, the healing response and the patients' oral hygiene. Comparison with other studies is complicated because of the number of different gingival inflammatory indices that have been used over the years. However the reduction in both patient groups agrees with published reports which indicate a reduction after non-surgical therapy (Becker et al., 1988; Singletary et al., 1982; Lavanchy et al., 1987).

Post-therapy there were significant improvements in plaque control in both groups, in keeping with reported data (Magnusson et al., 1984; Cercek et al., 1983; Badersten et al., 1984a; Nordland et al., 1987). Oral hygiene cannot significantly change the microflora especially in deeper sites without SRP (Kho et al., 1985; Beltrami et al., 1987; Smulow et al., 1983). However it is important

to prevent re-colonisation of pockets after SRP and could influence the subgingival flora (Dahlén et al., 1992; McNabb et al 1992; Katsulanos et al., 1992). Good oral hygiene is a pre-requisite for improvement in gingival inflammation.

The volume of GCF has been shown to be correlated with the degree of inflammation in the gingival tissue (Brill, 1960; Mann et al., 1963; Egelberg et al., 1964). The GCF volume increased slightly following therapy in contrast to a decrease in MGI and BOP. The decreased bacterial load which allows re-establishment of proper host responses and increased tissue turnover due to the healing process may account for the slight increases found.

As reported in previous studies, SRP is effective in improving clinical condition and in both AP and GEOP patients.

3.2.3 Effect of SRP on the microflora

Adult Periodontitis

Although the frequency of detection remained the same, reductions in the percentage of positive sites for *P. intermedia, B. forsythus* and *T. denticola* were found with the elimination of *A. actinomycetemcomitans*. However, SRP produced no significant decreases in the microflora in AP patients.

However on a site basis, there were decreases in the detection of all organisms. *P. intermedia, B. forsythus* and *T. denticola* had statistically significant

reductions of about 20%, *P. gingivalis* decreased by 10% and *A. actinomycetemcomitans* below the level of detection of PCR.

P. gingivalis has been reported to respond readily to SRP (Renvert et al., 1990a). Significant reductions in *P. gingivalis* detection generally occur after SRP (Renvert et al., 1990a; Lowenguth et al., 1995; Wikström et al., 1993; Renvert et al., 1997; Preber et al., 1995; Shiloah & Patters, 1994; Rosenburg et al., 1993; Ali et al., 1992; Goodson et al., 1991), although it is usually greater for sites than patients (Al-Yahfoufi et al., 1994; Wikström et al., 1993). Compared to these studies the decrease in *P. gingivalis* is low. Maiden et al. (1991) and Haffajee et al. (1997a) also reported a similarly low decrease of about 6-15% of sites. The differing results may reflect the microbiological test used, inadequate SRP, or the group of patients examined. A significant reduction in *P. gingivalis* has been correlated with clinical improvement and poorly responding sites still had high levels of *P. gingivalis* (Renvert et al., 1990a; Wikström et al., 1993; Newman et al., 1994). However this was not found in our study where continuing high prevalences were found irrespective of clinical improvement. Given the good clinical improvement, the response is unlikely to be the result of poor SRP. PCR was used to analyse subgingival plaque samples whereas culture or DNA probes were used in other studies quoted. The difference in the limits of detection may have resulted in the differing results. PCR has a much lower detection limit (10^2) compared to culture (10^{4-6}) and DNA probes (10^{4-5}), and what may be negative for these two tests may still be positive for PCR. Interestingly, both

Maiden et al. (1991) and Haffajee et al. (1997a) used DNA probes with a detection limit of about 10^4.

P. intermedia is readily reduced by treatment (Mombelli et al., 1995) and the response in our patient group supports previous studies (Ali et al., 1992; Renvert et al., 1990a; Bragd et al., 1987; Wikström et al., 1993).

Although *B. forsythus* is a named aetiological agent, there are relatively few studies that examine the effect of SRP on its prevalence. Haffajee et al. (1997a) reported its reduction from roughly 50% to 30% with SRP. The reduction in *B. forsythus* compares favourably with this study.

A. actinomycetemcomitans can be very difficult to eradicate from deep periodontal pockets (Renvert et al., 1990a, b, 1996; Bragd et al., 1987). Following treatment the decrease in *A. actinomycetemcomitans* is often not as great as that for other organisms (Flemmig et al., 1998; Renvert et al., 1997; Nieminen et al., 1996; Preber et al., 1995; Mombelli et al., 1994 a, b; Shiloah & Patters, 1994; Wikström et al., 1993; Goodson et al., 1991; Renvert et al. 1990 a, b; Maiden et al., 1991). SRP eliminated *A. actinomycetemcomitans* from AP patients. Previously Ali et al. (1992) and Rosenberg et al. (1993) had reported similar findings using SRP. In all three patient groups there were relatively low levels of *A. actinomycetemcomitans* before treatment compared to other studies where *A. actinomycetemcomitans* was not eliminated. This may account for the differences between the studies. Mombelli et al. (1994b) described a subset of

patients which was highly infected by *A. actinomycetemcomitans* and maintained high levels after treatment. Patients with only a few positive sites responded much better to treatment than those heavily infected patients. The current group of AP patients would seem to belong to Mombelli's lightly infected group. The elimination of *A. actinomycetemcomitans* was accompanied by clinical improvement, but given the low prevalence of the organism before treatment, the effect of *A. actinomycetemcomitans* on the periodontal condition was probably not as great as the other study organisms. It should be borne in mind that because the level of *A. actinomycetemcomitans* is low the results should be interpreted with some degree of caution.

T. denticola levels decreased readily with SRP. SRP has been shown to be very effective in reducing *T. denticola* numbers (Simonson et al., 1992; Loesche et al., 1992b; Katsanoulos et al., 1992; Haffajee et al., 1997a). The decrease in *T. denticola* prevalence is similar to that reported in these studies.

Although a decrease in the pathogenic flora accompanied an improvement in clinical condition, multiple regression with backward elimination did not link a decrease in any of the test organisms with this improvement. Previous studies have linked the reduction or elimination of *P. gingivalis* (Newman et al., 1994; Haffajee et al., 1997a), *P. intermedia* (Newman et al., 1994), *B. forsythus* (Haffajee 1997a; Socransky & Haffajee 1993), *A. actinomycetemcomitans* (Goene et al., 1990; Mombelli et al., 1994b; Renvert et al., 1990a) and *T. denticola* (Haffajee et al., 1997a; Simonson et al., 1992) with clinical

improvement in AP patients. The difference may be due again to the microbial test used. PCR used was not quantitative, and only gave a present or absent score. The studies that have found links have used quantitative or semi-quantitative techniques such as culture, ELISA or Checkerboard.

GEOP

In GEOP patients SRP resulted in a decrease in the prevalence of all bacteria which was significant for *P. intermedia* and *B. forsythus*. This pattern was repeated for the site analysis, where there were significant decreases for all the test organisms.

P. gingivalis reduced by over 10% in patients and about 30% in sites. Gunsolley et al. (1994) reported a decrease of about 6% in sites following SRP, but the prevalence of *P. gingivalis* was low at 6.7% before treatment. Rosenberg et al. (1993) found a decrease in *P. gingivalis* positive patients from 100% before treatment to 67% after treatment and Sigurdsson et al. (1994) demonstrated a 50% reduction in the number of positive sites in their study population aged 34-37 years.

P. intermedia reduced markedly in patients and sites, by 50% and 40% respectively, which suggests that SRP is very effective in reducing its numbers and supports the findings of Mombelli et al. (1995). Masunaga et al. (1990) similarly reported decreased *P. intermedia* detection frequencies after SRP.

B. forsythus also demonstrated significant decreases in both patients and sites. Comparison with published studies is difficult however. Studies on GEOP patients do not appear to have investigated the effect of SRP on its prevalence, perhaps due to its difficulty of culture, or have included this patient group with Adult or Advanced periodontitis groups. Our results show that in GEOP subjects *B. forsythus* responds readily to SRP, but prevalences are still high post-therapy compared to other organisms.

There were smaller reductions in the detection of *A. actinomycetemcomitans* compared to the other organisms, 12% of patients and 8% of sites. This is higher than reported in studies by Gunsolley et al. (1994), Sigurdsson et al. (1994) and Rosenberg et al. (1993). In all these studies *A. actinomycetemcomitans* was not eliminated from GEOP patients. Rosenberg et al. (1993) showed that SRP had no effect on the prevalence of *A. actinomycetemcomitans*, Gunsolley et al. (1994) reported a 3% reduction and Sigurdsson et al. (1994) a 5% decrease. The low reduction in *A. actinomycetemcomitans* may reflect its ability to invade gingival epithelial tissue (Meyer et al., 1991), which may act a source of re-infection.

The reductions in *T. denticola* were not as high as *P. gingivalis*, *P. intermedia* or *B. forsythus*, but there was still a significant reduction in the number of sites infected. Again there appear to be few studies which have examined the effect of SRP on *T. denticola* levels. However the spirochaete does seem to respond readily to treatment in GEOP patients.

Masunuga et al. (1990) linked the reductions in *P. gingivalis* and *P. intermedia* numbers with clinical improvement. Regression analysis of the clinical microbiological data from the GEOP patients failed to link reduction of any the organisms with clinical improvement, which may again be due to the microbiological test rather than the absence of a link.

Comparison of AP and GEOP

Before treatment, *P. gingivalis*, *P. intermedia*, *B. forsythus* and *A. actinomycetemcomitans* were more frequently detected in GEOP patients, but post-treatment *P. gingivalis* and *P. intermedia* were more common in AP patients. GEOP patients still had higher numbers of positive patients for *B. forsythus* and *A. actinomycetemcomitans*. *T. denticola* remained higher in AP subjects.

A. actinomycetemcomitans and *B. forsythus* were no longer significantly higher in GEOP patients following treatment, although both were still more prevalent. *A. actinomycetemcomitans* was eliminated from AP patients but not GEOP patients which may reflect the higher prevalence of the organism in GEOP patients before treatment.

Compared to AP sites, pre-treatment GEOP sites had higher prevalences of *P. gingivalis*, *B. forsythus* and *A. actinomycetemcomitans*, and lower *P. intermedia* and *T. denticola*. Post-treatment AP sites had a significantly higher percentage

of positive sites for *P. intermedia* and *T. denticola*, higher *P. gingivalis*, similar *B. forsythus* and lower *A. actinomycetemcomitans* compared to GEOP. GEOP patients had greater changes for all the test organisms except *T. denticola.* The change in *B. forsythus* in GEOP patients and sites was statistically significantly greater than AP patients and sites. GEOP sites also had significantly more reduction in *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* prevalences.

The differences may reflect the greater pocket depth change in GEOP patients. A deeper pocket before treatment or greater change in pocket depth may be accompanied by a greater decrease in the flora, though Haffajee et al. (1997a) showed similar decreases in the *P. gingivalis* numbers irrespective of initial pocket depth.

These results suggest that it is harder to reduce bacterial levels in AP, higher prevalences respond better to treatment and lower prevalences may be eliminated. Mombelli et al. (1991b, 1994b) also reported difficulties in eradicating high levels of organisms. The elimination of *A. actinomycetemcomitans* and continuing higher levels of *B. forsythus* in GEOP patients supports the hypothesis that these two have a greater role in the disease process in GEOP than AP.

3.2.4 Relationship of pocket depth and BOP, Suppuration and response to treatment

Deeper sites in both AP and GEOP patients had slightly more BOP than shallower sites. Both Cercek et al. (1983) and Badersten et al. (1985c) reported similar findings. Increased BOP at deep sites suggests a greater inflammatory response, which could be through an increased microbial load or the result of the greater destruction at these sites. The deeper sites in AP patients had a lower reduction in BOP than shallower sites, probably due to either difficulties in effectively scaling and root planing these sites or the continuing high numbers of the pathogens.

The increase in suppuration at deeper sites also reflects the increased bacterial numbers at these sites, and the resulting increased microbial challenge to the immune system as a result. Following therapy suppuration was linked with deeper AP sites but not in GEOP subjects. Increased prevalences were found for 4 of the 5 test organisms at deeper sites after therapy in AP patients and included *P. gingivalis* and *T. denticola* which are associated with suppuration (Socransky et al., 1991). There was no clear relationship between pocket depth and colonisation after treatment in GEOP sites, which may account for the difference.

The changes in pocket depth in both disease groups and attachment in AP subjects were greater in deeper pockets and similar to those reported in previous

studies. Interestingly the deeper pockets in GEOP subjects recorded lower attachment gains than the shallower pockets. The reasons for this are unclear.

3.2.5 Relationship of pocket depth and the microflora present and response to therapy

In AP subjects the presence of *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola* were related to pocket depth, both before and after treatment. *P. gingivalis* has been reported in increasing numbers in deeper pockets (Wolff et al., 1993; Wikström et al., 1993; Kojima et al., 1993; Christersson, 1992; Socransky et al., 1991) and its presence after therapy linked with deeper sites (Mombelli et al., 1991a; Wikström et al., 1993). Similarly *P. intermedia* numbers correlated with increasing pocket depth (Beck et al., 1992; Mombelli et al., 1991a; Haffajee et al., 1991b; Wolff et al., 1993). *B. forsythus* is also commonly found in increased numbers at deeper sites (Socransky et al., 1991; Dzink et al., 1985; Machtei et al., 1997), as have spirochaetes (Tanner et al., 1984; Savitt & Socransky, 1984).

It is harder to reduce bacterial numbers in deeper sites (Mombelli et al., 1991b, 1994b), as the results in this thesis also suggest. There is often a positive correlation between the residual probing depth following SRP and the presence of periodontal pathogens (Lindhe et al., 1985; Magnusson et al., 1984; Slots et al., 1985). This may be due to the more anaerobic conditions in deeper pockets, which may favour more rapid re-colonisation by anaerobic bacteria (Dzink et al., 1985, 1988; Tanner et al., 1979), or less effective treatment as the pocket depth

increases.

GEOP patients in general did not fit into this pattern. *T. denticola* and *P. intermedia* showed a trend for increasing numbers as the pocket depth increased but *P. gingivalis*, *B. forsythus* and *A. actinomycetemcomitans* did not. The low numbers of sites over 7 mm means that the results should be interpreted with caution. It may be that if the number of deep sites was increased this trend would become evident, or that the slightly better plaque control in these patients did not allow re-colonisation as quickly as AP patients and so the pattern did not develop.

The slight increase in *P. gingivalis* after treatment may have resulted from the time interval between the last SRP visit and sampling. If the sample had been taken within two weeks, there would probably have been much lower levels of *P. gingivalis* and other organisms. The three/four month delay may have allowed sufficient time for re-colonisation back to pre-treatment levels to have occurred (Magnusson et al., 1984; Sbordone et al., 1990a). Sbordone et al. (1990a) reported in their study that *P. gingivalis* prevalences returned to pre-treatment levels more quickly than other organisms studied in their 60 day investigation. The similar *P. gingivalis* prevalences in this study before and after SRP in AP patients may reflect this more rapid colonisation. Differing re-colonisation rates may also account for the differences between GEOP and AP sites.

3.2.6 Associations between the microflora and clinical measurements after treatment

A number of studies have indicated that after treatment persistent high levels of *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* may be associated with further disease progression or refractory periodontitis (Walker & Gordon, 1990; Tanner et al., 1987a; Mombelli et al., 1994b). Therefore it is worthwhile to assess the relationship of the microflora on the clinical measurements post-therapy.

A number of negative AP sites became infected after SRP by *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola.* The analysis of loser sites did not indicate any significant differences in the change in flora compared to gain sites. It is possible then that the increase at the sites resulted from an increase in bacterial numbers in response to SRP, somehow stimulated by SRP, contamination from outside the pocket perhaps as the result of SRP and the release of bacteria into the mouth, or natural fluctuation in bacterial numbers.

P. gingivalis, *B. forsythus* and *T. denticola* were associated with significantly more gingival inflammation and deeper pockets. Wikström et al. (1993) reported a similar association between levels of *P. gingivalis* and pocket depth and BOP after treatment. *B. forsythus* was also associated with significantly more suppuration and greater attachment loss. The presence of these organisms at these inflamed sites may indicate their pathogenicity or that these sites are an ideal environment. *P. intermedia* was associated with deeper pocketing, but not

BOP, which may suggest that, as some believe (Dahlén, 1993; Mombelli et al., 1991a), it is an opportunistic organism and not particularly related to disease. Post-treatment levels of *A. actinomycetemcomitans* reduced below detection, so no comments can be made about this organism.

In GEOP patients the results were quite different. *P. intermedia* was associated with deep inflamed sites and smoking, whereas *P. gingivalis*, *B. forsythus* and *T. denticola* were not. *T. denticola* was associated with poorer oral hygiene which may reflect the more rapid colonisation in these sites. *A. actinomycetemcomitans* was also associated with deep inflamed sites from smokers but as only four sites were positive after therapy this should be interpreted with caution. Therefore it seems that GEOP sites react differently than AP sites to the effects of treatment. Both groups responded well to treatment, but the effect of the flora post-therapy is dissimilar. Whether this reflects different conditions in the pockets perhaps due to differing host responses or different strains of infecting bacteria is yet to be determined, and would make an interesting investigation. Previous studies have reported a link between *P. gingivalis* and BOP (Albandar et al., 1990; Mombelli et al., 1991a; Socransky et al., 1991; Christersson, 1992; Kojima et al., 1993). In this study no such link could be found. This may reflect different methodologies for examining the microflora used or that there is variation in the response to pathogens between patients and study groups.

3.2.7 AP and GEOP loser/gain sites

Sites which lost or gained PD or AL in AP patients were generally not significantly different at baseline. This would suggest that these sites could not be predicted before treatment, and that measures other than clinical or microbial parameters are needed to predict breakdown. Sites which lost PD or AL had a lower reduction in microbial prevalence and an increase in *P. gingivalis*. In GEOP patients the low numbers of sites which increased in PD precluded analysis, but the sites that lost AL were not dissimilar to gain sites. There were no significant differences before and after SRP in clinical and microbial parameters between gain and loser sites, except for lower AL scores in loser sites at baseline. Loser sites also had a tendency for higher *P. intermedia*, *B. forsythus* and *T. denticola* prevalences post-therapy.

Wikström et al. (1993) also showed high levels of *P. gingivalis* in sites with continued deep pocketing and BOP. Similarly sites which lost AL were reported by Haffajee et al. (1995) and Socransky & Haffajee (1993) to have continuing high levels of *B. forsythus* after treatment compared to sites which gained attachment. In addition, Simonson et al. (1992) and Haffajee et al. (1997a) showed that a reduction in *T. denticola* accompanied clinical improvement. In the AP patients in this current study, there was also little change in *T. denticola* prevalence at sites which lost attachment or increased in pocket depth. All of these studies suggest an important role for the three organisms in AP.

As mentioned above, high levels of *P. gingivalis*, *P. intermedia* and *B. forsythus* have been linked with disease progression (Tanner et al., 1987a; Socransky & Haffajee, 1993), but not *T. denticola* (MacFarlane et al., 1988) which was a poor predictor of future attachment loss. It was not the intention of this study to monitor these sites post-SRP, so it is not known whether they experienced further breakdown. A further study would be to follow sites such as these over a period of time using PCR to investigate if high prevalences of *P. gingivalis*, *B. forsythus* and *T. denticola* are good predictors of further breakdown. The results suggest that there are differences between AP and GEOP loser sites. Unfortunately the low number of GEOP PD loser sites prevented comparison, and another study would be to increase patient numbers to examine any possible differences.

This is perhaps a situation where microbial assays could be useful. The assay could be used to determine whether further treatment such as re-SRP, surgery or antibiotics, may be required. Sites which responded poorly to SRP but with high levels of *P. gingivalis*, *B. forsythus* and *T. denticola*, as determined by the assay, would undergo further treatment whereas similar sites with low levels, and so less likelihood of further breakdown, could be monitored. Again this is an interesting area for further investigation.

3.2.8 Relationship between the presence or absence of the test bacteria and clinical parameters

Adult Periodontitis

Sites with *P. gingivalis* had greater mean PD which agrees with previous studies (Ali et al., 1996; Socransky et al., 1991; Wolff et al., 1993; Wikström et al., 1993; Beck et al., 1992; Mombelli et al., 1991a). Moore et al. (1983) and Kojima et al. (1993) have suggested that the presence of *P. gingivalis* is the result of deep pockets, which are an ideal environment for *P. gingivalis*, rather than the cause. However the fact that after therapy, *P. gingivalis* positive sites had higher pocket depths, bleeding on probing and gingival inflammation suggests that *P. gingivalis* has a role in the disease. The continuing high levels of *P. gingivalis* post-SRP may reflect the assay used or recolonisation by the organism. *P. gingivalis* has been shown to invade tissue (Sandros et al., 1993) and after removal of subgingival plaque, the bacteria within the tissue may have prevented satisfactory healing.

Previous studies have shown a link between *P. gingivalis* and BOP (Albandar et al., 1990; Socransky et al., 1991; Christersson, 1992; Mombelli et al., 1991a; Kojima et al., 1993). The large number of virulence factors produced by *P. gingivalis* probably result in the effective stimulation of an inflammatory response (Holt et al., 1999). The difference between these studies and the current study may reflect the effect of the other bacteria present in the pocket or aspects of the host response that disguised the effect of *P. gingivalis*, as after

treatment and reduction of the other bacteria, *P. gingivalis* positive sites showed a tendency for increased BOP.

Socransky et al. (1991) previously reported a tentative link between the presence of *P. gingivalis* and suppuration. In this study there was a significant link between the bacteria and suppuration, which reflects the organisms' ability to adversely interfere with the immune response, especially the cells. After SRP and the numbers of *P. gingivalis* were reduced slightly, these suppurating sites responded similarly to non-suppurating sites.

There was a tendency for *P. intermedia* sites to be more inflamed and display greater destruction, but not significantly. Both Christersson (1992) and Albandar et al. (1990) also failed to find a significant link between *P. intermedia* and disease. Both positive and negative sites responded similarly to treatment, which indicates that *P. intermedia* may not have had an important role in the disease, and is perhaps, as Mombelli et al. (1991a, b) suggested, an opportunistic comensal.

The role of *B. forsythus* in these patients in unclear and it is difficult to discern a pattern. *B. forsythus* was found in sites with less gingival inflammation but deeper pockets. The prevalence of *B. forsythus* decreased readily with treatment and both positive and negative sites responded similarly. This suggests that, similar to *P. intermedia*, *B. forsythus* may have a lesser role in the disease and its presence may be the result of the disease rather than the cause.

The low prevalence of *A. actinomycetemcomitans* in AP subjects means that no meaningful conclusions can be made about its effect on the severity of the disease. The low levels perhaps indicate that its role may be when the patients are younger and at the onset of disease (Slots et al., 1980; Zambon, 1985). The low levels give support to the concept that *A. actinomycetemcomitans* numbers drop as one ages (Rodenburg et al 1990; Slots et al 1990a). It is possible that *A. actinomycetemcomitans* initiates the disease process in patients allowing colonisation by opportunists such as *P. intermedia* and *B. forsythus*, and continuation of the disease process by *P. gingivalis*, once a suitable environment has been established. At what age this may occur is unclear and would make an interesting study.

The presence of *T. denticola* seemed to be related to smoking, but when corrected for multiple comparisons this was not found to be significant. The increased prevalence of *T. denticola* in smokers may indicate why some smokers are more prone to ANUG, and may reflect that *T. denticola* favours the more anaerobic environment found in pockets of smokers (Mettraux et al., 1984). The significantly increased suppuration at positive sites may suggest *T. denticola* is pathogenic in these patients and the host is ineffective in combating its presence. This is corroborated by the fact that positive sites responded less well to therapy. *T. denticola* is also able to invade tissues (Saglie et al., 1985; Riviere et al., 1991) and its continued presence may prevent a satisfactory healing response.

GEOP

In general the differences between positive and negative sites were not as pronounced as AP subjects. This may indicate that the host response rather than the pathogen is more important.

The presence or absence of *P. gingivalis*, *P. intermedia* and *T. denticola* had little effect on the clinical parameters, suggesting that these bacteria are not as important pathogens in GEOP as they may be in AP.

B. forsythus positive sites had lower gingival indices, GCF volumes and suppuration scores. The organism has been reported to be poorly immunogenic (Califano et al., 1997), and this may explain the differences.

Similarly *A. actinomycetemcomitans* positive sites had significantly lower gingival indices and GCF volumes. Again this may indicate lack of stimulation of an immune response. *A. actinomycetemcomitans* is implicated in LJP (Curtis & Darby, 2000), but its role in GEOP is less clear.

Ou Yang (1994) reported a negative correlation between *P. gingivalis* numbers and pocket depth, supporting the hypothesis that in some GEOP subjects, *P. gingivalis* is not a major pathogen. The presence of *P. gingivalis*, *P. intermedia*, and *T. denticola* could result from the presence of the pockets rather than the cause in this group of GEOP patients. Kamma et al. (1995) reported a correlation between pocket depth and numbers of *P. gingivalis*, *B. forsythus* and

A. actinomycetemcomitans, which was not found in this study. As mentioned before *P. gingivalis* predominated their GEOP patients and not *B. forsythus*. It is probable that a combination of a susceptible patient and a particular microflora are required to cause disease (Curtis & Darby, 2000). Differences between the two studies may reflect genetic differences between the study populations which predispose one group to *P. gingivalis* infection and the other to *B. forsythus* infection, or different pathogenicities of strains of these organisms found in the study populations. This would be similar to the differences in *A. actinomycetemcomitans* strains found between Europeans and Afro-Caribbean American LJP patients (Haubek et al., 1997). An interesting area of study would be to examine the pathogenicity of the strains of *P. gingivalis* and *B. forsythus* isolated from different periodontitis populations. The existing destruction without detectable levels of these organisms would indicate that other organisms may be involved or that only a small number of these pathogenic bacteria are required to initiate destruction. It may be that sites with no detectable organisms are not active and, when undergoing a period of breakdown, the numbers of organisms increases. With the design of this current study, this theory was not investigated further. This is again another exciting area for further research.

3.2.9 Clustering of bacteria

The fact that many different species can be found in the same pocket and patient, and that not all species are present in all pockets suggests that 1) different

species cause disease in different subjects, 2) periodontal disease is a mixed infection and 3) there may be microbial interaction and clustering involved.

Goodson et al. (1991), in their DNA probe study of the effect of tetracycline fibre therapy on the microflora, reported that the majority of subjects with detectable organisms had four or five present of the six investigated, but very few subjects one, two or three, suggesting that organisms tend to occur together. Bacterial combinations were examined by Söder et al. (1993) who reported the grouping of *P. gingivalis*, *P. intermedia*, *T. denticola*, *E. corrodens*, *F. nucleatum* and *C. rectus* at 24% of sites, and *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *T. denticola* at 16% of sites. *A.. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* and *T. denticola* were absent in 25% of sites examined. *T. denticola*, *E. corrodens*, *F. nucleatum* and *C. rectus* were much more prevalent in the presence of either *P. gingivalis* or *P. intermedia*. These two studies support findings by other techniques that organisms occur in combinations.

Socransky et al. (1998) reported the presence of five cluster patterns. The first was *P. gingivalis*, *B. forsythus*, and *T. denticola*, and previous studies have hinted at parts of this relationship (Gmür et al., 1989; Simonson et al., 1992; Umeda et al., 1996; Kigure et al., 1995; Söder et al., 1993). Lotufo et al. (1994) closely related the occurrence of *B. forsythus* with the presence of *P. gingivalis*. These three organisms, *P. gingivalis*, *B. forsythus* and *T. denticola*, are known to coaggregate *in vitro* (Grenier et al., 1992a; Onagawa et al., 1994; Yao et al.,

1991) and produce growth factors required by each other (Grenier et al., 1992b; Nilius et al., 1993). This first cluster was found to be related to disease levels, pocket depth and bleeding on probing. After treatment clinical improvement was linked to a reduction in these organisms (Simonson et al., 1992; Haffajee et al., 1997a). This trio form the basis of the BANA and SK-013 enzyme tests which have shown good correlation with disease status also (Loesche et al., 1992b; Seida et al., 1992).

The second cluster was *F. nucleatum*, *P. intermedia*, *P. nigrescens*, *P. micros*, *E. nodatum*, and *C. rectus*. This grouping has also been suggested in part by previous studies (Ali et al., 1994; Wikström et al., 1993; Socransky et al., 1988; Söder et al., 1993). Ali et al. (1994) reported *P. intermedia* in all *F. nucleatum* positive sites, as well as increases in *T. denticola* and *P. gingivalis*.

S. sanguis, *S. oralis*, *S. mitis*, *S. gordonii* and *S. intermedia* comprised the third complex. A previous study by Socransky et al. (1988) had again reported parts of this group and linked it with less periodontal disease and better response to treatment.

The fourth and fifth groupings were *Capnocytophaga* species, *C. concisus*, *E. corrodens*, *A. actinomycetemcomitans* serotype a and *V. parvula*, *A. odontolyticus*, *A. actinomycetemcomitans* serotype b, *S. noxia* and *A. naeslundii* respectively. Wikström et al. (1993) had again previously reported

aspects of the fourth grouping, but had also found associations between *P. gingivalis*, *P. intermedia*, and *Capnocytophaga* species.

In this current study, *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola* showed increased prevalences when one of the others was present in AP subjects, although this may reflect the deeper pocketing associated with these organisms rather than clustering. The low levels of *A. actinomycetemcomitans* prevent any determination of its effect on the other bacteria. Interestingly when one of *P. gingivalis*, *B. forsythus* and *T. denticola* is reduced following SRP, the other two also decreased indicating a grouping of these bacteria, which is suggestive of the first cluster of Socransky et al. (1998). Haffajee et al. (1997a) found that following SRP these three organisms decreased significantly, but did not report on the correlation between the reduction of the bacteria. In GEOP patients *B. forsythus* was significantly higher at *P. intermedia* negative sites and vice versa, suggesting an antagonistic relationship. *B. forsythus* was found in all *T. denticola* positive sites, confirming earlier reports by Socransky et al. (1998) and Umeda et al (1996) of a relationship between these bacteria. Contrary to these reports there was no discernible relationship with *P. gingivalis*. *B. forsythus* was also found at all *A. actinomycetemcomitans* positive sites and *T. denticola* significantly increased suggesting a grouping between these three bacteria as well.

It is possible that one of the differences between AP and GEOP, apart from microflora and host response, is the manner in which the subgingival flora

interact to form different groupings. It would also appear that, in addition to each subject having a unique flora, there may be unique cluster patterns.

Evidence from animal studies has shown that combinations of species were capable of inducing experimental abscesses, even though individual components in the mixture could not (Haffajee & Socransky 1994; Dahlén et al., 1989b).

The concept of microbial groupings, shown in this study and by previous studies, is an exciting area requiring further investigation. A number of aetiological agents have been identified, but we are still far from understanding the role of these organisms in periodontal disease. An examination of bacterial interactions should improve our understanding and perhaps also elucidate some aspects of the immune response to these organisms. It is probable that interaction between organisms is mutually beneficial in terms of survival, but may be also in evading or combating host defences. The formation of a biofilm is one example of this (Darveau et al., 1997).

3.2.10 Absence of organisms

Socransky et al. (1991) and Wolff et al. (1993) reported that a sizeable percentage of sites with severe periodontal destruction did not contain any of the organisms under investigation. In both studies shallower sites had a greater percentage of sites without detectable study organisms (37.5% and 80% respectively), moderate sites fewer (30.5% and 70%) and the deepest sites fewest (21.9% and 48%). In addition, Goodson et al. (1991) reported that in 12.6% of

sites tested (about 1% of subjects) none of the test organisms were detected. In this current study 23.5% of AP sites were without detectable organisms and 3.5% of GEOP sites. This difference between AP and GEOP could be a factor in the greater destruction seen in GEOP patients. In AP patients 24.5% of shallower sites were not positive for any of the test bacteria, and this decreased to 16.7% in sites over 7mm. As the pocket depth increased in GEOP subjects the percentage of sites with no detectable test bacteria decreased, shallower sites 5.8% and sites over 7mm 0%. The percentages recorded in this study are less than those noted by Socransky et al. (1991) and Wolff et al. (1993). The differences in percentages between these two studies are due to the much smaller number of organisms investigated by Wolff et al. (1993). Wolff et al. (1993) examined the presence of 5 organisms, similar to this current study, and one would perhaps expect the results to be similar also. However, the presence of the bacteria in this thesis was determined by PCR, which is more sensitive than the method used by Wolff et al. (1993) and probably accounts for the differences. In all three studies the percentage of sites without detectable bacteria decreased as the pocket depth increased, which presumably reflects the suitability of deeper sites for colonisation by these anaerobic organisms. The lack of organisms at detectable levels in some sites, indicates that either small numbers of organisms are sufficient to cause disease in these sites or, more likely, other organisms are responsible, which supports the hypothesis that a wide range of organisms can cause disease. Exactly which organisms these may be, whether other bacteria, or viruses is yet to be determined. Studies which

examine a wide range of bacteria present in samples are required and the Checkerboard would seem to be an ideal technique for this.

CHAPTER 4

COMPARISON OF PCR AND THE CHECKERBOARD TECHNIQUE

This section deals with the comparison of PCR and Checkerboard diagnostic assays. A comparison of the two techniques is presented first and then the effect of SRP on the microflora using Checkerboard to determine prevalences is discussed. In addition a comparison of the prevalences of the bacteria with other studies using DNA probes, Checkerboard and PCR is made. All patient plaque samples were used in the analysis of PCR and Checkerboard. Both AP and GEOP patients had a third set of plaque samples taken four months after the post-SRP samples. These samples are only included in this section and have not been used elsewhere in this thesis. A total of 620 plaque samples were available for comparison.

4.1 Comparison of PCR and the Checkerboard technique: Results

Figures 4.1 to 4.5 show the typical analysis of the PCR products for each of the test bacteria. In addition, figures 4.6 and 4.7 show the typical results of the Checkerboard technique. After the first run (Figure 4.6), it became apparent that there were problems with the controls applied in Glasgow and new controls were applied to the membranes in Gothenberg (Figure 4.7).

The percentage of positive sites for all samples and all test organisms using PCR and the Checkerboard technique is shown in table 4.1. *P. gingivalis* was detected at 38.4% of sites by PCR compared to 20.5% by the Checkerboard technique. *B. forsythus* was found significantly more by the Checkerboard technique than PCR, 46.6% compared to 25.5%. The detection rates for *A. actinomycetemcomitans* were similar at 3.1% and 3.2% for PCR and the

Figure 4.1 *P. gingivalis* PCR product analysis. Base pair ladder (BP), lanes 1-20 and positive (+) and negative (-) controls

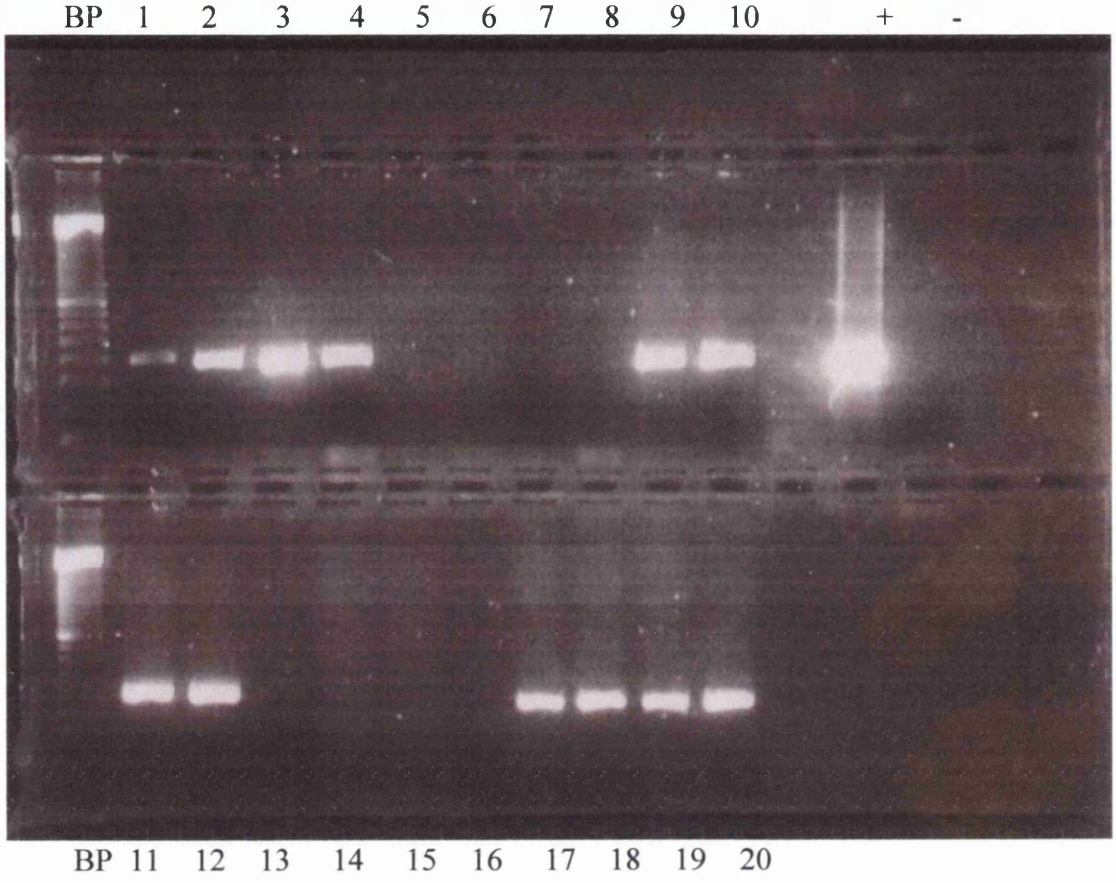


Figure 4.2 *P. intermedia* PCR product analysis. Base pair ladder (BP), lanes 1-20 and positive (+) and negative (-) controls

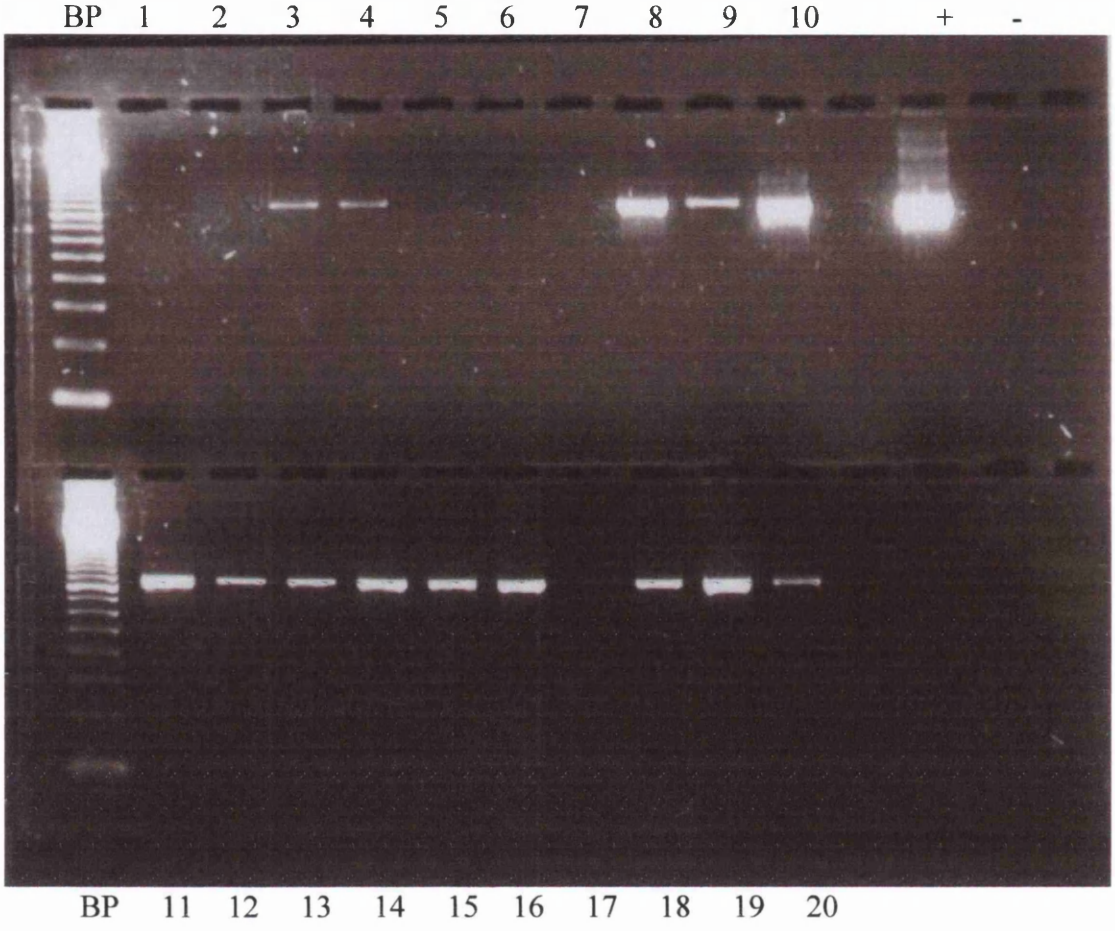


Figure 4.3 *B. forsythus* PCR product analysis. Base pair ladder (BP), lanes 1-20 and positive (+) and negative (-) controls

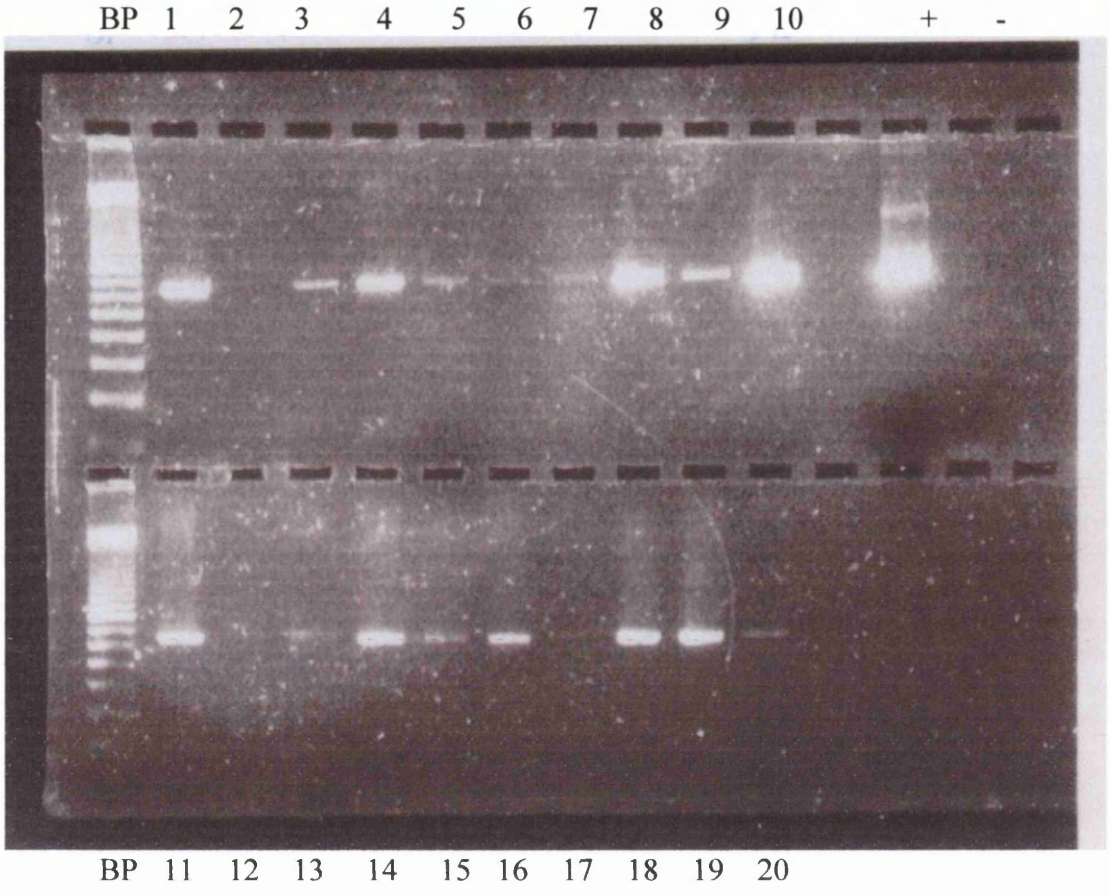


Figure 4.4 *A. actinomycetemcomitans* PCR product analysis. Base pair ladder (BP), lanes 1-22 and positive (+) and negative (-) controls

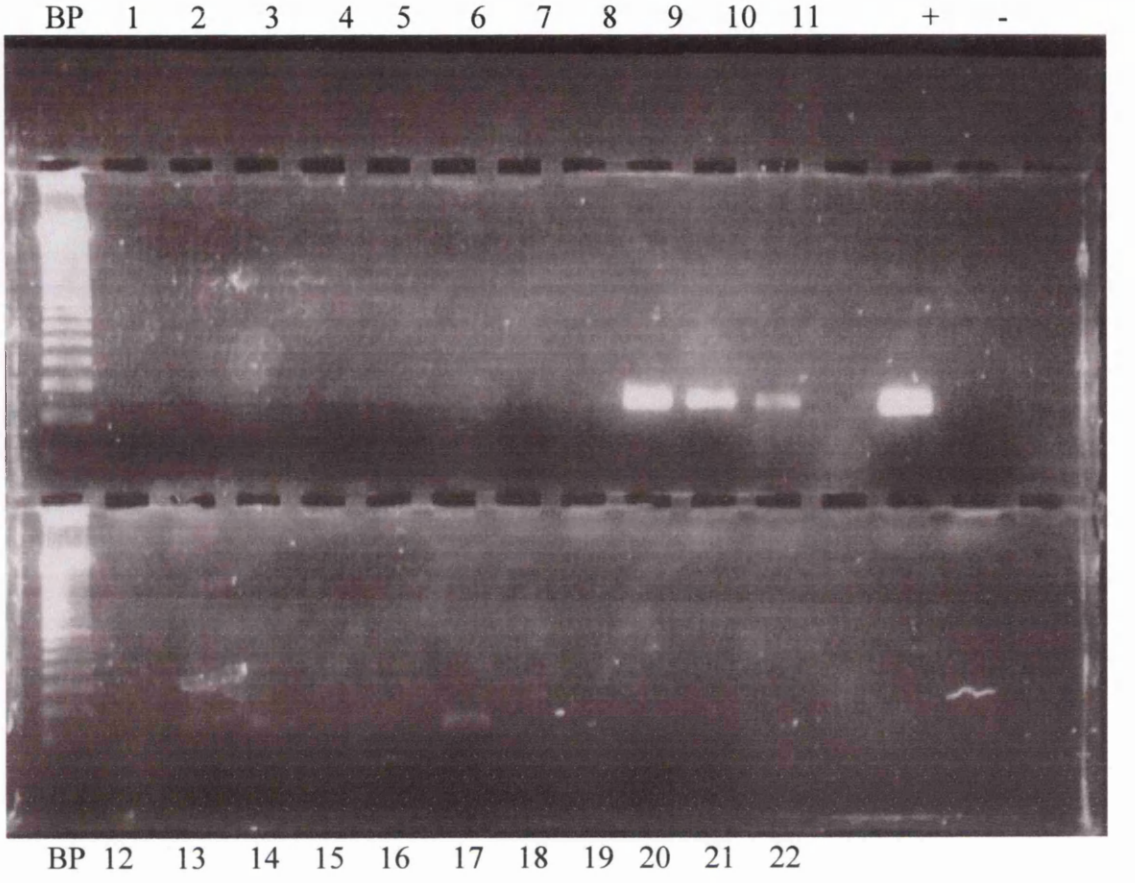


Figure 4.5 *T. denticola* PCR product analysis. Base pair ladder (BP), lanes 1-20 and positive (+) and negative (-) controls

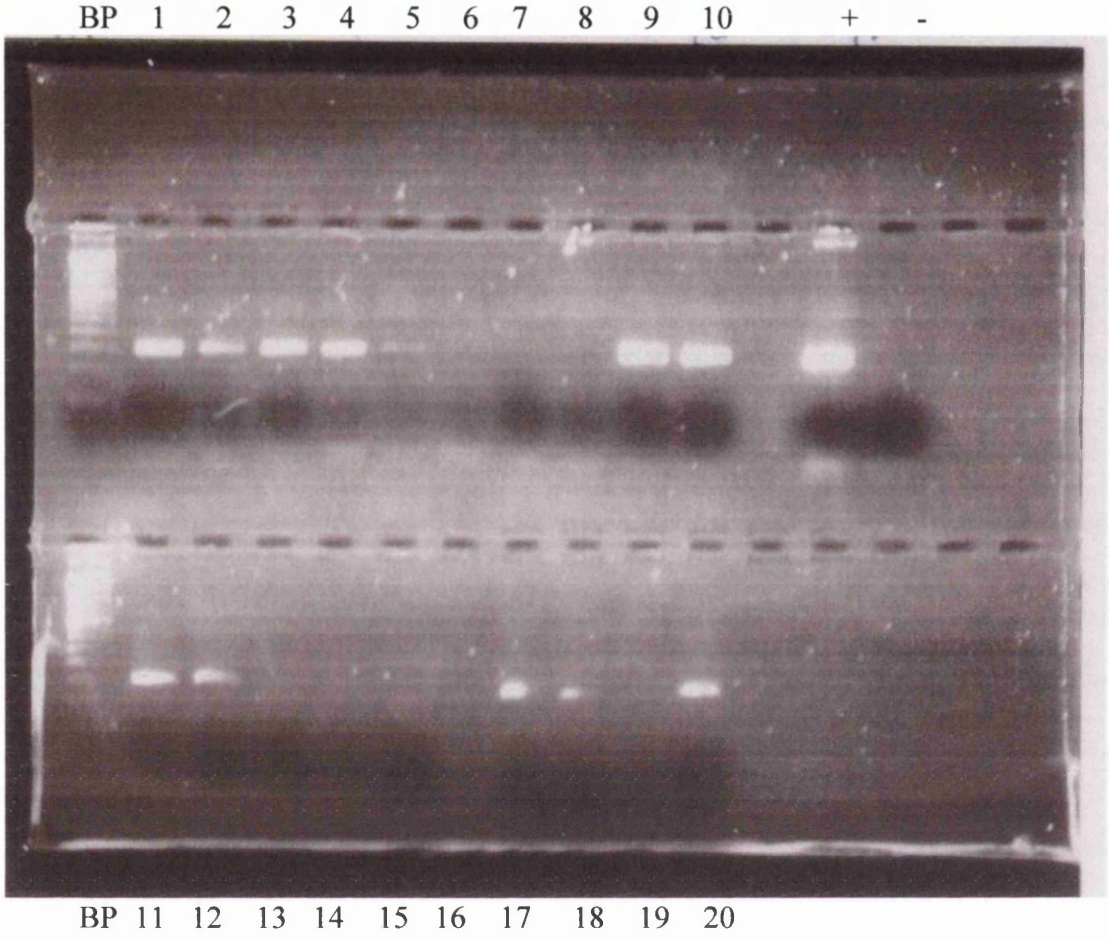


Figure 4.6 Results of Checkerboard technique (without controls). Lanes used for each probe and black dots indicate hybridisation reactions.

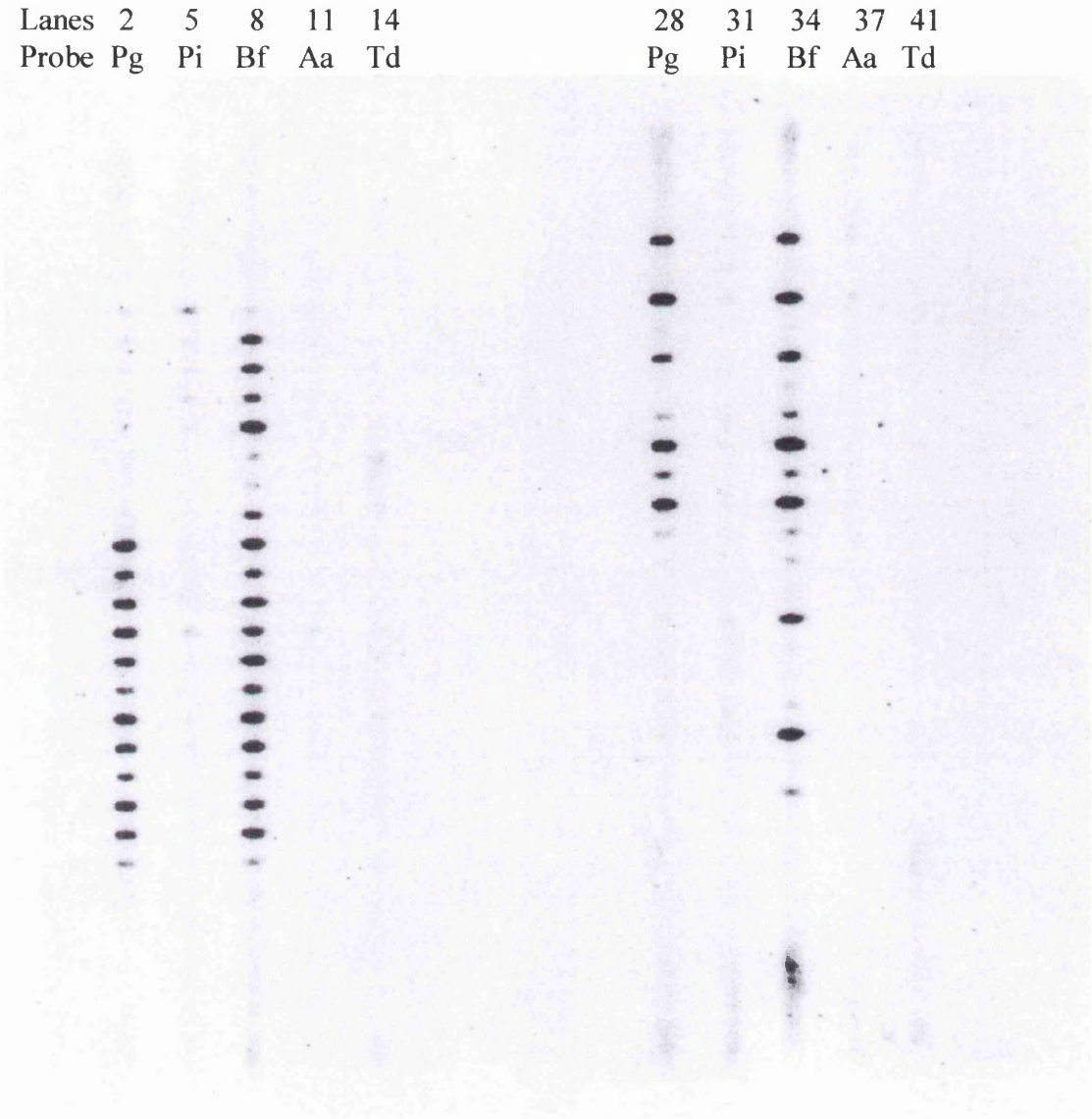


Figure 4.7 Results of Checkerboard technique (with controls). Lanes (Lan) used for each probe (Pro) and black dots indicate hybridisation reactions. Controls were mixed bacteria of 10^5 and 10^6 concentrations and placed at the bottom of each membrane (10^5 above 10^6).

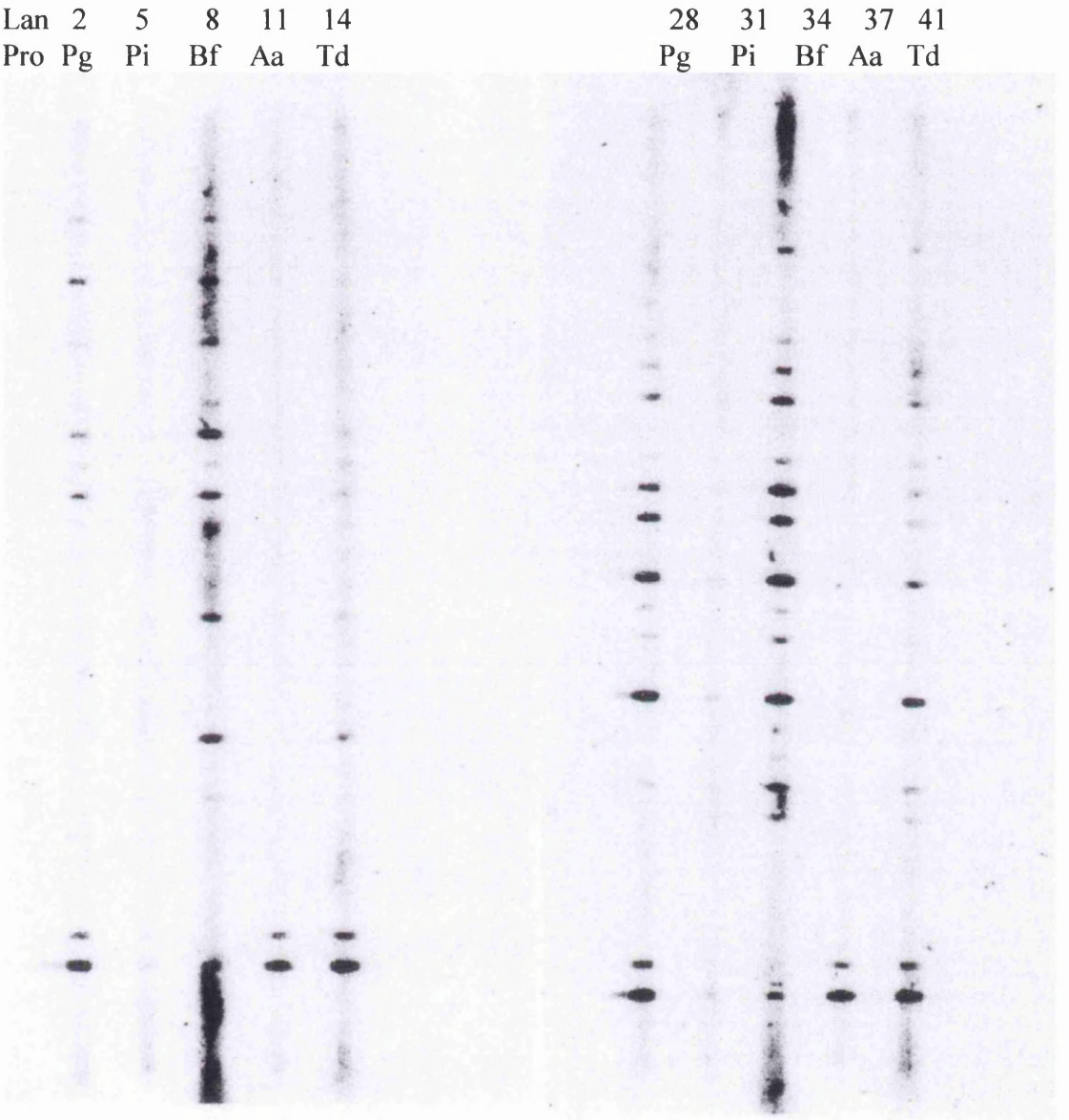


Table 4.1 Agreement and disagreement between PCR and the Checkerboard technique for all samples with results expressed as a percentage

Organism	%PCR+	%XB+	%Agreement	%Disagree	%PCR+/XB-	%PCR-/XB+	p value
Pg	38.4	20.5	63.4	36.6	27.2	9.4	<0.0001
Bf	25.5	46.6	59.2	40.8	9.8	31.0	<0.0001
Aa	3.1	3.2	94.4	5.6	2.7	2.9	1.0
Td	43.7	22.4	59.7	40.3	30.8	9.5	<0.0001

Checkerboard technique respectively. PCR detected *T. denticola* at 43.7% of sites compared to 22.4% by the Checkerboard technique. The differences in the detection frequencies for all test organisms except *A. actinomycetemcomitans* were statistically significant ($p<0.0001$).

The agreement and disagreement of the techniques is shown in tables 4.1 and 4.2. There was 63.4% agreement in the detection of *P. gingivalis*. 27.2% of sites were PCR positive and Checkerboard technique negative, compared to 9.4% Checkerboard technique positive and PCR negative. The detection of *B. forsythus* had the lowest agreement at 59.2%. The Checkerboard technique detected *B. forsythus* at 31% of sites that were PCR negative and PCR at 9.8% of sites that were Checkerboard technique negative. The agreement between the techniques in the detection of *A. actinomycetemcomitans* was the highest at 94.4% with similar percentages of PCR positive/Checkerboard technique negative and Checkerboard technique positive/PCR negative sites. The techniques agreed at 59.7% of sites in the detection of *T. denticola*. PCR detected *T. denticola* at just over 30% of Checkerboard technique negative sites and Checkerboard technique at 9.5% of PCR negative sites.

Specificity and sensitivity of the Checkerboard technique is shown in table 4.3, using PCR as the gold standard. Sensitivity ranges from 0.1 for *A. actinomycetemcomitans* to 0.61 for *B. forsythus*, and sensitivity from 0.58 for *B. forsythus* to 0.97 for *A. actinomycetemcomitans*.

Table 4.2 Agreement and disagreement between PCR and the Checkerboard technique (XB) for a samples with results expressed on a sample basis

Microorganism	Both +ve	PCR+/XB-	PCR-/XB+	Both -ve
Pg	68	169	58	325
Bf	97	61	192	270
Aa	2	17	18	583
Td	80	190	60	290

Table 4.3 Specificity and sensitivity of the Checkerboard technique compared to PCR. PCR was used as the "gold standard".

Organism	Sensitivity	Specificity
Pg	0.29	0.85
Bf	0.61	0.58
Aa	0.1	0.97
Td	0.30	0.83

Table 4.4 Effect of SRP on the AP and GEOP microflora using the Checkerboard technique. Percentage of sites positive for each organism before and after SRP

Organism	AP Pre	AP Post	p value	GEOP Pre	GEOP Post	p value
Pg	50	11.6	<0.0001	15.6	7.3	0.16
Bf	79.6	33.9	<0.0001	57.3	26.0	0.0001
Aa	5.3	2.7	0.29	3.1	1.0	0.63
Td	35.6	8.9	<0.0001	40.6	12.5	0.0001

4.1.1 Effect of SRP on microbial prevalences as determined by the Checkerboard technique

Adult Periodontitis

Table 4.4 shows the effect of treatment of the AP flora using the Checkerboard technique to identify the test bacteria. At baseline 50% of sites were positive for *P. gingivalis*, 79.6% for *B. forsythus*, 5.3% for *A. actinomycetemcomitans* and 35.6% for *T. denticola.* After treatment there were significant reductions in the detection of *P. gingivalis* (11.6%, <0.0001), *B. forsythus* (33.9%, <0.0001) and *T. denticola* (8.9%, <0.0001). *A. actinomycetemcomitans* detection was reduced by approximately half following treatment but this was not statistically significant.

GEOP

There were fewer significant reductions in the prevalence of the test organisms before and after treatment in GEOP patients using the Checkerboard technique to identify the test organisms (table 4.4). *B. forsythus* decreased from 57.3% to 26%, and *T. denticola* to 12.5% from 40.6%. Both were significant at p=0.0001. *P. gingivalis* was reduced from 15.6% to 7.3%, and *A. actinomycetemcomitans* reduced by 2% from 3.1% to 1%. These were not significant.

4.2 Comparison of PCR and the Checkerboard technique : Discussion

4.2.1 Comparison of the two techniques

The development of new technologies or diagnostic tests always raises the issue as to what is the appropriate primary test, the so-called "gold standard". The

definition of the "gold standard" is essential, in order to evaluate the diagnostic performance of the new technology (Papapanou et al., 1997a). In assessment of the composition of plaque microbiota, culture procedures have traditionally been used as the reference (Moore & Moore, 1994). It has been recognised that culture can no longer be regarded as devoid of methodological errors and is not a suitable "gold standard" (Loesche et al., 1992a; Riggio et al., 1996).

Both polymerase chain reaction and the Checkerboard technique offer significant advantages over culture. They are quicker, with results available in a few hours, cheaper and less labour intensive (Riggio et al., 1996; Papapanou et al., 1997a). The use of DNA probes and primers enables a more accurate identification of bacteria and do not rely on cell viability. Both have lower detection limits, which is important in detecting such organisms as *A. actinomycetemcomitans* which occur in low levels, even in severely diseased patients (Papapanou et al., 1997b). It is difficult to detect by culture organisms which represent 1% or less of the flora (Moncla et al., 1991).

A comparison of the two techniques is warranted because of the possibility of cross-reactivity of whole genomic probes, non-optimal stringency of the Checkerboard technique and the accuracy of PCR. With the lower detection limit of PCR some disagreement would be expected, in favour of PCR. However the analysis showed a number of unexpected discrepancies. Agreement between the two techniques for *P. gingivalis*, *B. forsythus* and *T. denticola* ranged from 59.2% to 63.4% percent and perhaps could be accounted

for by the lower detection limit for PCR. However a much lower frequency for *P. intermedia* was recorded by the Checkerboard technique suggesting more than just the variation in detection limits. Hence the results for *P. intermedia* probes were not included in the subsequent data analysis.

Sample analysis using the Checkerboard technique suffered a number of problems. The controls that were applied initially were not detected on probing and new controls had to be reapplied. This involved re-baking the membranes, which would have affected the DNA on the membrane, probably resulting in loss of material. The controls and samples consistently came up faintly with the *P. intermedia* probes, even after stripping of the membranes and re-probing. This suggested that the probes were not binding properly, but subsequent testing on other samples on different membranes could not find fault with them. The reasons for the *P. intermedia* probes not performing optimally are still unclear.

B. forsythus was detected much more frequently by the Checkerboard technique than PCR. This may have resulted from cross-reactivity of the probe or non-specific binding. Cross-reactivity of the probe is unlikely as the probes have been rigorously tested and there are no reports of previous problems of *B. forsythus* probes cross-reacting to such an extent. The stringency of the technique is quite high and there were no problems with background contamination on the membranes for the other probes. Again it is possible that the re-baking of the membrane may have affected the results by denaturing the DNA further and allowing non-specific binding to this newly exposed DNA.

A. actinomycetemcomitans was detected equally by both techniques. There were differences between AP and GEOP samples but overall the results were similar. The low prevalence of *A. actinomycetemcomitans* by both techniques suggests that *A. actinomycetemcomitans* was actually present in low frequencies in this patient group. However *A. actinomycetemcomitans* has been shown to clump together and form chains that cannot be broken up by vortexing (Fives-Taylor et al., 1999). If this is the case both PCR and the Checkerboard technique would underestimate the presence of this organism.

The plaque samples were thoroughly mixed and divided in two in an attempt to produce two identical samples. Robrish et al. (1976) and Olsen & Socransky (1981) showed that a fully homogeneous suspension of plaque samples is not achievable. Consequently it is likely that the plaque samples were not identical. However, Papapanou et al. (1997a) suggested that these errors must be considered random and could not introduce a systemic bias towards a specific direction. The results from the Checkerboard technique were read by eye and correlated with computer printouts of intensity of the chemiluminescence of the reactions, which automatically took into account the background intensity and controls. It is therefore unlikely to be an error in assessing the reactions visible by eye. Another possible explanation may be an increasing degree of non-specific binding of whole genomic probes in the presence of plenty of target DNA and may reflect impaired specificity of the technology in heavy plaque samples. However, this would be equally applicable to both PCR and the

Checkerboard technique, and would affect all samples. Given the discrepancies between these two techniques, the problems with the Checkerboard technique analysis and also bearing in mind the absence of an indisputable reference standard, no conclusions may be drawn from the comparison of these two techniques.

The Checkerboard technique reported greater and more significant reductions in the flora following therapy. A comparison with the PCR results showed that PCR sites had higher numbers of positive sites before treatment. This is to be expected given the lower detection limits of PCR and the decreased microbial load in pockets after treatment, which may have allowed positive detection by only PCR. The relatively poor reduction in the flora after treatment as monitored by PCR compared rather unfavourably to studies by culture and DNA probes. This suggests that PCR might be too sensitive, and was detecting low numbers of bacteria that were now associated with health rather than disease. Mombelli et al. (1991b) questioned the use of techniques with higher diagnostic value, and suggested that they may detect an organism at a level too low to have any pathogenic impact. In such a situation one would wish that the threshold for detection would coincide with the threshold for initiation of pathology. The problem is compounded by the fact that different organisms may have different thresholds for causing disease (Bragd et al., 1987; Moncla et al., 1991). As yet an ideal technique for the accurate detection of pathogens in subgingival plaque samples has yet to be developed. The Checkerboard technique is promising and allows the detection of many bacteria at the same time. PCR is the most accurate

technique to date, but limited in that it can only detect a few organisms at the same time and as yet is not quantitative. At the moment it would seem that PCR is best suited for molecular analysis rather than microbial diagnosis.

4.2.2 Prevalence of periodontal microflora using DNA probe and the Checkerboard technique diagnostic assays

A number of studies have analysed the presence of periodontal bacteria using DNA probes and the Checkerboard technique. The results of the Checkerboard technique analysis compare favourably, except for the low prevalence of *P. intermedia.*

4.2.2.1 Prevalence of periodontal microflora using DNA probes

Using DNA probes, *P. gingivalis* frequencies on a subject basis range from 61% (Lowenguth et al., 1995) to 95% (Söder et al., 1993) for AP subjects and 68.2% for GEOP (Albandar et al., 1997). The organism has been detected in 31% of AP sites (Kojima et al., 1993) to 89% (Maiden et al., 1991). Generally *P. gingivalis* is found at high rates of detection (Savitt & Socransky 1984; Loesche et al., 1992b; Goodson et al., 1991; Zappa et al., 1990; Ali et al., 1994; Yasui et al., 1993). The number of positive sites for *P. gingivalis* is related to increasing pocket depth and the presence of bleeding on probing (Kojima et al., 1993). The prevalence of *P. gingivalis* in AP patients reported in this study using the Checkerboard technique was 50% and is within the range of the studies reported above. The prevalence of *P. gingivalis* in GEOP was much lower than

that reported by Albandar et al. (1997). This may reflect variations in microbial colonisation and aetiology between study populations.

P. intermedia has been reported in 86% (Goodson et al., 1991) to 98% (Haffajee et al., 1992) in AP subjects and 82% of GEOP subjects (Albandar et al., 1997), and from 42% to 92% of AP sites, with frequencies generally above 60% (Ali et al., 1994; Shiloah & Patters, 1994; Savitt & Socransky 1984; Zappa et al., 1990; Maiden et al., 1991; Gersdorf et al., 1993; Lippke et al., 1991; Lowenguth et al., 1995; Söder et al.; 1993). The technical problems with *P. intermedia* in this study prevent comparison with these studies.

On a subject basis, Haffajee et al. (1992) detected *B. forsythus* in 26% of AP patients and from 52% (Tanner et al., 1997a) of AP sites to 83% (Lippke et al., 1991). *B. forsythus* prevalences for AP and GEOP groups in this thesis are also within the range previously reported.

Using DNA probes the prevalence of *A. actinomycetemcomitans* in AP subjects ranges from 0% (Zappa et al., 1990) to 75% (Söder et al., 1993), and has been detected in 21% of GEOP subjects (Albandar et al., 1997). On an AP site basis the range is from 0% (Lowenguth et al., 1995; Zappa et al., 1990) to 44% (Savitt & Socransky, 1984) though it is generally low (Ali et al., 1994; Maiden et al., 1991; Loesche et al., 1992b; Lowenguth et al., 1995; Goodson et al., 1991). The low prevalences of *A. actinomycetemcomitans* in both disease groups found in this thesis is in keeping with previous reports.

Söder et al. (1993) reported that one hundred percent of AP subjects were positive for *T. denticola* and Albandar et al. (1997) 86.5% of GEOP subjects. Detection on a site basis ranged from 61% (Söder et al., 1993) to 87% (Loesche et al., 1992b). In comparison with these studies, *T. denticola* was found at lower prevalences. This may reflect differences in study populations, sampling techniques or detection limits of the probes used.

The majority of the site based studies used deep pockets (5mm or greater) and there are generally high levels for most of the above organisms. *A. actinomycetemcomitans* is found in much lower levels than the other organisms. Overall there is good agreement between the results presented in this thesis and published studies. The results indicate that high prevalences of *P. gingivalis*, *B. forsythus* and *T. denticola* are found in deep sites in advanced periodontitis patients. The infrequent detection of *A. actinomycetemcomitans* may indicate a lesser role in advanced disease in AP subjects compared to the other three bacteria mentioned above.

4.2.2.2 Prevalence of periodontal microflora using the Checkerboard technique

The Checkerboard technique has been used to investigate the presence of a number of periodontal bacteria in subgingival plaque samples. Haffajee et al. (1995) reported the most frequently detected organisms in AP patients were *S. mitis* (49%), *F. nucleatum* subsp *vincentii* (48%), *P. micros* (41%), *V. parvula*

(40%), *B. forsythus* (39%), *P. intermedia* (37%), *S. oralis* (36%), *F. nucleatum* subsp *nucleatum* (36%), *P. nigrescens* (35%) and *E. corrodens* (35%). *T. denticola* was detected in 18% of sites and *A. actinomycetemcomitans* serotype b in 10%. The presence of *P. gingivalis*, *P. micros*, *T. denticola*, *P. nigrescens*, and *F. nucleatum* subsp *nucleatum*, *B. forsythus* and *P. intermedia* were positively correlated with pocket depth.

In a later study, Haffajee et al. (1997a) reported that *P. gingivalis* and *B. forsythus* colonised over 50% of sites over 6mm in AP patients. Overall *B. forsythus* was found at roughly 45% of sites, *T. denticola* 35% and *P. gingivalis* 20%. By far the most abundant organism was *A. viscosus*, which was detected in 48% of sites.

Tanner et al. (1998a) used the Checkerboard technique to examine the microbiota in health, gingivitis and initial periodontitis. Sites which lost attachment of more than 1.5mm were deemed active and higher mean levels of *B. forsythus* and *C. rectus* were found in these sites compared to inactive periodontitis. *P. gingivalis* and *A. actinomycetemcomitans* were detected infrequently.

Recently, Dibart et al. (1998) used the Checkerboard technique to identify bacterial species present in or on crevicular epithelial cells in healthy and diseased sites. The most frequently detected species in healthy subjects were *S. oralis* (63%), *S. intermedius* (26%), and *T. denticola* (26%), and in periodontitis

subjects *B. forsythus* (75%), *P. intermedia* (54%), and *F. nucleatum* (50%). *P. intermedia*, *C. ochracea*, *C. rectus* and *B. forsythus* were significantly more common in periodontitis sites compared to health.

In comparison with these studies from the Forsyth Dental Centre, *P. gingivalis* and *B. forsythus* prevalences reported in this thesis are higher. However these studies examined a range of pocket depths from shallow to deep, and when the results were stratified according to pocket depth (Haffajee et al., 1997a), at sites over 6mm, similar prevalences were found. In all studies *A. actinomycetemcomitans* was detected infrequently, and *T. denticola* prevalences were similar.

However, Papapanou et al. (1997b), in their examination of the subgingival microbiota in adult Chinese subjects, reported *P. gingivalis*, *P. intermedia*, *F. nucleatum*, *P. micros* and *C. rectus* in all patients. *A. actinomycetemcomitans* was found in 83% of subjects, *B. forsythus* 99% and *T. denticola* 98%. *P. gingivalis*, *P. nigrescens*, *P. intermedia*, *T. denticola* and *B. forsythus* were associated with deep pockets. The high levels of detection for all organisms may reflect the lack of dental care in general. Oral hygiene and dental attendance were poor. The relatively better dental attendance and oral hygiene standards in Western populations may account for the differences between the study by Papapanou et al. (1997b) and the results in this thesis and those from Boston. Despite much higher prevalences in Chinese patients similar results were

produced in all studies associating *P. gingivalis*, *B. forsythus* and *T. denticola* with deeper pockets.

4.2.3 Analysis of the response of the microflora to SRP using DNA probes and the Checkerboard technique

Very few studies have investigated the effect of SRP on the subgingival microflora using DNA probes. After SRP there seems to be a marked reduction in the frequency of detection for *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia* (Shiloah & Patters, 1994; Lowenguth et al., 1995) and also for *E. corrodens*, *F. nucleatum* and *C. rectus* (Lowenguth et al., 1995). This reduction is maintained for up to twelve months after treatment except for *P. gingivalis* which started to increase after six months (Lowenguth et al., 1995).

Haffajee et al. (1997a) investigated the effect of SRP on 57 patients and compared the flora before and after SRP using the Checkerboard technique. The mean prevalences of *P. gingivalis*, *T. denticola* and *B. forsythus* were significantly reduced after SRP, while *A. viscosus* showed a significant increase. *P. gingivalis*, *T. denticola* and *B. forsythus* were equally prevalent among current, former and never smokers, and decreased significantly post-SRP in never and former smokers, but increased in current smokers. Clinical improvement was accompanied by a modest change in the subgingival microflora, primarily a reduction in *P. gingivalis*, *T. denticola* and *B. forsythus*.

From this study the group of patients that did not respond to treatment was examined in more detail (Haffajee et al., 1997b). In subjects which responded poorly, compared to those that responded well, *A. naeslundii* genospecies 2 (*A. viscosus*), *P. gingivalis*, *T. denticola*, *B. forsythus*, *C. gracilis* and *C. rectus* were all found at lower levels pre-treatment.

In keeping with these previous studies, SRP produced significant reductions in the percentage of samples positive for *P. gingivalis*, *B. forsythus* and *T. denticola* in AP subjects and *P. gingivalis* and *T. denticola* in GEOP subjects. The reduction in these bacteria was also accompanied by a significant improvement in clinical condition, as also shown by Haffajee et al. (1997a). However studies using DNA probes also produced significant reductions in *A. actinomycetemcomitans* prevalence, whereas the reduction observed in this thesis and the other Checkerboard technique reports are not as great. This may reflect the lower prevalences of *A. actinomycetemcomitans* reported using the Checkerboard technique or differences between assay techniques.

4.2.4 Prevalence of periodontopathogens using PCR

The prevalences of the bacteria discussed in this section are those which have been previously mentioned in Chapter 3 and not the overall prevalence for all samples as mentioned above.

Varying prevalences of periodontopathogens have been reported using PCR. In adult periodontitis, *P. gingivalis* has been detected in 28% to 79% of subjects

(Riggio et al., 1996; Griffen et al., 1998; Wahlfors et al., 1995; Leys et al., 1994; Amano et al., 1999). *P. gingivalis* was much more prevalent in diseased patients than healthy subjects (Griffen et al., 1998). Similarly varying prevalences of *A. actinomycetemcomitans* have been reported, from 40% to 60% in AP patients (Riggio et al., 1996; Wahlfors et al., 1995; Leys et al., 1994). However these two organisms were not often found together (Riggio et al., 1996). *P. intermedia* had been detected in 39% of AP patients using primers designed to prevent cross-reactivity with *P. nigrescens* (Riggio et al., 1998), and *B. forsythus* in 89.7% of subjects (Meurman et al., 1997).

The prevalence of periodontal pathogens in advanced periodontitis (age 15-75 years) has been investigated in two reports from Slots' group (Slots et al., 1995; Ashimoto et al., 1996). The prevalences of *A. actinomycetemcomitans* ranged from between 30% to 53%, *B. forsythus* 86-91%, *C. rectus* 74-77%, *E. corrodens* 76-80%, *P. gingivalis* 63-70%, and *P. intermedia* 58-67%. Ashimoto et al. (1996) also detected *P. nigrescens* in 52% of sites and *T. denticola* in 54% of sites. The prevalence of all these organisms except *E. corrodens* was significantly higher in periodontitis patients than gingivitis patients (Ashimoto et al., 1996).

The prevalences of the bacteria reported in this study are generally within the range of the reports above. *A. actinomycetemcomitans* prevalences were low and may reflect differences in study populations, primers used, cycling conditions, sample collection or the reagents and thermocycler used. A comparison of

prevalences between GEOP groups is difficult because the author is unaware of any studies that have used PCR to study GEOP bacterial prevalences. However, the comments made with regard to the AP prevalences are equally relevant to the GEOP prevalences.

One report studied the change in *A. actinomycetemcomitans* detection with SRP and found that there was no significant change (Takamatsu, 1997). The results in this thesis also produced a non-significant change in *A. actinomycetemcomitans*, but prevalences were very low to begin with.

CHAPTER 5

THE HUMORAL IMMUNE RESPONSE IN ADULT PERIODONTITIS AND EFFECT OF SRP

This chapter discusses the results obtained from the analysis of the AP patient antibody titres and avidity. Only the PCR analysis of the plaque samples was used in this chapter in the determination of the microflora.

5.1 Humoral immune response in adult periodontitis and effect of SRP: Results

5.1.1 Effect of SRP on serum and GCF antibody titres and serum antibody avidity

Tables 5.1, 5.2 and 5.3 show the effect of SRP on serum antibody titres and avidity and GCF antibody titres. Post-treatment *P. gingivalis* median serum titres increased slightly, but for the other test organisms there was a decrease. None of these changes were statistically significant. The median change in titres indicated slight rises in titres for *P. intermedia*, *B. forsythus* and *T. denticola*, but no changes for *P. gingivalis* and *A. actinomycetemcomitans*.

Antibody avidity decreased after therapy for four of the test organisms. The avidity of *B. forsythus* antibodies increased slightly. The decrease in avidity for *P. gingivalis* and *P. intermedia* antibodies was significant ($p = 0.024$ and 0.025 respectively).

Post-SRP there was a slight rise in *P. gingivalis* median GCF antibody titres, but there were no statistically significant changes in the other titres.

Table 5.1 Comparison of median serum titres before and after SRP in AP patients. Interquartile ranges shown in brackets. Titres shown as EU.

Organism	Serum Pre	Serum Post	Change	p value
Pg	219 (8, 6229)	246 (9, 24506)	0 (-105, 201)	1
Pi	216 (5, 1278)	177 (3, 741)	-3 (-138, 22)	0.62
Bf	753 (12, $1x10^7$)	72 (10, $1x10^6$)	-9 (-7544, 7)	0.69
Aa	36 (1, 306)	17 (1, 241)	0 (-32, 4)	0.88
Td	2458 (1, $7.8x10^8$)	239 (1, 814159)	-5 ($-7x10^7$, 0)	0.4

Table 5.2 Comparison of mean antibody avidity before and after SRP in AP patients. SD shown in brackets. Avidity shown as concentration (M) at ID_{50}.

Organism	Avidity Pre	Avidity Post	Change	p value
Pg	1.36 (±0.7)	1.1 (±0.5)	0.21 (±0.47)	0.024
Pi	0.86 (±0.3)	0.75 (±0.25)	0.1 (±0.23)	0.025
Bf	1.14 (±0.7)	1.2 (±0.7)	-0.06 (±0.66)	0.62
Aa	0.68 (±0.4)	0.65 (±0.3)	0.03 (±0.23)	0.44
Td	0.64 (±0.3)	0.61 (±0.4)	0.03 (±0.52)	0.76

Table 5.3 Comparison of median GCF titres before and after SRP in AP patients treatment. Interquartile ranges shown in brackets. GCF titres shown as EU/30s.

Organism	GCF Pre	GCF Post	Change	p value
Pg	26 (12, 63.3)	31 (13, 125)	3 (-3, 34)	0.2
Pi	6 (5, 8)	6 (5, 8)	0 (-1, 1)	0.84
Bf	0 (0, 1)	0 (0, 1)	0 (0, 0)	0.6
Aa	0 (0, 13)	0 (0, 34)	0 (0, 14)	0.1
Td	3 (0, 9)	1 (0, 7)	0 (-3, 2)	0.73

5.1.2 Correlations between serum titres and PD

The correlation between serum titres and PD is shown in table 5.4. *P. gingivalis* titres correlated positively with PD and this was statistically significant (R 0.52, p 0.005). *P. intermedia* and *A. actinomycetemcomitans* had non-significant positive correlations with PD. *B. forsythus* and *T. denticola* showed negative correlations, which were not significant with PD.

There were four significant positive correlations between serum antibody titres to the test organisms and no significant negative correlations. Antibody titres to *P. intermedia* and *P. gingivalis* (R 0.53, p 0.04), *P. intermedia* and *B. forsythus* (R 0.43, p 0.02), *P. intermedia* and *T. denticola* (R 0.38, p 0.047), and *B. forsythus* and *T. denticola* (R 0.48, 0.009) correlated positively with each other.

5.1.3 Correlations between GCF titres and PD

Correlations between GCF titres and PD are displayed in table 5.5. There was a significant correlation between *P. gingivalis* titres and PD (R 0.42, p 0.025). The correlations between *B. forsythus* and *T. denticola* titres approached significance. Local antibody titres to *P. gingivalis* correlated positively and significantly with *B. forsythus*, *A, actinomycetemcomitans* and *T. denticola* titres, and *P. intermedia* titres significantly with *A. actinomycetemcomitans* titres. *B. forsythus* titres correlated significantly and positively with *A. actinomycetemcomitans* and *T. denticola* titres, and *A. actinomycetemcomitans* titres with *T. denticola* titres.

Table 5.4 Correlations between serum titres and PD using Spearman's rank correlation (R/p value)

	pd	pg sera	pi sera	bf sera	aa sera
pg sera	**0.52/0.005**				
pi sera	0.23/0.25	**0.53/0.04**			
bf sera	-0.016/0.93	-0.12/0.55	**0.43/0.02**		
aa sera	0.26/0.18	0.28/0.15	0.19/0.92	-0.26/0.18	
td sera	-0.16/0.93	0.24/0.22	**0.38/0.047**	**0.48/0.009**	0.03/0.88

Table 5.5 Correlations between GCF titres and PD using Spearman's rank (R/p value)

	PD	pg gcf	pi gcf	bf gcf	aa gcf
pg gcf	**0.42/0.04**				
pi gcf	-0.03/0.89	0.18/0.054			
bf gcf	0.32/0.09	**0.28/0.003**	0.15/0.12		
aa gcf	0.22/0.26	**0.22/0.021**	**0.38/0.001**	**0.18/0.048**	
td gcf	0.33/0.09	**0.21/0.026**	0.15/0.12	**0.57/0.001**	**0.21/0.027**

Table 5.6 Correlation between local (across) and systemic (down) antibody titres (R/p value)

Sera\GCF	*P.gingivalis*	*P. intermedi*	*B. forsythus*	*A. actino*	*T. denticola*
P. gingivalis	**0.48/0.01**	-0.06/0.77	**0.6/0.001**	0.1/0.63	**0.61/0.001**
P. intermedia	0.22/0.25	0.05/0.8	0.27/0.17	-0.05/0.8	**0.57/0.002**
B. forsythus	-0.04/0.85	-0.21/0.28	-0.23/0.25	-0.3/0.12	0.04/0.85
A. actinomycetemcomitans	0.15/0.44	0.32/0.09	0.35/0.07	**0.75/0.000**	0.22/0.26
T. denticola	-0.09/0.65	-0.05/0.8	0.22/0.25	-0.09/0.97	**0.39/0.04**

5.1.4 Local and systemic antibody titre correlations

Table 5.6 shows the correlations between local and systemic antibody titres. *P. gingivalis* local antibody titres significantly and positively correlated with *P. gingivalis* serum titres (R 0.48, p 0.01). *P. intermedia* GCF titres did not correlate with any serum titre, although the correlation with *A. actinomycetemcomitans* titres approached significance (R 0.32, p 0.09). *B. forsythus* GCF antibody levels significantly and positively correlated with *P. gingivalis* serum titres (R 0.6, p 0.001) and approached significant correlation with *A. actinomycetemcomitans* serum titres (R 0.35, p 0.07). *A. actinomycetemcomitans* local and systemic titres correlated significantly and positively (R 0.75, $p<0.001$), as did *T. denticola* local and systemic titres (R 0.39, p 0.04). In addition, *T. denticola* GCF titres correlated positively and significantly with systemic *P. gingivalis* (R 0.61, p 0.001) and *P. intermedia* titres (R 0.57, p 0.002).

5.1.5 Relationship between presence or absence of each organism and antibody titres and avidity

5.1.5.1 Serum titres

The comparison of median serum titres in patients with and without each organism is shown in table 5.7. Patients were scored as positive for the organism if one or more sites was positive and negative if all four sites were negative.

Table 5.7 Comparison of median serum antibody titres at AP sites with (+) and without (-) each organism. Number of patients shown in brackets beside column title and interquartile range beside each value.

Antibody Titre	PG+ (15)	PG- (13)	p value	PI+ (20)	PI- (8)	p value	BF+ (17)	BF- (11)	p value
P. gingivalis	3067 (112, 220772)	114 (5, 301)	0.036	278 (113, 56221)	9 (2, 349)	0.08	345 (113, 138776)	11 (4, 350)	0.09
P. intermedia	198 (1, 1417)	225 (9, 970)	0.98	223 (39, 1567)	120 (1, 562)	0.26	198 (10, 1190)	527 (1, 3132)	0.52
B. forsythus	104 (6, 445407)	4541 (30, $5x10^{11}$)	0.06	70 (10, 338397)	$5x10^{9}$ (338, $7.5x10^{11}$)	0.06	104 (8, 3565)	1000000 (14, $1x10^{12}$)	0.045
A. actinomycetemcom	86 (3, 333)	4 (1, 2928)	0.43	101 (3, 106)	1 (1, 4243)	0.16	52 (2, 211)	4 (1, 587039)	0.77
T. denticola	976 (1, $1x10^{6}$)	$1x10^{7}$ (4, $5x10^{5}$)	0.25	1301 (1, $1x10^{8}$)	56773 (3, $7.5x10^{10}$)	0.68	1622 (1, $1x10^{8}$)	23198 (1, $1x10^{12}$)	0.58

Antibody Titre	AA+ (1)	AA- (27)	TD+ (16)	TD- (12)	p value
P. gingivalis	3067	182 (7, 6754)	264 (6, 44274)	196 (10, 3577)	0.76
P. intermedia	94	225 (1, 1367)	136 (1, 555)	613 (64, 3021)	0.07
B. forsythus	5	849 (13, $1x10^{7}$)	64 (7, 14160)	722704 (239, $7.5x10^{11}$)	0.03
A. actinomycetemcom	19	116 (1, 333)	36 (2, 216)	98 (1, 4337)	0.87
T. denticola	53	3294 (1, $1x10^{9}$)	2458 (1, $1x10^{8}$)	12087 (2, $7.75x10^{11}$)	0.81

P. gingivalis positive patients had significantly higher titres to *P. gingivalis* than negative patients. Although there were slight differences between titres to the other organisms, there were no other significant differences.

In *P. intermedia* positive patients *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* titres were higher and *B. forsythus* and *T. denticola* lower. There were no statistically significant differences though *P. gingivalis* titres approached significance.

B. forsythus positive patients had significantly lower *B. forsythus* antibody titres, near significantly increased *P. gingivalis* titres (p 0.09), and higher median titres for *A. actinomycetemcomitans*. Lower titres for *P. intermedia* and *T. denticola* were recorded in these patients.

Only one patient was positive for *A. actinomycetemcomitans*, and there are insufficient numbers of patients to make any valid statistical conclusions.

Patients who were positive for *T. denticola* had significantly lower titres to *B. forsythus*, and almost significantly to *P. intermedia*. Lower titres to *A. actinomycetemcomitans* and *T. denticola*, whereas *P. gingivalis* titres were higher, but these were not significant.

5.1.5.2 GCF titres

A comparison of local titres at sites with and without each organism is shown in table 5.8. Sites that were positive for *P. gingivalis* had significantly higher titres to *P. gingivalis* and significantly lower *P. intermedia* titres. The titres for the other bacteria were similar. *P. intermedia* positive sites had similar titres to negative sites, and there were no significant differences. The difference between *B. forsythus* titres approached significance at *B. forsythus* positive sites. *P. gingivalis* titres were increased at these sites and *P. intermedia* titres decreased, but neither were significant. Only two sites were positive for *A. actinomycetemcomitans*, and so no statistical analysis would be valid. *T. denticola* positive sites had higher *P. gingivalis*, lower *P. intermedia* and similar titres for the other bacteria. There were no significant differences.

5.1.5.3 Antibody Avidity

A comparison of antibody avidity in patients with and without each organism is shown in table 5.9. Patients were scored positive and negative using the same criteria that were used for the serum titres. At *P. gingivalis* positive sites *A. actinomycetemcomitans* antibody avidity was significantly higher than negative sites. The avidities for the antibodies to the other organisms were similar and not significantly different. *A. actinomycetemcomitans* and *T. denticola* antibody avidities at *P. intermedia* positive sites were significantly higher than negative sites. The other antibody avidities were similar. At sites positive for *B. forsythus*, *P. gingivalis* and *P. intermedia* antibody avidities were significantly lower than negative sites. *B. forsythus*, *A. actinomycetemcomitans*

Table 5.8 Comparison of median local antibody titres at AP sites with (+) and without (-) each organism. Number of sites shown in brackets beside column title and interquartile range beside each value.

Antibody Titre	Pg + (49)	Pg- (63)	p value	Pi + (62)	Pi- (50)	p value	Bf+ (62)	Bf- (50)	p value
P. gingivalis	34 (17, 88)	18 (6, 49)	0.007	29 (12, 66)	23 (12, 62)	0.56	32 (11, 80)	24 (13, 54)	0.67
P. intermedia	6 (5, 7)	7 (5, 11)	0.04	6 (5 ,8)	7 (5, 9)	0.84	6 (5, 8)	7 (5, 14)	0.09
B. forsythus	1 (0, 1)	0 (0, 1)	0.17	1 (0, 1)	0 (0, 1)	0.06	1 (0, 1)	0 (0, 1)	0.055
A. actinomycetemcom	0 (0, 12)	0 (0, 13)	0.68	0 (0, 6)	0 (0, 46)	0.12	0 (0, 6)	0 (0, 46)	0.12
T. denticola	5 (1, 17)	0 (0, 5)	0.26	5 (0, 17)	0 (0, 4)	0.27	5 (0, 15)	1.5 (0, 4.3)	0.37

Antibody Titre	Aa+ (2)	Aa- (100)	Td+ (46)	Td- (56)	p value
P. gingivalis	75	26 (12, 60)	33 (11, 74)	23 (13, 58)	0.65
P. intermedia	5.5	6.5 (5, 8.3)	6 (5, 7)	7 (5, 11)	0.067
B. forsythus	1	0 (0, 1)	1 (0, 1)	0 (0, 1)	0.42
A. actinomycetemcom	6.5	0 (0, 11)	0 (0, 10.3)	0 (0, 32)	0.48
T. denticola	7.5	3 (0, 8)	3.5 (0, 14)	3 (0, 6)	0.4

Table 5.9 Comparison of mean antibody avidity in AP with (+) or without (-) each organism at one or more sites. Number of patients shown in brackets beside column title and SD beside each value.

Antibody Avidity	Pg + (49)	Pg- (63)	p value	Pi + (62)	Pi- (50)	p value	Bf+ (62)	Bf- (50)	p value
P. gingivalis	1.2 (±0.6)	1.3 (±0.7)	0.18	1.2 (±0.5)	1.3 (±0.8)	0.32	1.1 (±0.7)	1.4 (±0.6)	0.02
P. intermedia	0.9 (±0.4)	0.8 (±0.3)	0.23	0.9 (±0.3)	0.8 (±0.3)	0.1	0.8 (±0.3)	0.9 (±0.3)	0.03
B. forsythus	1.2 (±0.7)	1.1 (±0.6)	0.38	1.1 (±0.7)	1.1 (±0.6)	0.97	1.1 (±0.7)	1.2 (±0.6)	0.7
A. actinomycetemcom	0.8 (±0.4)	0.6 (±0.3)	0.013	0.75 (±0.4)	0.6 (±0.3)	0.02	0.7 (±0.4)	0.7 (±0.3)	0.79
T. denticola	0.6 (±0.2)	0.6 (±0.3)	0.62	0.7 (±0.3)	0.6 (±0.1)	0.002	0.7 (±0.3)	0.6 (±0.2)	0.19

Antibody Avidity	Aa+ (2)	Aa- (100)	Td+ (46)	Td- (66)	p value
P. gingivalis	1.3 (±0)	1.3 (±0.7)	1.0 (±0.6)	1.4 (±0.6)	0.0007
P. intermedia	0.8 (±0)	0.9 (±0.3)	0.7 (±0.3)	0.9 (±0.3)	0.0002
B. forsythus	1.0 (±0)	1.1 (±0.7)	1.1 (±0.7)	1.2 (±0.6)	0.53
A. actinomycetemcom	0.4 (±0)	0.7 (±0.4)	0.6 (±0.4)	0.7 (±0.3)	0.57
T. denticola	0.4 (±0)	0.6 (±0.3)	0.7 (±0.4)	0.6 (±0.2)	0.22

and *T. denticola* antibody avidities were similar. There were too few sites positive for *A. actinomycetemcomitans* for meaningful statistical analysis. At *T. denticola* positive sites, antibodies to *P. gingivalis* and *P. intermedia* had significantly lower avidity to those in negative sites. Similar avidities were recorded for the other antibodies.

5.1.6 Systemic antibody serostatus

5.1.6.1 Percentage of high responder and low responder patients

The percentage of patients with antibody titres two-times greater than the median control titres is shown in table 5.10. In descending order, 71.4% were high responder for *T. denticola* antibody titres, 67.9% for *P. gingivalis*, 60.7% for *B. forsythus*, 57.1% for *A. actinomycetemcomitans* and 25% for *P. intermedia.*

5.1.6.2 Comparison of high responder and low responder patients: *P. gingivalis* antibody titre

A comparison of baseline, post-SRP and change in clinical parameters, microbiological parameters, serum antibody titres, local antibody titres and antibody avidity in high responder and low responder patients for *P. gingivalis* antibody titres is shown in table 5.11.

Clinical parameters

High responder patients have significantly lower gingival inflammation scores and deeper pockets 6.2 ±1.4 mm compared to 5.3 ±0.9 mm, (p=0.0001). Attachment, GCF volume, and bleeding on probing were slightly higher and

Table 5.10 Percentage of AP patients seropositive and seronegative for antibody titres to each organism.

Antibody	Seropositive (%)	Seronegative (%)
P. gingivalis	67.9	32.1
P. intermedia	25	75
B. forsythus	60.7	39.3
A. actino	57.1	42.9
T. denticola	71.4	28.6

Table 5.11 Comparison of mean clinical and microbial parameters in high responder and low responder AP patients for *P. gingivalis* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and SD beside each value.

Clin. Param.	High Pre (19)	Low Pre (9)	p value	High Pre (19)	Low Pre (9)	p value	High Pre (19)	Low Pre (9)	p value
MGI	2.25 (±0.7)	2.6 (±0.6)	0.004	1.0 (±1.0)	1.5 (±1.1)	0.027	1.2 (±0.9)	1.2 (±0.8)	0.74
PLI	1.5 (±0.9)	1.6 (±1.0)	0.53	0.3 (±1.0)	0.8 (±1.2)	0.032	1.2 (±0.8)	0.75 (±0.8)	0.015
BOP (%)	88.2	80.6	0.28	44.7	25	0.053	43.4	55.6	0.23
Supp (%)	27.6	30.6	0.075	21	19.4	0.86	6.6	11.1	0.41
PD (mm)	6.2 (±1.4)	5.3 (±0.9)	0.0001	1.6 (±1.2)	1.2(±1.6)	0.19	4.6 (±1.5)	4.0 (±1.5)	0.039
AL (mm)	13.7 (±2.4)	13.3 (±1.5)	0.31	0.7 (±1.2)	0.2 (±1.3)	0.065	13.1 (±2.6)	12.8 (±1.6)	0.51
GCFVol (nl/30s)	425.3 (±348.5)	337.4 (±294.6)	0.17	-56.3 (±393.7)	45.2 (±351.2)	0.17	460.4 (±384)	337 (±384)	0.073
Smoker (%)	10.5	66.7	<0.001						
Micro. Param. (%)									
P. gingivalis	48.7	33.3	0.13	14.5	0	0.013	40.8	19.4	0.026
P. intermedia	65.8	33.3	0.001	22.4	22.2	0.19	36.8	25	0.21
B. forsythus	69.7	25	<0.001	30.3	-5.6	0.015	32.9	44.4	0.24
A. actinomycetem	2.6	0	0.32	2.6	0	0.32	0	0	1
T. denticola	40.8	41.7	0.93	29	8.3	0.15	21.1	13.9	0.36

Table 5.11 (con'd) Comparison of median serum and gcf titres and mean antibody avidity in high responder and low responder AP patients for *P. gingivalis* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and interquartile range or SD beside each value.

Median Serum Titres	High Pre (19)	Low Pre (9)	p value	High Pre (19)	Low Pre (9)	p value	High Pre (19)	Low Pre (9)	p value
P. gingivalis	**637 (182, 56781)**	**4 (1, 7)**	<0.001	23 (-380, 3843)	-1 (-6, 0)	0.18	1465 (170, 219886)	3 (1, 12)	<0.001
P. intermedia	499 (137, 1617)	1 (1, 16)	<0.001	18 (-140, 204)	0 (0, 0)	0.01	347 (53, 995)	1 (1, 10)	<0.001
B. forsythus	4541 (10, $1x10^7$)	168 (13, $1x10^7$)	0.8	1 (-21, 8592)	12 (0, 405)	0.15	143 (11, $1x10^6$)	15 (4, 444)	0.014
A. actinomycetem	116 (1, 333)	4 (1, 16)	0.048	0 (-5, 57)	0 (0, 3)	0.02	54 (3, 267)	1 (1, 1)	0.001
T. denticola	$1x10^7$ (53, $1x10^{11}$)	1 (1, 23198)	<0.001	24 (0, $1x10^7$)	0 (0, 23197)	0.58	15204 (29, $1x10^6$)	1 (1,1)	<0.001
Median GCF Titres									
P. gingivalis	42 (15, 119)	14.5 (11, 23.8)	<0.001	-10 (-95, 6)	-2 (-6, 0.75)	0.13	69 (20, 303)	15 (12, 24)	<0.001
P. intermedia	6 (5, 8)	7 (0.75, 14)	0.92	0 (-1, 1)	0 (-2.5, 1)	0.72	6 (6, 7.8)	7 (3, 14)	0.89
B. forsythus	1 (0, 1)	0 (0, 0)	<0.001	0 (0, 0)	0 (0, 1)	0.3	1 (0, 1)	0 (0, 0)	<0.001
A. actinomycetem	0 (0, 11)	0 (0, 31.8)	0.87	0 (-10, 0)	0 (-31, 0)	0.99	0 (0, 28.8)	0 (0, 34)	0.88
T. denticola	5 (0, 17)	0 (0, 1.8)	<0.001	0 (-3, 5)	0 (-0.75, 0.75)	0.39	4.5 (0, 10.8)	0 (0, 2)	0.001
Mean Avidity									
P. gingivalis	1.4 (±0.7)	1.0 (±0.5)	0.07	0.3 (±0.5)	0.08 (±0.3)	0.21	1.1 (±0.5)	0.9 (±0.4)	0.18
P. intermedia	0.9 (±0.2)	0.8 (±0.4)	0.78	0.1 (±0.2)	0.1 (±0.3)	0.9	0.8 (±0.2)	0.7 (±0.3)	0.56
B. forsythus	1.2 (±0.6)	0.9 (±0.8)	0.32	-0.1 (±0.8)	0.04 (±0.3)	0.46	1.3 (±0.7)	0.9 (±0.7)	0.11
A. actinomycetem	0.7 (±0.3)	0.7 (±0.4)	0.77	0.02 (±0.2)	0.06 (±0.2)	0.63	0.6 (±0.4)	0.6 (±0.3)	0.99
T. denticola	0.7 (±0.3)	0.6 (±0.1)	0.16	0.07 (±0.48)	-0.06 (±0.3)	0.48	0.6 (±0.5)	0.6 (±0.3)	0.94

plaque levels and suppuration slightly lower than low responder patients, but not significantly. There were significantly fewer high responder smoker patients (10.5% compared to 66.7%, $p<0.001$). The change in clinical parameters in response to SRP is also shown in table 5.11. High responder patients had significantly lower decreases in gingival inflammation and plaque scores. These patients also recorded greater reductions in bleeding, suppuration and attachment, but these were not significant, though the reductions for bleeding and attachment approached significance. High responder patients recorded an increase in GCF volume compared to a decrease in low responder patients. Following therapy high responder patients recorded significantly higher plaque scores and deeper pockets. There were no other significant differences although the different GCF volumes approached significance.

Microbiological parameters

At baseline high responder patients showed increased prevalences for all test bacteria except *T. denticola* compared to low responder patients. *P. intermedia* and *B. forsythus* were both found at significantly higher percentages in high responder patients. *T. denticola* was found at similar levels. High responder patients generally recorded greater reductions in the prevalences of the test bacteria in response to SRP. Both *P. gingivalis* and *B. forsythus* reductions were significantly higher than low responder patients. Low responder patients gained *B. forsythus*. Post-therapy *P. gingivalis* was detected in significantly more high responder patients, and *P. intermedia* and *T. denticola* found in more high responder patients. *B. forsythus* was more common in low responder patients.

Serum titres

The median titre for patients with high responder titres to *P. gingivalis* was 637 EU and for low responder patients 4 EU. Titres to the other organisms were higher in high responder patients, and this was significant for *P. intermedia*, *A. actinomycetemcomitans* and *T. denticola* titres. In response to SRP, high responder patients recorded a decrease in antibody titre whereas low responder patients reported a slight decrease. A significant reduction in *P. intermedia* titre was recorded in high responder patients. Post-treatment titres to all organisms were significantly higher in high responder patients.

GCF titres

High responder patients recorded significantly higher GCF antibody titres to *P. gingivalis*, *B. forsythus* and *T. denticola*. In both high responder and low responder there was little change in GCF titre in response to SRP and no significant differences. Titres to these three bacteria remained significantly higher post-treatment in high responder patients.

Avidity

P. gingivalis antibody avidity was higher in high responder patients at baseline and this approached significance. The avidities of the other antibodies were similar. There were no significant differences in the change in antibody avidity between high responder and low responder patients in response to SRP. High responder patients had a slight increase in *B. forsythus* antibody avidity, and low

responder a slight increase in *T. denticola* antibody avidity. Other antibody avidities decreased slightly. After treatment avidity was slightly higher for *P. gingivalis*, *P. intermedia* and *B. forsythus* antibodies in high responder patients, but there were no significant differences.

5.1.6.3 Comparison of high responder and low responder patients: *P. intermedia* antibody titres

Table 5.12 displays the comparison of AP patients with high responder and low responder antibody titres to *P. intermedia.*

Clinical parameters

The comparison of baseline clinical measurements showed significantly greater attachment loss in high responder patients and deeper pockets in these patients. The difference in plaque scores and the smaller number of high responder smokers compared to low responder patients approached significance. There were no significant differences in the change in parameters in response to SRP between the two groups. The greater reduction in plaque levels and suppuration in low responder patients approached significance. High responder patients showed an increase in mean GCF volume compared to a slight decrease in low responder patients. Post-therapy, high responder patients had significantly greater attachment loss and GCF volume. There were no significant differences between the other parameters.

Table 5.12 Comparison of mean clinical and microbial parameters in high responder and low responder AP patients for *P. intermedia* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and SD beside each value.

Clin. Param.	High Pre (7)	Low Pre (21)	p value	High Pre (7)	Low Pre (21)	p value	High Pre (7)	Low Pre (21)	p value
MGI	2.4 (±0.6)	2.4 (±0.8)	0.8	1.2 (±1.0)	1.2 (±1.0)	0.83	1.2 (±1.0)	1.2 (±0.9)	0.93
PLI	1.2 (±0.8)	1.6 (±0.9)	0.052	0.1 (±1.0)	0.6 (±1.0)	0.058	1.1 (±0.6)	1.0 (±0.9)	0.45
BOP (%)	85.7	85.7	1	42.9	36.9	0.85	42.9	48.8	0.59
Supp (%)	28.6	28.6	1	17.9	21.4	0.08	10.7	7.1	0.55
PD (mm)	6.2 (±1.3)	5.8 (±1.3)	0.14	1.6 (±1.2)	1.5 (±1.4)	0.83	4.6 (±1.4)	4.3 (±1.6)	0.39
AL (mm)	14.6 (±2.1)	13.2 (±2.1)	0.005	0.6 (±1.2)	0.5 (±1.3)	0.63	13.8 (±2.2)	12.7 (±2.3)	0.033
GCFVol (nl/30s)	408.1 (±338.9)	393.4 (±333.5)	0.84	-98.4 (±436.2)	1.2 (±361.7)	0.28	561.3 (±374.5)	373.9 (±351.8)	0.025
Smoker (%)	14.3	33.3	0.053						
Micro. Param. (%)									
P. gingivalis	32.1	47.6	0.15	7.1	10.7	0.23	35.7	33.3	0.82
P. intermedia	67.9	51.2	0.12	32.1	19.1	0.16	39.3	31.0	0.42
B. forsythus	50	57.1	0.51	21.4	17.9	<0.001	28.6	30.3	0.31
A. actinomycetem	0	2.4	0.41	0	2.4	0.41	0	0	1
T. denticola	14.3	50	0.001	28.6	20.2	0.58	17.9	19.1	0.89

Table 5.12 (con'd) Comparison of median serum and gcf titres and mean antibody avidity in high responder and low responder AP patients for *P. intermedia* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and interquartile range or SD beside each value.

Median Serum Titres	High Pre (7)	Low Pre (21)	p value	High Pre (7)	Low Pre (21)	p value	High Pre (7)	Low Pre (21)	p value
P. gingivalis	4653 (299, 432707)	114 (6, 637)	<0.001	23 (-26912, 212821)	0 (-23, 47)	0.71	31565 (322, 660570)	32 (5, 1465)	<0.001
P. intermedia	**2695 (1417, 6632)**	**135 (1, 240)**	<0.001	608 (-320, 2944)	0 (0, 24)	0.18	1576 (425, 7235)	36 (1, 347)	<0.001
B. forsythus	445407 ($10,1x10^{10}$)	168 (13, $1x10^{6}$)	0.15	0 (-$1x10^{9}$, 8592)	19 (-3, 4398)	0.018	$1x10^{6}$ (11, $1x10^{12}$)	43 (9, 6318)	0.005
A. actinomycetem	222 (1, 380)	19 (1, 200)	0.62	-2 (-45, 0)	3 (0, 32)	0.004	164 (1, 385)	14 (1, 134)	0.067
T. denticola	1622 (1, $1x10^{12}$)	3294 (1, $1x10^{8}$)	0.87	0 (-44459, $1x10^{8}$)	24 (0, 9000000)	0.14	46081 (1, $1x10^{6}$)	86 (1, 92545)	0.054
Median GCF Titres									
P. gingivalis	49 (22, 173)	23 (11, 48.5)	0.002	-26 (-315, 16)	-2 (-15, 2)	0.14	96 (32, 516)	24 (12,70)	<0.001
P. intermedia	8 (5, 13)	6 (5, 8)	0.063	-1 (-4, 2)	0 (-1, 1)	0.12	8 (6, 11.8)	6 (5, 7)	0.008
B. forsythus	1 (0, 1)	0 (0, 1)	0.032	0 (0, 1)	0 (0, 0)	0.79	1 (0, 1)	0 (0, 1)	0.022
A. actinomycetem	0 (0, 36)	0 (0, 9.3)	0.36	0 (-71, 0)	0 (-6.8, 0)	0.2	6.5 (0, 466.8)	0 (0, 19.7)	0.065
T. denticola	5 (3, 15)	1 (0, 8)	0.007	-2 (-8, 3)	0 (0, 4)	0.014	8 (0, 19.5)	0 (0, 4.75)	<0.001
Mean Avidity									
P. gingivalis	2.0 (±0.6)	1.0 (±0.5)	0.005	0.5 (±0.3)	0.1 (±0.5)	0.01	1.4 (±0.4)	0.9 (±0.5)	0.016
P. intermedia	1.1 (±0.3)	0.8 (±0.3)	0.06	0.2 (±0.2)	0.08 (±0.2)	0.32	0.9 (±0.3)	0.7 (±0.2)	0.23
B. forsythus	1.1 (±0.8)	1.2 (±0.6)	0.77	-0.05 (±1.0)	-0.07 (±0.5)	0.97	1.1 (±0.8)	1.2 (±0.7)	0.73
A. actinomycetem	0.9 (±0.5)	0.6 (±0.3)	0.2	0.08 (±0.4)	0.02 (±0.2)	0.67	0.8 (±0.5)	0.6 (±0.2)	0.34
T. denticola	0.6 (±0.1)	0.6 (±0.3)	0.66	-0.2 (±0.7)	0.1 (±0.4)	0.39	0.8 (±0.7)	0.5 (±0.2)	0.46

Microbiological parameters

At baseline high responder patients had lower prevalences of *P. gingivalis*, *B. forsythus* and *A. actinomycetemcomitans* and higher *P. intermedia*. These were not significantly different. *T. denticola* prevalence was significantly lower in high responder patients. In response to treatment, high responder patients showed greater reductions in *P. intermedia*, *B. forsythus*, and *T. denticola*, and lower reductions in *P. gingivalis* and *A. actinomycetemcomitans*. The difference in *B. forsythus* reduction was significant. Post-SRP, the prevalences for all bacteria were similar with no significant differences.

Serum titres

Baseline median titre for *P. intermedia* high responder patients was 2, 695 EU compared to 135 EU for low responder patients. *P. gingivalis* titres were also significantly higher in high responder patients. *B. forsythus* and *A. actinomycetemcomitans* median titres were higher and *T. denticola* titres were lower in high responder patients, but these were not statistically significant. In response to SRP, *P. intermedia* high responder patients showed a decrease in *P. gingivalis* and *P. intermedia* titres but these were not significant when compared to low responder patients. There were significant reductions for *B. forsythus* and *A. actinomycetemcomitans* titres in low responder patients. High responder titres showed a slight increase in median *A. actinomycetemcomitans* titres. *T. denticola* titres showed a reduction in low responder patients and remained the same in high responder patients. Post-SRP, *P. gingivalis*,

P. intermedia and *B. forsythus* titres were significantly higher in high responder patients. Titres for *A. actinomycetemcomitans* and *T. denticola* were also higher and approached significance.

GCF titres

Pre-treatment, high responder patients displayed significantly greater antibody titres to *P. gingivalis*, *B. forsythus* and *T. denticola.* The higher GCF titres to *P. intermedia* in high responder patients approached significance. High responder patients showed an increase in *P. gingivalis*, *P. intermedia* and *T. denticola* titres in response to SRP, but only the increase in *T. denticola* antibody levels was statistically significant compared to low responder patients. Post-treatment, high responder patients recorded significantly higher titres for *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola*, with the increased *A. actinomycetemcomitans* titres approaching significance.

Avidity

P. gingivalis antibody avidity was significantly higher in high responder patients than low responder. *P. intermedia* antibody avidity was also greater but not significantly. In response to SRP, high responder patients had a significantly greater decrease in *P. gingivalis* antibody avidity. Both groups had a slight increase in *B. forsythus* antibody avidity, and *T. denticola* antibody avidity increased in high responder patients. Post-treatment *P. gingivalis* antibody avidity remained significantly higher, and, although avidities were slightly higher in general, there were no other significant differences.

5.1.6.4 Comparison of high responder and low responder patients: *B. forsythus* antibody titres

Table 5.13 shows the comparison of *B. forsythus* antibody titre high responder and low responder patients.

Clinical parameters

At baseline, high responder patients has significantly lower plaque scores, and slightly, but not significantly, greater attachment loss and pocket depth. The other parameters were similar when compared to low responder patients. In response to SRP high responder patients had significantly greater reduction in MGI, and a significantly lower decrease in plaque levels. High responder patients showed an increase in mean GCF volume compared to a decrease in low responder patients, which was significant. The changes in the other parameters were similar. Post-SRP, high responder patients had significantly lower MGI and pocket depth, and significantly higher GCF volume. Low responder patients had higher bleeding and suppuration percentages but these were not significantly different.

Microbiological parameters

High responder patients generally recorded lower prevalences for the organisms studied at baseline. Significantly higher prevalences were found for *P. gingivalis* and *T. denticola* in low responder patients and the prevalences for *B. forsythus* and *A. actinomycetemcomitans* approached significance. In

Table 5.13 Comparison of mean clinical and microbial parameters in high responder and low responder AP patients for *B. forsythus* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and SD beside each value.

Clin. Param.	High Pre (17)	Low Pre (11)	p value	High Pre (17)	Low Pre (11)	p value	High Pre (17)	Low Pre (11)	p value
MGI	2.4 (±0.7)	2.3 (±0.7)	0.75	1.4 (±1.0)	0.7 (±1.0)	0.001	1.0 (±0.9)	1.6 (±0.7)	0.001
PLI	1.2 (±0.9)	1.9 (±0.8)	<0.001	0.3 (±1.1)	0.75 (±1.1)	0.027	1.0 (±0.9)	1.1 (±0.8)	0.76
BOP (%)	83.8	88.6	0.48	42.7	31.8	0.46	41.2	56.8	0.1
Supp (%)	29.4	27.3	0.8	23.5	15.9	0.57	5.9	11.4	0.3
PD (mm)	6.0 (±1.3)	5.8 (±1.3)	0.41	1.5 (±1.4)	1.5 (±1.4)	0.94	4.2 (±1.6)	4.8 (±1.4)	0.03
AL (mm)	13.6 (±2.3)	13.4 (±2.0)	0.65	0.6 (±1.2)	0.4 (±1.2)	0.24	12.9(±2.3)	13.2 (±2.3)	0.4
GCFVol (nl/30s)	402.7 (±343.6)	388.2 (320.5)	0.82	-91.7 (±375.6)	81.4 (±372)	0.019	486.3 (±370.1)	319.5 (±336.6)	0.016
Smoker (%)	17.7	45.5	0.001						
Micro. Param. (%)									
P. gingivalis	33.8	59.1	0.008	16.2	0	0.06	32.4	36.4	0.66
P. intermedia	50	63.6	0.16	19.1	27.3	0.55	25	45.5	0.025
B. forsythus	48.5	65.9	0.07	30.9	0	0.035	33.8	40.9	0.45
A. actinomycetem	0	4.5	0.076	0	4.6	0.076	0	0	1
T. denticola	30.9	56.8	0.006	17.7	29.6	0.31	23.5	11.4	0.11

Table 5.13 (con'd) Comparison of median serum and gcf titres and mean antibody avidity in high responder and low responder AP patients for *B. forsythus* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and interquartile range or SD beside each value.

Median Serum Titres	High Pre (17)	Low Pre (11)	p value	High Pre (17)	Low Pre (11)	p value	High Pre (17)	Low Pre (11)	p value
P. gingivalis	257 (11, 637)	182 (7, 54541)	0.74	8 (-8, 107)	-1 (-263, 12)	0.036	88 (12, 1465)	358 (8, 321409)	0.9
P. intermedia	527 (198, 1367)	20 (1, 207)	<0.001	18 (0, 196)	0 (-30, 9)	0.019	425 (36, 786)	11 (1, 188)	<0.001
B. forsythus	**100000 (4541, $1x10^{10}$)**	**10 (5, 14)**	<0.001	625 (0, 853765)	-3 (-21, 5)	<0.001	6318 (43, $1x10^{8}$)	11 (2, 56)	<0.001
A. actinomycetem	4 (1, 333)	86 (4, 222)	0.15	0 (-2, 15)	3 (-45, 32)	0.74	4 (1, 164)	54 (1, 267)	0.38
T. denticola	90348 (976, $1x10^{11}$)	1 (1, $1x10^{8}$)	<0.001	2906 (0, $1x10^{7}$)	0 (0, 24)	0.001	388 (18, 1000000)	1 (1, 92545)	0.015
Median GCF Titres									
P. gingivalis	26 (12, 89)	26 (11, 41)	0.39	-3 (-75, 3)	-4 (-33, 2)	0.67	32 (13, 255)	31 (13.5, 74.5)	0.29
P. intermedia	6 (5, 8)	7 (5, 13)	0.17	0 (-2, 0)	0 (-1, 4)	0.025	6 (5, 8)	6 (5.25, 8)	0.71
B. forsythus	1 (0, 1)	0 (0, 1)	0.22	0 (0, 0.75)	0 (0, 0)	0.31	0 (0, 1)	0 (0, 1)	0.99
A. actinomycetem	0 (0, 10.8)	0 (0, 25)	0.49	0 (-5, 0)	0 (-19, 3)	0.8	0 (0, 73.5)	5 (0, 30.75)	0.95
T. denticola	4 (0, 9)	1 (0, 5)	0.055	0 (-3, 4)	0 (0, 2)	0.33	3 (0, 9)	0.5 (0, 3.5)	0.14
Mean Avidity									
P. gingivalis	1.4 (±0.7)	1.1 (±0.6)	0.23	0.3 (±0.5)	0.06 (±0.5)	0.15	1.1 (±0.4)	1.0 (±0.6)	0.84
P. intermedia	0.9 (±0.3)	0.8 (±0.4)	0.42	0.1 (±0.2)	0.08 (±0.2)	0.71	0.8 (±0.3)	0.7 (±0.2)	0.4
B. forsythus	1.3 (±0.6)	1.0 (±0.8)	0.3	-0.15 (±0.7)	0.08 (±0.6)	0.37	1.4 (±0.7)	0.9 (±0.6)	0.056
A. actinomycetem	0.7 (±0.3)	0.7 (±0.4)	0.86	0.04 (±0.2)	0.03 (±0.2)	0.98	0.6 (±0.2)	0.7 (±0.5)	0.85
T. denticola	0.6 (±0.3)	0.6 (±0.2)	0.83	-0.03 (±0.6)	0.1 (±0.4)	0.44	0.7 (±0.5)	0.5 (±0.2)	0.23

response to SRP, *B. forsythus* significantly decreased in high responder patients compared with no change in low responder patients. *P. gingivalis* also decreased in high responder patients and did not change in low responder patients but this was not significant. The reduction of *P. intermedia*, *A. actinomycetemcomitans* and *T. denticola* was greater in low responder patients but not significant. Post-therapy, *P. gingivalis*, *P. intermedia* and *B. forsythus* prevalences were lower in high responder patients, with *P. intermedia* significantly lower. *T. denticola* prevalence was higher in high responder patients.

Serum titres

The median antibody titre to *B. forsythus* in high responder patients was 100, 000 EU compared to 10 EU in low responder patients. *P. intermedia* and *T. denticola* titres were also significantly higher at baseline in high responder patients. *P. gingivalis* titres were slightly higher and *A. actinomycetemcomitans* slightly lower in these patients. In response to SRP, high responder patients recorded significantly greater reductions in *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola* titres than low responder patients. *P. gingivalis* and *B. forsythus* titres in low responder patients showed a slight increase whereas *P. intermedia* and *T. denticola* median titres did not change. Post-treatment, *P. intermedia*, *B. forsythus* and *T. denticola* titres remained significantly higher in high responder patients. *P. gingivalis* and *A. actinomycetemcomitans* titres were higher but not significantly in low responder patients.

GCF titres

At baseline, local titres were similar with only *T. denticola* titres significantly higher in high responder patients. *P. intermedia* antibody titres showed a tendency to increase slightly in high responder patients in response to SRP and a tendency to decrease slightly in low responder patients in response to SRP. This was statistically significant. There were no other significant differences. *P. gingivalis* titres increased in both patient groups with little change in the other antibody titres. Post-therapy, there were no significant differences between GCF titres to all organisms and antibody levels were similar.

Avidity

Before treatment, there were no significant differences in antibody avidity, although *P. gingivalis*, *P. intermedia*, and *B. forsythus* antibody avidity were slightly higher in high responder patients. Avidities were similar for the other antibodies. Both *B. forsythus* and *T. denticola* antibody avidities increased in high responder patients, but there were no significant differences in response to SRP. Following therapy, *B. forsythus* antibodies had higher avidity in high responder patients, and this approached significance.

5.1.6.5 Comparison of high responder and low responder patients: *A. actinomycetemcomitans* antibody titres

The comparison of high responder and low responder *A. actinomycetemcomitans* patients is shown in table 5.14.

Table 5.14 Comparison of mean clinical and microbial parameters in high responder and low responder AP patients for *A. actinomycetemcomitans* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and SD beside each value.

Clin. Param.	High Pre (16)	Low Pre (12)	p value	High Pre (16)	Low Pre (12)	p value	High Pre (16)	Low Pre (12)	p value
MGI	2.0 (±0.7)	2.6 (±0.6)	<0.001	0.9 (±1.0)	1.3 (±1.0)	0.054	1.1 (±0.9)	1.3 (±0.9)	0.21
PLI	1.4 (±1.0)	1.6 (±0.9)	0.22	0.2 (±1.1)	0.6 (±1.1)	0.097	1.1 (±1.0)	1.0 (±0.8)	0.42
BOP (%)	81.3	89.1	0.24	33.3	42.2	0.51	47.9	46.9	0.91
Supp (%)	27.1	29.7	0.76	22.9	18.8	0.89	4.2	10.9	0.19
PD (mm)	6.2 (±1.5)	5.7 (±1.1)	0.066	1.6 (±1.4)	1.5 (±1.4)	0.73	4.4 (±1.6)	4.4 (±1.5)	0.89
AL (mm)	13.8 (±2.4)	13.4 (±2.0)	0.4	0.7 (±1.0)	0.4 (±1.4)	0.17	13.1 (±2.8)	12.9 (±1.9)	0.64
GCFVol (nl/30s)	449 (±373.5)	358.1 (±296.8)	0.17	-41.5 (±375.5)	-10.4 (±389.3)	0.67	483.2 (±391.2)	373.9 (±339.8)	0.12
Smoker (%)	25	31.3	0.47						
Micro. Param. (%)									
P. gingivalis	43.8	43.8	1	27.1	-3.1	0.024	45.8	25	0.02
P. intermedia	68.8	45.3	0.014	22.9	21.8	0.78	35.4	31.3	0.64
B. forsythus	56.3	54.7	0.87	43.8	0	<0.001	33.3	39.1	0.53
A. actinomycetem	4.2	0	0.1	4.2	0	0.1	0	0	1
T. denticola	37.5	43.8	0.51	39.6	9.4	0.011	20.8	17.2	0.63

Table 5.14 (con'd) Comparison of median serum and gcf titres and mean antibody avidity in high responder and low responder AP patients for *A. actinomycetemcomitans* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and interquartile range or SD beside each value.

Median Serum Titres	High Pre (16)	Low Pre (12)	p value	High Pre (16)	Low Pre (12)	p value	High Pre (16)	Low Pre (12)	p value
P. gingivalis	3860 (117, 338726)	123 (6, 333)	<0.001	-1 (-258, 162830)	0 (-19, 41)	0.6	17447 (109, 334080)	54 (6, 590)	<0.001
P. intermedia	362 (104, 1567)	168 (1, 668)	0.037	21 (-105, 185)	0 (-22, 76)	0.13	268 (35, 852)	102 (1, 741)	0.3
B. forsythus	476 (10, 15465)	500328 (13, $1x10^{10}$)	0.034	31 (-17, 7543)	9 (-6, 640480)	0.96	294 (22, 751580)	24 (6, $7.5x10^{11}$)	0.3
A. actinomycetem	**357 (205, 441693)**	**1 (1, 13)**	<0.001	13 (-85376,142)	0 (0, 12)	1	275 (136, 525734)	1 (1, 3.75)	<0.001
T. denticola	2458 (3, $7.5x10^{10}$)	12089 (1, $7.7x10^{8}$)	1	0 (-33344, 2185)	12077 (0, $7.7x10^{8}$)	<0.001	23235 (8, $7.5x10^{8}$)	52 (1, 73210)	0.004
Median GCF Titres									
P. gingivalis	35 (12, 83)	23 (12, 42.8)	0.073	-3 (-46, 5)	-3 (-31, 2)	0.53	43 (8, 184)	27 (13, 93)	0.4
P. intermedia	7 (6, 11)	6 (4, 8)	0.008	0 (-1, 2)	0 (-1, 0.75)	0.58	7 (6, 10)	6 (5, 8)	0.053
B. forsythus	1 (0, 1)	0 (0, 0)	0.001	0 (0, 0)	0 (0, 0)	0.17	1 (0, 1)	0 (0, 1)	0.05
A. actinomycetem	6 (0, 0)	0 (0, 38)	<0.001	0 (-8, 5)	0 (-31, 0)	0.21	5.5 (0, 31.8)	0 (0, 34)	0.18
T. denticola	5 (0, 5)	1 (0, 15)	0.022	0 (-2, 6)	0 (-1.8, 3)	0.61	2 (0, 10.5)	0 (0, 5)	0.12
Mean Avidity									
P. gingivalis	1.3 (±0.7)	1.3 (±0.7)	0.79	0.3 (±0.4)	0.1 (±0.5)	0.18	1.0 (±0.5)	1.1 (±0.5)	0.38
P. intermedia	0.9 (±0.2)	0.8 (±0.4)	0.4	0.09 (±0.2)	0.1 (±0.2)	0.73	0.8 (±0.2)	0.7 (±0.3)	0.15
B. forsythus	1.2 (±0.7)	1.1 (±0.6)	0.51	-0.1 (±0.9)	-0.006 (±0.4)	0.65	1.4 (±0.8)	1.1 (±0.6)	0.3
A. actinomycetem	0.8 (±0.4)	0.6 (±0.3)	0.14	0.05 (±0.3)	0.02 (±0.2)	0.73	0.8 (±0.4)	0.6 (±0.3)	0.16
T. denticola	0.7 (±0.3)	0.6 (±0.2)	0.61	0.1 (±0.4)	-0.06 (±0.6)	0.3	0.5 (±0.1)	0.7 (±0.5)	0.33

Clinical parameters

Pre-treatment, high responder patients had significantly lower MGI and the increased pocket depth approached significance. The other parameters were not significantly dissimilar. Slightly more low responder patients were smokers. In response to SRP, high responder patients recorded significantly lower reductions in MGI and plaque levels. There were no significant differences in the reductions of the other parameters, or, in the case of GCF volume, increases. Following therapy, there were no significant differences between high responder and low responder patients.

Microbiological parameters

High responder patients had significantly higher prevalences of *P. intermedia*, and higher *B. forsythus* and *A. actinomycetemcomitans* percentages. Both groups had similar *P. gingivalis* prevalences and high responder patients lower *T. denticola*. In response to SRP, the reductions for *P. gingivalis*, *B. forsythus* and *T. denticola* were significantly greater in high responder patients. The decreases in *P. intermedia* and *A. actinomycetemcomitans* were also higher in these patients. Low responder patients recorded a slight increase in *P. gingivalis* prevalence. Post-treatment high responder patients had significantly higher prevalence for *P. gingivalis*. The prevalences of the other species were not significantly different.

Serum titres

Median titres for *A. actinomycetemcomitans* at baseline were, for high responder patients 357 EU, and 1 EU for low responder. *P. gingivalis* and *P. intermedia* antibody titres were also significantly higher. *B. forsythus* titres were significantly lower in high responder patients. *T. denticola* titres were similar. High responder patients recorded greater reductions in *P. intermedia*, *B. forsythus* and *A. actinomycetemcomitans* titres in response to SRP compared to low responder patients, where titres remained the same or decreased very slightly. The changes in *P. gingivalis* titres were similar for both groups. Low responder patients had a significantly greater decrease in *T. denticola* titre, with median titres unchanging in high responder patients. Post-SRP median titres for all organisms were higher in high responder patients but only significantly for *P. gingivalis*, *A. actinomycetemcomitans* and *T. denticola.*

GCF titres

Local titres for all organisms were higher in high responder patients, and this was significant for all except *P. gingivalis*, which approached significance. There were no significant differences in the changes in GCF titres in response to SRP. In both groups, *P. gingivalis* titres increased slightly and there was little change in the other antibody levels. Post-therapy, high responder patients' titres were higher for all bacteria but only significantly for *B. forsythus*. The difference in *P. intermedia* titres approached significance.

Avidity

At baseline, antibody avidities were similar for both high responder and low responder subjects. *B. forsythus* antibody avidity increased slightly in both groups in response to SRP. Apart from a slight increase in low responder *T. denticola* antibody avidity, there was a general decrease in avidity. There were no significant differences between groups. Post-SRP, high responder patients had slightly, but not significantly, higher avidity for *B. forsythus* and *A. actinomycetemcomitans* antibodies, and lower avidity for *T. denticola* antibodies.

5.1.6.6 Comparison of high responder and low responder patients: *T. denticola* antibody titres

The comparison of *T. denticola* antibody titre high responder and low responder patients is shown in table 5.15.

Clinical parameters

At baseline, high responder patients had deeper pockets 6.0 ±1.4 mm compared to 5.6 ±0.9 mm, ($p=0.08$), but there were no statistical differences in the clinical parameters. 10% of high responder patients smoked compared to 75% of low responder patients who were non-smokers, and this was significant at $p<0.001$. There were no significant differences in the reductions of the clinical parameters between the two groups in response to SRP. Both groups recorded an increase in GCF volume. High responder patients had slightly greater reductions in

Table5.15 Comparison of mean clinical and microbial parameters in high responder and low responder AP patients for *T. denticola* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and SD beside each value.

Clin. Param.	High Pre (20)	Low Pre (8)	p value	High Pre (20)	Low Pre (8)	p value	High Pre (20)	Low Pre (8)	p value
MGI	2.3 (±0.7)	2.5 (±0.6)	0.17	1.2 (±1.0)	1.0 (±1.0)	0.33	1.1 (±0.9)	1.6 (±0.8)	0.009
PLI	1.5 (±0.8)	1.4 (±1.1)	0.75	0.5 (±1.0)	0.5 (±1.3)	0.79	1.1 (±0.9)	0.9 (±0.8)	0.56
BOP (%)	85	87.5	0.73	40	34.4	0.74	45	53	0.44
Supp (%)	28.8	28.1	0.95	22.5	15.6	0.47	6.3	12.5	0.27
PD (mm)	6.0 (±1.4)	5.6 (±0.9)	0.082	1.6 (±1.3)	1.3 (±1.5)	0.3	4.2 (±1.6)	4.9 (±1.4)	0.03
AL (mm)	13.4 (±2.4)	13.9 (±1.6)	0.25	0.6 (±1.3)	0.4 (±1.1)	0.3	12.7 (±2.4)	13.7 (±2.0)	0.04
GCFVol (nl/30s)	404.2 (±340.1)	379.2 (±320.5)	0.71	-30.5 (±409.9)	-6.8 (±306.6)	0.74	447.2 (±363.7)	354 (±365.6)	0.23
Smoker (%)	10	75	<0.001						
Micro. Param. (%)									
P. gingivalis	41.3	50	0.4	13.8	0	0.51	35	31.3	0.7
P. intermedia	52.5	62.5	0.34	21.3	25	0.07	27.5	46.9	0.049
B. forsythus	56.3	53.1	0.76	27.5	-3	0.002	33.8	43.8	0.32
A. actinomycetem	2.5	0	0.36	2.5	0	0.19	0	0	1
T. denticola	36.3	53.1	0.1	20	28.1	0.67	21.3	12.5	0.28

Table 5.15 (con'd) Comparison of median serum and gcf titres and mean antibody avidity in high responder and low responder AP patients for *T. denticola* antibody titres at baseline, change in response to SRP, and post-SRP. Number of patients shown in brackets beside column title and interquartile range or SD beside each value.

Median Serum Titres	High Pre (20)	Low Pre (8)	p value	High Pre (20)	Low Pre (8)	p value	High Pre (20)	Low Pre (8)	p value
P. gingivalis	278 (113, 4257)	8 (1, 42594)	0.02	4 (-258, 105)	0 (-18, 2882)	0.88	340 (16, 24506)	20 (1, 165642)	0.03
P. intermedia	370 (136, 1278)	1 (1, 1213)	<0.001	11 (-154, 138)	0 (0, 894)	0.76	356 (40, 943)	1 (1, 141.3)	<0.001
B. forsythus	231386 (52, $1x10^{10}$)	13 (7, 130)	<0.001	40 (-7, 643086)	1 (-57, 122)	0.039	294 (12, $7.5x10^{7}$)	12 (4, 900)	0.001
A. actinomycetem	69 (1, 225)	10 (1, 440362)	0.96	0 (-4, 34)	0 (-85358, 12)	0.27	34 (1, 235)	1 (1, 525679)	0.22
T. denticola	**$5.5x10^{7}$ (1140, $7.7x10^{11}$)**	**1 (1, 1)**	<0.001	13051 (1, $7.7x10^{8}$)	0 (0, 0)	<0.001	11555 (43, 7750000)	1 (1, 1)	<0.001
Median GCF Titres									
P. gingivalis	27 (11, 83)	25 (12.5, 35.5)	0.49	-1 (-31, 6)	-7 (-40, 0)	0.12	31 (12, 147)	31 (13, 93)	0.96
P. intermedia	6 (5, 8)	6.5 (5, 14.75)	0.53	0 (-1, 1)	0 (-2, 10)	0.61	6 (6, 7.8)	7 (1.25, 13.25)	0.68
B. forsythus	1 (0, 1)	0 (0, 0)	<0.01	0 (0, 1)	0 (0, 0)	0.008	0 (0, 1)	0 (0, 1)	0.28
A. actinomycetem	0 (0, 11)	0 (0, 31)	0.88	0 (-6, 0)	-0.5 (-31, 0)	0.23	0 (0, 15)	9.5 (0, 34)	0.59
T. denticola	5 (0, 15)	0.5 (0, 2)	<0.001	0 (-2, 5)	0 (-1.75, 1)	0.21	3 (0, 9)	0.5 (0, 2)	0.08
Mean Avidity									
P. gingivalis	1.4 (±0.7)	0.9 (±0.4)	0.04	0.3 (±0.5)	0.1 (±0.3)	0.32	1.1 (±0.5)	0.8 (±0.3)	0.038
P. intermedia	0.9 (±0.2)	0.8 (±0.5)	0.86	0.1 (±0.2)	0.1 (±0.3)	0.91	0.8 (±0.2)	0.7 (±0.3)	0.86
B. forsythus	1.4 (±0.5)	0.5 (±0.7)	0.012	-0.002 (±0.6)	-0.2 (±0.7)	0.49	1.4 (±0.6)	0.7 (±0.7)	0.046
A. actinomycetem	0.6 (±0.3)	0.8 (±0.4)	0.52	-0.02 (±0.2)	0.2 (±0.3)	0.15	0.7 (±0.4)	0.6 (±0.3)	0.63
T. denticola	0.7 (±0.3)	0.6 (±0.1)	0.35	0.008 (±0.6)	0.09 (±0.2)	0.6	0.6 (±0.5)	0.5 (±0.1)	0.2

bleeding, suppuration, pocket depth and attachment loss. Post-therapy, high responder patients had significantly lower MGI, pocket depth and attachment loss. These patients also had slightly lower bleeding and suppuration.

Microbiological parameters

For all bacteria except *B. forsythus* and *A. actinomycetemcomitans*, high responder patients had lower prevalences before SRP. These were not significant, however. In response to SRP high responder patients recorded greater reductions in all bacteria except *T. denticola* and *P. intermedia*. *B. forsythus* reduced significantly more in high responder patients compared to a slight increase in low responder patients. The slightly greater reduction in *P. intermedia* in low responder patients than high responder approached significance. Post-therapy, high responder patients had significantly lower levels of *P. intermedia*. *P. gingivalis* and *T. denticola* were detected more frequently in high responder patients, and there was also a lower prevalence for *B. forsythus* but these were not statistically significant.

Serum titres

The median titre for high responder patients for *T. denticola* was 5.5×10^7 EU compared to 1 EU for low responder patients. *P. gingivalis*, *P. intermedia* and *B. forsythus* titres were also significantly greater with *A. actinomycetemcomitans* titres increased compared to low responder patients. Low responder patients generally showed little change in median titres in response to SRP compared to reductions in the titres in high responder patients. The changes in *P. intermedia*

and *T. denticola* titres in high responder patients were significant when compared to low responder patients. Post-treatment, all titres were higher and this was significant for all titres except *A. actinomycetemcomitans* titres in high responder patients.

GCF titres

Pre-treatment local titres for *B. forsythus* and *T. denticola* were significantly higher in high responder patients when compared to low responder patients. The other titres were similar and not significantly different. In response to SRP, *P. gingivalis* titres increased slightly more in high responder patients but not significantly. There was a tendency for high responder *B. forsythus* titres to decrease in high responder patients and this was significant. There was little change in the other antibody levels and no other significant differences. Post-therapy, there were no significant differences in titres although the higher *T. denticola* titre in high responder patients approached significance.

Avidity

Patients high responder for *T. denticola* antibody titres had antibodies to *P. gingivalis* and *B. forsythus* of significantly greater avidity than low responder patients at baseline. There were no other significant differences before treatment. In response to SRP, in high responder subjects *B. forsythus* and *A. actinomycetemcomitans* antibody avidity increased slightly as did *B. forsythus* antibody avidity in low responder patients. However, most antibody avidities decreased and there were no significant differences between the two groups.

After treatment *P. gingivalis* and *B. forsythus* antibody avidity remained higher in high responder patients with similar avidities for the antibodies to the other organisms in both groups.

5.2 Humoral immune response in adult periodontitis and effect of SRP: Discussion

5.2.1 Titres and avidity at baseline

Serum titres

There were comparatively high titres to *T. denticola* and *B. forsythus*, similar titres for *P. gingivalis* and *P. intermedia* and comparatively low *A. actinomycetemcomitans* titres. The high percentage of high responder titres for *T. denticola* and *B. forsythus* may indicate a greater previous exposure of these patients to these organisms or greater pathogenicity. Just over twice as many patients were high responder for *P. gingivalis* than *P. intermedia* titres, and this also indicates the pathogencity of *P. gingivalis* and perhaps also the lack of pathogenicity of *P. intermedia.*

GCF titres

In general GCF titres were low. *P. gingivalis* produced the greatest antibody titre which may reflect its importance in the disease. The low titres to the other organisms may reflect a lack of local immune response or a lack of pathogenicity. There is a considerable response to *B. forsythus* and *T. denticola*, but this did not appear locally. The failure of the local response may allow these organisms to be pathogenic or the response is absorbed or degraded by the

plaque biofilm (Kilian, 1981). The low serum and GCF titres to *A. actinomycetemcomitans* coupled with its low prevalence are consistent with previous reports that *A. actinomycetemcomitans* is not a major pathogen in AP. However, it suggests a previous role in these patients. The organism may have been involved in the onset or initial stages of the disease process, resulting in the residual antibody levels.

It should be noted that the low GCF titres may reflect the dilution of the sample in 1ml of IB to allow analysis of the IgG response to five organisms. Previous studies in our laboratory have diluted GCF samples in 0.5ml to allow for four analyses. Tests using GCF samples from withdrawn patients indicated that dilution to eight samples rather than four did not affect the ELISA or dilute the antibody titres to a level below detection.

Avidity

It was not the intent of this study to compare titres or avidity to control patients and so control serum were used only to establish serostatus and not as age/sex matched controls for comparison to health. However in the current patient group, avidity of all antibodies was low, especially when compared to the inoculation studies of Ebersole et al. (1990) and Lopatin et al. (1991). These studies examined the avidity of antibodies after immunisation of tetanus toxoid and streptokinase, and found that avidities were significantly higher than those of antibodies to periodontal pathogens.

The overall failure of the humoral immune response to react may be due to oral tolerance (Lopatin et al., 1991), where long term exposure to an antigen produces poor quality antibodies.

5.2.2 Effect of SRP on antibody titre and avidity

Treatment resulted in little change in systemic and local antibody titres. A significant decrease in *P. gingivalis* and *P. intermedia* antibody avidity followed therapy, but there was little response in the avidity of the other antibodies.

Previous reports have shown mixed responses to SRP on antibody titre. Tolo et al. (1982) reported slight changes, but only antibodies to *P. gingivalis* produced a clear pattern. Ebersole et al. (1985b) showed marked increases for *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans* antibody titres. In contrast Aukhil et al. (1988) found significant reduction in *P. gingivalis* titres but little change in *T. denticola.* A significant decrease in *P. gingivalis* and *P. intermedia* titres was shown after SRP and surgery by Horibe et al. (1995). They also reported a slight but not significant decrease in *A. actinomycetemcomitans* titres. Murray et al. (1989) showed significantly lower titres in treated patients than untreated. More recently Mooney et al. (1995), before they split their patients by serostatus, reported significant increases in *A. actinomycetemcomitans* IgG and *P. gingivalis* IgG titres post-therapy. However, Mouton et al. (1987) found little change in antibody levels. It appears that the response to therapy can produce variable responses in antibody titre. Previous studies on a West of Scotland patient group (Mooney et

al., 1995) produced conflicting results to this current study. However, there was wide variation in subject response to treatment, as shown by the interquartile range. Some patients had marked increases while other decreases but together it appeared there was little response.

The changes in antibody level have been related to suppression of pathogens in plaque (Horibe et al., 1995). In this current study there were few significant differences in microbial prevalence before and after treatment, which may explain the lack of response. It should be remembered that only four sites per patient were examined and not the whole mouth. Together with the use of a very sensitive diagnostic assay, this may have disguised any significant response. SRP produces an inoculation effect, but this appeared to produce little response. Mouton et al. (1987) had previously suggested that although there is inoculation, it may not provoke active immunisation. The lack of response to the presumed reduction in microbial load suggests a failure of the host response, which may have been a factor in the onset of disease in the first place. Alternatively the poor response may reflect the fact that all five test organisms were not involved in the disease process. This seems unlikely given the relationship of some of the bacteria with clinical parameters and antibody titres.

Murray et al. (1989) also reported a concomitant reduction in local *P. gingivalis* titres and that this was a result of removal of the subgingival flora. Ebersole, et al (1984b) also reported a decrease in local titres in response to treatment. Although there was significant reduction in bacterial prevalences, there was not a

concomitant drop in local titres. Local antibody consumption may be reduced after removal of *P. gingivalis* (Ou Yang, 1993). If this were the case, there should have been an increase in antibody titres. However this was not found and the response more likely reflects a poor host reaction rather than microbial consumption. Again there was wide individual site reaction to treatment, and the grouping together of sites showed little response overall.

The response of antibody avidity following SRP has again produced mixed reports. Mooney et al. (1995) found no significant change in *A. actinomycetemcomitans* IgG antibody avidity, but a significant increase in *P. gingivalis* avidity. Holbrook et al. (1996) reported no change in *A. actinomycetemcomitans* antibody avidity during therapy. Chen et al. (1991) showed an increase in *P. gingivalis* antibody avidity after therapy in GEOP patients. The authors concluded that many GEOP patients do not produce protective levels of biologically functional antibody during the course of natural infection, but they may be stimulated to do so by treatment. It appears that in AP subjects, without regard for serostatus, these patients do not also produce adequate levels of biologically functional antibodies and that treatment does not stimulate these patients to do so. This may result from differences in the host response between GEOP and AP patients or the longer chronic exposure to antigens in AP patients. Further studies are required to investigate the response of avidity in the different patient categories.

The reduction in antigen load is known to result in selection of B-cell clones producing higher avidity antibodies (Mooney, 1995). In this study this did not appear to happen, and instead lower avidity antibodies were produced. However there was little change in the microflora on a patient basis and this may have affected the result, although it is presumed that the microbial load did decrease with treatment.

The low levels of *A. actinomycetemcomitans* preclude much comment but may reflect the organisms' lack of pathogenicity in AP. Hence the immune response would not be expected to change significantly. The fact that there are high responder patients to *A. actinomycetemcomitans* indicates previous antigenic challenge. It is possible that *A. actinomycetemcomitans* had a role earlier in the disease process and the titres reflect this. It is also possible that the high *A. actinomycetemcomitans* titres keep levels of the organism low preventing it from having further effect in these patients. However, from the clinical and microbiological results, it appears that *P. gingivalis*, *B. forsythus* and *T. denticola* are pathogenic, yet there is little response locally. This lack of response may allow these organisms to be pathogenic and cause periodontal destruction without recompense. Studies analysing the local and systemic response in patients where the microflora has been analysed from a large number of sites are required to clarify the effect of the flora on the humoral immune response.

In this study only the serum from AP patients was included and it would be worthwhile to analyse the serum from GEOP patients. Mooney & Kinane (1994) showed that GEOP patients had higher titres but lower avidity antibodies than AP subjects, and that the quality of the immune response may have bearing in the aetiology. The lack of response may reflect that whole cells were used. If particular antigens had been studied, there may have been a more marked response. Podmore et al. (1999), using serum from these AP patients, reported a similar lack of response to whole cells, but found a decrease in *P. gingivalis* W50 protease antibody titres post-SRP. Some studies have shown that certain antigens may be more immunogenic (Kinane et al., 1999). One example is the *Rag* antigen on the outer surface of *P. gingivalis* (Hanley et al., 1999). Further investigation would be to examine the effect of SRP on the antibody response to this and other immunodominant antigens, and to relate the response to the presence of the antigen before and after treatment. Using the *Rag* antigen as an example again, this antigen seems to be found more frequently on *P. gingivalis* isolated from deeper pockets (Hanley et al., 1999) and may be related to survival of the organism in these deeper sites. Would the reduction in *P. gingivalis* and pocket depth in response to SRP have an effect on this antigen and the humoral immune response towards it?

5.2.3 Serostatus

In this study 67.9% of patients were high responder for *P. gingivalis* antibody titres and is higher than previously reported by Mooney et al. (1995) (53.9%) and Chen et al. (1991) (33%), although Chen et al. (1991) analysed GEOP

patients. The percentage of high responder patients for *A. actinomycetemcomitans* titres is also higher than that reported by Mooney et al. (1995) (41.7%). Zafiropoulos et al. (1992) reported similar percentages of patients with elevated antibody levels to those in this study, and also similarly low levels for *P. intermedia.* The low number of high responder patients for *P. intermedia* titres could indicate that it is either not particularly immunogenic, it is not pathogenic, or that it is immunogenic/pathogenic only in these high responder patients. In this study a high percentage of patients were high responder for *A. actinomycetemcomitans*, but there was low *A. actinomycetemcomitans*. This suggests previous exposure to the organisms, which was probably pathogenic.

High responder patients for *P. gingivalis* had significantly greater pocketing and higher bleeding scores. Mooney et al. (1995) reported a similar trend. However they did not correlate serostatus with the microflora. *P. gingivalis* was significantly more prevalent in sites of high responder patients. Thus the increased immune response and greater pocketing may have resulted from higher prevalence of the organism. Interestingly Mouton et al. (1987) found high prevalences of *P. gingivalis* in their high responder group, which would support this hypothesis.

Serostatus generally had little significant effect on change in pocket depth. *P. gingivalis* high responder patients had a greater decrease in pocket depth, but generally the reduction was similar for high responder and low responder

patients. This finding disagrees with previous observations by Mouton et al. (1987), Mooney et al. (1995), Ebersole et al. (1992) and Danielsen et al. (1993). They suggested that the prior development of a protective humoral immune response had a positive contribution to disease resolution during and after therapy. The differences between this study and those above may reflect differences between patient groups and the averaging of individual variations in the immune response. These reported studies concentrated on titres to *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans*. It could be that the response to *B. forsythus* and *T. denticola* was similar to this current study.

Each different antibody serostatus reflected varying responses to the microflora. *P. gingivalis* high responder patients generally had higher prevalences of the test bacteria. The increased immune response to all organisms in these patients may be a reflection of this or the deeper sites found in these patients. Interestingly *P. intermedia* and *B. forsythus* were significantly higher at sites in high responder *P. gingivalis* antibody titre patients which may indicate that these sites are a more suitable environment or that, although the species are related and may share similar antigens (Vasel et al., 1996), both P. *intermedia* and *B. forsythus* are not targets of the antibody response to *P. gingivalis* in these patients. *P. gingivalis* is known to release outer membrane vesicles (Grenier & Belanger, 1991) which can act as immunostimulants. This may have increased the response to the organism and so the number of subjects who were high responder. Sites in *P. intermedia* high responder patients had significantly higher titres to but lower prevalences of *P. gingivalis* and *T. denticola*, which

may indicate that the immune response in these patients while not effective against *P. intermedia*, is effective against *P. gingivalis* and *T. denticola.* Similarly sites in *B. forsythus* high responder patients had lower prevalences for these two organisms, and similar prevalences for the other test species. Sites in *A. actinomycetemcomitans* high responder patients had significantly higher *P. intermedia* prevalence, but otherwise similar prevalences. Generally lower prevalences were found in sites from *T. denticola* high responder patients. The lower prevalences at sites in high responder patients may suggest an effective immune response. The effect is unlikely to be due to differences in pocket depth as there were no significant differences between high responder and low responder groups. However the lack of a clear pattern suggests that serostatus may have little effect on microbial colonisation.

5.2.3.1 Relationship between serostatus and serum titres

High responder subjects had higher serum titres to all test organisms in general. Given the protective nature of antibodies, it is surprising then that these patients did not respond significantly better clinically than low responder patients to treatment. This would suggest that there are other underlying mechanisms involved in the resolution of periodontal disease. Low responder patients had a tendency to show little change or no response in median titres to treatment compared with a reduction in high responder titres. The decrease in titre may reflect the decrease in microbial load (Horibe et al., 1995). Chen et al. (1991) and Mooney et al. (1995) also reported a decrease in titre in high responder patients for *P. gingivalis*. In these two studies, low responder patients showed an

increase in titre after treatment, but in this current study low responder patients responded poorly. Mooney et al. (1995) suggested that high responder patients respond to treatment by production of similar titres but higher avidity antibody, and low responder by increased titres of similar avidity. The results of this current study do not support this result. However these authors only examined *P. gingivalis* and *A. actinomycetemcomitans* titres and did not examine *P. intermedia*, *B. forsythus* and *T. denticola.* Further studies are required to clarify the role of serostatus in response to treatment, and to examine titres and antibody avidity to other pathogens than *P. gingivalis* and *A. actinomycetemcomitans.*

5.2.3.2 Relationship between serostatus and local titres

High responder patients were found to have higher local titres to all organisms. This suggests that serostatus is not only a systemic response but also applies to the local response. This would be expected given that the local response is a mixture of systemic and local antibody production (Mooney & Kinane, 1997). High responder patients may in general be more responsive to antigenic challenge than low responder patients. The greater local titres may also reflect a greater input from the systemic immune response.

It would be unrealistic to try to determine serostatus from local samples. Healthy subjects do not have high GCF volumes, which would make collection of samples difficult. In addition, these patients do not have the high numbers of plasma cells infiltrating the gingival epithelium or increased permeability of the

junctional epithelium from the inflammatory reaction. Therefore they would not be expected to have high levels of antibodies which could be measured.

Treatment resulted in little change in local titres irrespective of serostatus. This failure of the humoral immune response may account for the similar clinical response in these patients in this study and the differences with previous studies (Mooney et al., 1995; Mouton et al., 1987; Ebersole et al., 1992; Danielsen et al., 1993).

Changes at a local level may be more important in determining treatment outcome than the systemic response. Individual sites vary in microbial composition (Gunsolley et al., 1992), and presumably this is reflected in the local immune response. The varying immunogenicity of the flora (Califano et al., 1997; Lamster et al., 1990), and the consequent immune response or lack of response may determine the rate of disease progression and response to treatment. Further investigation is required to examine the relationship between the flora and immune response at a site level, and also the effect of the systemic response on the local response.

5.2.3.3 Relationship between serostatus and avidity

Papers by Mooney et al. (1995) and Chen et al. (1991) showed that avidity increased after treatment in high responder patients for *P. gingivalis* antibodies. In this current study *B. forsythus* subjects had a slight increase in avidity, but generally there was a decrease in avidity which was not significantly different

from low responder patients. Interestingly Mooney et al. (1995) reported a non-significant decrease in *A. actinomycetemcomitans* antibody avidity. Both subjects in the study by Mooney et al. (1995) and this current study responded well to treatment, irrespective of serostatus. It would appear that there is great variation in the response to treatment. High responder status to different organisms may produce different results, even within the same patient, and patient groups respond differently. Larger studies are required to try to determine a pattern. It could be suggested that the numbers of subjects in the studies reported are too small to present a representative picture. However finding a large number of suitable and similar patients would be difficult.

In general, although high responder patients had a higher antibody titre to all test organisms regardless of which organism the subject was high responder to, both groups responded similarly to treatment in terms of clinical condition and changes in avidity and local titres. The reasons for this are unclear, given the findings of previous studies (Mooney et al., 1995; Chen et al., 1991), and suggests other mechanisms in addition to the humoral response are involved. These mechanisms could include the the innate immune response, the inflammatory reaction, the T-cell response or particularly virulent strains of bacteria. Further investigation is required to clarify the situation. In addition, this study suggested that serostatus may have little effect on microbial colonisation. Another interesting area of investigation would be to expand on this using a greater number of patients and also different patient populations.

5.2.4 Relationship between the presence and absence of bacteria and serum antibody titres

The classification of the presence or absence on a patient basis of an organism from the evidence of four sites may not be representative. Clearly the organism may be present at other sites, and so any such classification is likely to be incorrect. Previous studies by Mombelli et al. (1991a, 1994a) and Gunsolley et al. (1992) have suggested that if deep bleeding sites are selected, it is possible to accurately determine if the patient harbours a particular organism. However although presence can be determined with some degree of certainty, a negative result does not mean the organism is absent, just not detected from those samples. In spite of this, patients that had detectable *P. gingivalis* had a significantly higher antibody titre to the organism. Interestingly, patients positive for *B. forsythus* had significantly lower titres to the organism. Ebersole et al. (1984b, 1986) suggested that increased titres to *P. gingivalis* may reflect colonisation and indicate pathogenicity. In this study *P. gingivalis* was associated with deeper pocketing, and supports the findings by Ebersole et al. (1984b, 1986). The higher titres of *B. forsythus* antibodies in *B. forsythus* negative patients and sites may indicate that an adequate response is effective in reducing the level of *B. forsythus*. However low titres at positive sites may indicate that *B. forsythus* is poorly immunogenic (Califano et al., 1997) or that the antibody response is reduced in some manner.

5.2.5 Relationship between the presence and absence of bacteria and local antibody titres

The presence of *P. gingivalis* in a subgingival sample resulted in a significantly higher local antibody response, and provides further evidence for the pathogenicity of this organism. *B. forsythus* titres were slightly higher at *B. forsythus* positive sites and again may suggest pathogenicity. Titres in general were low for *B. forsythus*, compared to the other organisms. The low response at sites suggests that the host may only mount a feeble response to this organism or that it only stimulates a small response locally. Ebersole et al. (1985a) and Ebersole & Cappelli (1994) showed that the frequency and distribution of antibody in GCF is related to colonisation by the target organism. The later study showed that the pattern of antibody response to *A. actinomycetemcomitans* was characteristic of localised host-parasite interactions. Therefore they suggested that antibodies may play an important role in the gingival sulcus in relationship to colonisation and clinical presentation. In this current study, *A. actinomycetemcomitans* was present at low levels and did provoke an immune response similar to that found by Ebersole et al. (1985a). However *P. gingivalis* seemed to produce a similar response. The lack of a response in general may have allowed colonisation and disease progression.

5.2.6 Relationship between the presence and absence of bacteria and antibody avidity

Again for the reasons mentioned above, it is probably incorrect to examine the effect of the presence or absence of organisms from four sites per patient on avidity. This is reflected in the results, in that there seems to be little difference. The presence of *B. forsythus* and *T. denticola* seems to adversely affect the immune system to produce low avidity antibodies to *P. gingivalis* and *P. intermedia.* This may be an artefact produced from the analysis of only four sites per patient or an actual effect. The effect of periodontal bacteria on the systemic immune system is unclear. Periodontal bacteria especially *P. gingivalis* and *A. actinomycetemcomitans* can affect the local host cells and impair function (Holt et al., 1999; Fives-Taylor et al., 1999). In this study *T. denticola* was found frequently at suppurating sites, which would suggest that the bacterium is able to adversely affect white cells. It is more likely that the bacteria produce a local effect on the plasma cells in the gingival tissue rather than a systemic effect. Further work is required to clarify this, and the effect of periodontal pathogens on plasma cells.

5.2.7 Antibody titre correlations

Antibody titres for *P. gingivalis*, *A. actinomycetemcomitans* and *T. denticola* correlated systemically and locally, indicating that these local titres have a greater systemic input. The non-correlation of *P. intermedia* and *B. forsythus* titres may suggest a greater local production of antibodies or destruction of these antibodies in the gingival crevice and not the other antibodies. Mooney and

Kinane (1997) showed that systemic and local antibody production contributes to the overall GCF antibody profile and is supported by these results. It appears that the contribution to the GCF antibody profile from systemic and local antibody response varies between each organism. This is in keeping with the variations in the flora in each site and the variation in the immunogenicity of each organism. The predominant cellular infiltrate in the periodontitis lesion comprises IgG producing plasma cells, and so one would expect local production of antibodies in addition to a systemic input (Mooney & Kinane, 1997).

P. gingivalis serum and local titres correlated with pocket depth. Ebersole et al. (1984a, 1986) reported similar findings and correlated both antibody titre and pocket depth with the presence of the organism. They suggested that this may indicate a role for *P. gingivalis* in the disease. Mooney and Kinane (1997) reported a negative correlation with pocket depth and *P. gingivalis* antibodies. These authors suggested that the response is protective because patients with greater disease had lower antibody levels. In this study the positive correlation suggests that the antibody response may not be protective and is ineffectual at preventing destruction. Alternatively the correlation may reflect the increased prevalence of *P. gingivalis* at deep sites, and increased titres where the organism is present.

Systemically, high antibody titres to *B. forsythus* are evident, but locally there is little response. This suggests that *B. forsythus* may be poorly immunogenic locally and supports the findings of Califano et al. (1997), who suggested the

lack of an immune response to *B. forsythus* may allow it to be pathogenic. The reduced local titres compared to serum titres might result from immunoglobulin degradation. Kilian (1981) and Frandsen et al. (1991) have shown that periodontal bacteria are capable of degrading immunoglobulins. Lamster et al. (1990) reported a similarly poor response to *P. gingivalis* and suggested that deficiency in local response may lead to disease.

A number of serum titres positively and significantly correlated with other serum titres. Similar results were found for local titres also. This could suggest that the different antibodies are responding to similar or shared antigens (Vasel et al., 1996, Hinode et al., 1998). The correlations may also reflect clustering of micro-organisms. Frequently *P. gingivalis*, *B. forsythus* and *T. denticola* titres correlated. In this study these three organisms have been shown to be frequently detected together. The immune response to these three pathogens may have produced high titres against all three and so produced the correlations. High responder patients for one antibody titre were often high responder for another. This general increased responsiveness may also have produced the titre correlations. The correlations also indicate the complex nature of the interactions between the host response and also the subgingival microflora.

CHAPTER 6

EFFECT OF SMOKING ON PERIODONTAL THERAPY

This chapter examines the effect of smoking on periodontal therapy in all smoker and non-smoker patients, and also AP and GEOP smokers and non-smokers. In addition a comparison of baseline parameters is made. The effect of smoking on the antibody titres and avidity in AP patients is also discussed. There were a total of 20 smokers (8 AP, 12 GEOP) in the 52 patients that completed SRP in this study. A Chi-squared analysis of the number of smokers in AP and GEOP groups returned a non-significant p value, 0.11. The results of the PCR assay were solely used in this chapter for determination of the microflora in smokers and non-smokers.

6.1 Effect of smoking on periodontal therapy: Results

6.1.1 Comparison of smokers and non-smokers: All subjects

6.1.1.1 Baseline

Table 6.1 compares the clinical measurements between all non-smoker and smoker patients. MGI scores for non-smokers and smokers were 2.4 (±0.8) and 2.0 (±1.1) respectively, and these were significantly different (p=0.005). The BOP scores for each group were 90% and 66% respectively, and were significantly different (p 0.001). There were no other significant differences between the two groups.

Table 6.1 also shows the comparison of the percentage of positive non-smoker and smoker patients for each microorganism. Although there were differences in prevalence between the groups, none was statistically significant. The comparison of the percentage of positive sites for non-smokers and smokers for

Table 6.1 Comparison of mean clinical and microbial parameters in all smokers and non-smoker patients at baseline, change in response to SRP, and post-SRP. Number of sites or patients shown brackets beside column title and SD beside values.

Clin. Parameters	Smoker Pre (80)	Non-smoker Pre (128)	p value	Smoker Chan (80)	Non-smoker Chan (128)	p value	Smoker Post (80)	Non-smoker Post (128)	p value
MGI	1.9 (±1.0)	2.4 (±0.8)	<0.001	0.9 (±1.0)	1.2 (±1.0)	0.1	0.9 (±0.9)	1.1 (±0.9)	0.06
PLI	1.2 (±1.1)	1.4 (±1)	0.2	0.6 (±1.1)	0.9 (±1.3)	0.42	0.7 (±0.8)	1 (±0.9)	0.02
BOP (%)	64	91	<0.001	22.5	49	0.01	41	41	0.98
Supp (%)	32.5	30.5	0.76	24	26	0.3	9	5	0.24
PD (mm)	6.3 (±1.4)	6.4 (±1.2)	0.72	1.2 (±1.2)	2.1 (±1.4)	<0.0001	5.1 (±1.3)	4.3 (±1.4)	0.0001
AL (mm)	13.9 (±1.9)	13.5 (±1.9)	0.16	0.7 (±1.0)	0.9 (±1.3)	0.28	13.2 (±1.9)	12.6 (±2.1)	0.05
GCFVol (nl/30s)	327 (±292)	367 (±305)	0.35	-26 (±357)	-34 (±380)	0.88	353 (±343)	401 (±335)	0.32
Micro. Parameters Patient based (%)	Smoker (20)	Non-smoker (32)	p value	Smoker (20)	Non-smoker (32)	p value	Smoker (20)	Non-smoker (32)	p value
P. gingivalis	55	59	0.76	20	-3	0.058	35	62.5	0.053
P. intermedia	85	69	0.19	30	34	0.62	55	34	0.14
B. forsythus	80	72	0.51	15	19	0.17	65	53	0.4
A. actinomycetem	10	12.5	0.78	0	12.5	0.1	10	0	0.93
T. denticola	50	53	0.83	25	25	1	25	28	0.81
Micro. Parameters Site based (%)	Smoker (80)	Non-smoker (128)	p value	Smoker (80)	Non-smoker (128)	p value	Smoker (80)	Non-smoker (128)	p value
P. gingivalis	47.5	49.2	0.81	23.8	14.8	0.57	34.4	23.8	0.1
P. intermedia	60	49.2	0.13	27.5	31.3	0.75	32.5	17.8	0.02
B. forsythus	70	67.2	0.67	23.8	35.2	0.002	46.3	32	0.04
A. actinomycetem	7.5	6.3	0.73	2.5	6.2	0.93	5	0	0.01
T. denticola	38.8	38.3	0.65	25	26.6	0.72	13.8	11.7	0.67

each organism is shown in Table 6.1. Again although there were some differences between the groups, none were significant.

6.1.1.2 Change in clinical and microbiological parameters in response to SRP

The change in clinical parameters in response to SRP is shown in table 6.1. Non-smokers had a greater reduction in all scores except GCF volume, where smokers have a slightly greater increase in volume. Non-smokers had significantly greater reductions in BOP (49% compared to 22.5%, $p=0.01$) and PD (2.1 ±1.4 mm compared to 1.2 ±1.2 mm, $p<0.0001$).

For all except *P. gingivalis* and *T. denticola*, smokers had less of a reduction in the microflora on a patient basis. The prevalence of *T. denticola* was the same for both groups and, while *P. gingivalis* decreased in smokers, there was a slight increase in non-smokers. There were no statistically significant differences however.

Comparing the test organisms on a site basis shows that non-smokers had greater reductions for all except *P. gingivalis* in response to SRP. Only the reduction in *B. forsythus* was statistically significant compared to smokers (35.2% compared to 23.8%, $p=0.002$). *P. gingivalis* decreased more in smokers than non-smokers, but not significantly.

6.1.1.3 Post-SRP

Post-treatment, compared to non-smokers, smokers had lower MGI and GCF volume scores, and a higher number of suppurating sites but these were not significant, though the difference in MGI scores approached significance (table 6.1). BOP scores were the same. Smoking subjects had statistically significantly lower plaque scores, and higher PD (5.1 ±1.3 compared to 4.3 ±1.4 mm, p=0.0001) and AL (13.2 ±1.9 mm compared to 12.6 ±2.1 mm, p=0.05) scores.

After SRP, smokers had higher prevalences of *P. intermedia*, *B. forsythus*, *A. actinomycetemcomitans* and lower *T. denticola* and *P. gingivalis*, but none of these were statistically significant. The difference between the *P. gingivalis* frequencies was only just above significance (p=0.053). *A. actinomycetemcomitans* was eliminated in non-smokers but not smokers.

Sites in smoking subjects had significantly higher numbers of positive sites for *P. intermedia* (32.5% compared to 17.8%, p=0.02), *B. forsythus* (46.3% compared to 32%, p=0.04), and *A. actinomycetemcomitans* (5% compared to 0%, p=0.01). *A. actinomycetemcomitans* was eliminated or reduced below the level of detection in non-smokers. *P. gingivalis* and *T. denticola* were found in more sites in smokers, but not significantly so.

6.1.2 Comparison of smokers and non-smokers: AP subjects

6.1.2.1 Baseline

Table 6.2 shows the comparison of the clinical and microbiological data for AP smokers and non-smokers. The only significant difference between the groups was that smokers have significantly less BOP (p=0.001). When the difference in the prevalence of *T. denticola* between AP smokers and non-smokers was corrected using the Bonferroni correction it was not found to be significant.

6.1.2.2 Change in response to SRP

Non-smokers had significantly greater reductions in BOP (49% compared to 12.5%, p=0.02) and PD (1.7 ±1.4 mm compared to 1.0 ±1.3 mm, p=0.007). Non-smokers also had greater improvements in MGI, PLI, Supp and AL. The GCF volume decreased in smokers compared to an increase in non-smokers. These differences were not significant however.

In response to SRP, *P. intermedia* and *T. denticola* reduced more in smokers, *B. forsythus* and *A. actinomycetemcomitans* in non-smokers, and *P. gingivalis* did not change. *B. forsythus* was found to increase in smokers rather than decrease. None of these differences was statistically significant.

The site analysis shows similar changes in *P. gingivalis* percentages for both groups and a significantly greater reduction in non-smokers for *P. intermedia* (24% compared to 19%, p=0.02). There was an increase in *B. forsythus* in smokers and a decrease in non-smokers which was significant (p<0.001). There

Table 6.2 Comparison of mean clinical and microbial parameters in AP smoker and non-smoker patients at baseline, change in response, and post-SRP. Number of sites or patients shown in brackets beside column title and SD beside values.

Clin. Param.	Smoker Pre (32)	Non-smoker Pre (80)	p value	Smoker Change (32)	Non-smoker Change (80)	p value	Smoker Post (32)	Non-smoker Post (80)	p value
MGI	2.3 (±0.74)	2.4 (±0.7)	0.56	0.97 (±1.1)	1.2 (±1.0)	0.37	1.3 (±0.8)	1.2 (±0.9)	0.35
PLI	1.5 (±1.1)	1.5 (±0.9)	0.96	0.26 (±0.96)	0.38 (±1.1)	0.17	0.8 (±0.8)	1.1 (±0.9)	0.1
BOP (%)	69	92.5	0.001	12.5	49	0.017	56	44	0.23
Supp (%)	28	29	0.95	15.6	22.5	0.86	12.5	6	0.27
PD (mm)	5.86 (±1.55)	5.94 (±1.2)	0.79	0.97 (±1.3)	1.7 (±1.4)	0.007	4.9 (±1.4)	4.2 (±1.6)	0.028
AL (mm)	14.1 (±2.2)	13.3 (±2.2)	0.12	0.26 (±0.96)	0.65 (±1.3)	0.085	13.8 (±2.1)	12.7 (±2.3)	0.017
GCFVol (nl/30s)	403.4 (±324)	394.5 (±339)	0.9	56 (±307)	-56 (±406)	0.12	346.9 (±347)	450.3 (±370)	0.17
Micro. Parameters Patient based (%)	Smoker (8)	Non-smoker (20)	p value	Smoker (8)	Non-smoker (20)	p value	Smoker (8)	Non-smoker (20)	p value
P. gingivalis	50	55	0.81	0	0	0.61	50	55	0.81
P. intermedia	75	70	0.79	25	15	0.78	50	55	0.81
B. forsythus	50	65	0.46	-25	20	0.057	75	45	0.15
A. actinomycetem	0	5	0.48	0	5	0.48	0	0	1
T. denticola	62.5	55	0.72	37.5	20	0.5	25	35	0.61
Micro. Parameters Site based (%)	Smoker (32)	Non-smoker (80)	p value	Smoker (32)	Non-smoker (80)	p value	Smoker (32)	Non-smoker (80)	p value
P. gingivalis	50	41.3	0.4	9.4	10	0.7	40.6	31.3	0.34
P. intermedia	62.5	52.5	0.34	18.8	23.8	0.015	43.8	28.8	0.13
B. forsythus	40.6	61.3	0.05	-25	36.3	<0.001	65.6	25	<0.001
A. actinomycetem	0	2.5	0.63	0	2.5	0.63	0	0	1
T. denticola	56.3	35	0.04	31.3	18.8	0.22	25	16.3	0.28

was no change in *A. actinomycetemcomitans* for smokers and only a slight change for non-smokers. A greater but non-significant reduction of *T. denticola* in smokers was noted.

6.1.2.3 Post-SRP

Post treatment AP smoking and non-smoking clinical and microbial parameters are shown in table 6.2 also. Smokers had deeper pockets (4.9 ±1.4 mm compared to 4.2 ±1.6 mm, p=0.03), but non-significant when Bonferroni corrected, and significantly greater attachment loss (13.8 ±2.1 mm compared to 12.7 ±2.3 mm, p=0.02). This group also had slightly more gingival inflammation, BOP and Supp, but less plaque and GCF volume than non-smokers. However, these differences were not significant.

The prevalence of the test organisms in patients after treatment in the two groups was not significantly different, although smokers had higher detection rates for *B. forsythus* and lower for *P. gingivalis*, *P. intermedia* and *T. denticola*. *A. actinomycetemcomitans* was eliminated in both groups.

However, a site analysis showed a significantly increased prevalence of *B. forsythus* in smokers (65.6% compared to 25%, p<0.0001). *P. gingivalis*, *P. intermedia* and *T. denticola* were also more frequently detected but not significantly. *A. actinomycetemcomitans* was absent.

6.1.3 Comparison of smokers and non-smokers: GEOP subjects

6.1.3.1 Baseline

The clinical and microbiological data for EOP non-smoker and smoker groups are shown in table 6.3. The only significant difference in the clinical measurements between the two groups was that smokers again had significantly lower BOP (P=0.003). When the difference in pocket depth between EOP smokers and non-smokers is Bonferroni corrected, it is not significant.

6.1.3.2 Change in response to SRP

The change in pocket depth was the only statistically significantly different clinical parameter between GEOP smokers and non-smokers (1.4 ±1.1 mm and 2.6 ±1.2 mm, $p<0.0001$).

In smoker patients *P. gingivalis* decreased, but increased slightly in non-smokers, which was significant (p=0.04). However, when corrected for multiple comparison using the Bonferroni method, it was not. Non-smoker patients had greater reductions for *P. intermedia*, *A. actinomycetemcomitans* and *T. denticola.* In smokers there was no reduction in *A. actinomycetemcomitans* and a greater decrease in *B. forsythus* compared to non-smokers, but these were not significantly different.

There were no significant differences when the changes in the test organisms were analysed on a site basis, though greater reductions were noted for *P. intermedia*, *A. actinomycetemcomitans* and *T. denticola* in non-smokers.

Table 6.3 Comparison of mean clinical and microbial parameters in GEOP smoker and non-smoker patients at baseline, change in response to SRP, and post-SRP. Number of sites or patients shown brackets beside column title and SD beside values.

Clin. Param.	Smoker Pre-SRP (48)	Non-smoker Pre-SRP (48)	p value	Smoker Change (48)	Non-smoker Change (48)	p value	Smoker Post-SRP (48)	Non-smoker Post-SRP (48)	p value
MGI	1.6 (±1)	2.3 (±1.0)	0.001	1.0 (±0.9)	1.3 (±1.0)	0.21	0.6 (±0.8)	1.0 (±1.0)	0.304
PLI	1.0 (±1.0)	1.2 (±1.1)	0.43	0.5 (±1.1)	0.4 (±1.2)	0.87	0.6 (±0.6)	0.8 (±1.0)	0.38
BOP (%)	60	87.5	0.003	29	50	0.25	31	37.5	0.52
Supp (%)	35	33	0.83	29	31.3	0.69	6	2	0.31
PD (mm)	6.6 (±1.2)	7 (±0.7)	0.023	1.4 (±1.1)	2.6 (±1.2)	<0.0001	5.2 (±1.3)	4.4 (±1.1)	0.0024
AL (mm)	13.8 (±1.6)	13.9 (±1.4)	0.88	1.0 (±1.0)	1.3 (±1.2)	0.22	12.8 (±1.6)	12.6 (±1.8)	0.26
GCFVol (nl/30s)	276 (±260)	321 (±232)	0.38	-82 (±379)	1 (±333)	0.26	358 (±344)	319 (±250)	0.54
Micro. Parameters Patient based (%)	Smoker (12)	Non-smoker (12)	p value	Smoker (12)	Non-smoker (12)	p value	Smoker (12)	Non-smoker (12)	p value
P. gingivalis	58.3	66.7	0.67	33.3	-8.3	0.039	25	75	0.014
P. intermedia	91.7	66.7	0.13	33.3	66.7	0.85	58.3	0	0.002
B. forsythus	100	83.3	0.14	41.7	16.7	0.18	58.3	66.7	0.67
A. actinomycetem	16.7	25	0.62	0	25	0.064	16.7	0	0.14
T. denticola	41.7	50	0.68	16.7	33.3	0.32	25	16.7	0.62
Micro. Parameters Site based (%)	Smoker (48)	Non-smoker (48)	p value	Smoker (48)	Non-smoker (48)	p value	Smoker (48)	Non-smoker (48)	p value
P. gingivalis	45.8	62.5	0.1	33.3	22.9	0.46	12.5	39.6	0.003
P. intermedia	58.3	43.8	0.15	33.3	43.8	0.06	25	0	<0.001
B. forsythus	89.6	77	0.1	56.3	33.3	0.15	33.3	43.8	0.29
A. actinomycetem	12.5	12.5	1	4.2	12.5	0.3	8.3	0	0.04
T. denticola	27.1	43.8	0.09	20.8	39.6	0.12	6.3	4.2	0.65

6.1.3.3 Post-SRP

GEOP smokers demonstrated significantly higher pocket depths after treatment (5.2 ±1.3 mm and 4.4 ±1.1 mm, p=0.0024). This group also showed higher Supp, AL and GCF volume and lower MGI and PLI scores compared to non-smokers, which were not significantly different.

After therapy smoker patients had significantly lower *P. gingivalis* prevalence (25% and 75%, p=0.01) and higher *P. intermedia* (58.3% and 0%, p=0.002). *P. intermedia* and *A. actinomycetemcomitans* were eliminated in non-smoker subjects. Smokers also showed higher *T. denticola* percentages and non-smokers higher *B. forsythus*, but these were not significant.

The site analysis also showed lower *P. gingivalis* and higher *P. intermedia* in smokers, which was significant. *P. gingivalis* was found in 12.5% of smokers and 39.6% of non-smokers, p=0.003. *P. intermedia* was found at 25% of smoker sites but absent in non-smoker sites and the p value was <0.001. *P. intermedia* and *A. actinomycetemcomitans* were eliminated in non-smokers. The difference between *A. actinomycetemcomitans* was significant, p=0.04, but when corrected for multiple comparisons became non-significant. *B. forsythus* was detected more frequently in smokers and *T. denticola* in non-smokers, but not significantly.

6.1.4 Smoking and serum antibody titres

Before treatment, smoker subjects had lower median titres for all the five test organisms, though this was only significant for *P. intermedia* (table 6.4). The differences between *P. gingivalis* and *T. denticola* titres approached significance. However there were no significant differences in the change in median antibody titres between smokers and non-smokers in response to treatment. There was a similar pattern after treatment to that pre-therapy. Again *P. intermedia* titres were significantly higher in non-smokers.

6.1.5 Smoking and GCF antibody titres

Smokers generally had lower median GCF antibody titres at baseline (table 6.4), with *B. forsythus* antibody titres significantly lower ($p<0.0001$). There were no significant differences in the change in median titres in response to SRP when smokers and non-smokers were compared. Post-therapy titres were significantly lower in smokers for *B. forsythus* and *T. denticola*, and also lower for *P. gingivalis*. *A. actinomycetemcomitans* titres were slightly higher in smokers post-therapy.

6.1.6 Smoking and antibody avidity

Table 6.4 also shows the differences between smokers and non-smokers for antibody avidity. Non-smokers had higher avidity scores for all organism antibodies tested. *P. gingivalis* avidity was significantly higher in non-smokers compared to smokers (1.4 ± 0.73 compared to 0.9 ± 0.38, $p=0.023$). In response to SRP, non-smokers had greater reductions in *P. gingivalis*, *P. intermedia*, and

Table 6.4 Comparison of median serum and GCF titres and mean antibody avidity to each organism in smoker and non-smoker AP patients at baseline, change in response to SRP, and post-SRP. Interquartile range shown beside each value.

Median Serum Titres	Smokers Pre	Non-smok Pre	p val.	Smokers Chan	Non-smok Chan	p val.	Smokers Post	Non-smok Post	p val
P. gingivalis	8 (1, 40909)	322 (117, 6229)	0.06	0 (-6, 18)	-6 (-162, 258)	0.76	6 (1, 32950)	391 (39, 24506)	0.06
P. intermedia	1 (1, 105)	513 (152, 1405)	0.01	0 (-63, 0)	-11.5 (-185, 154)	0.98	1 (1, 42.2)	395 (11, 100000)	0.002
B. forsythus	14 (10, 152)	13565 (27, $7.5x^{8}$)	0.11	-3 (-49, 57)	-212 (-43270, 7)	0.21	29 (4, $7.5x10^{8}$)	293 (11, 100000)	0.7
A. actinomycetem	10 (2, 440372)	69 (1, 225)	0.3	-2 (-12, 85500)	0 (-34, 4))	0.14	1 (1, 525821)	34 (1, 158)	0.14
T. denticola	1 (1, 2468)	5045174 (284, $7.5x10^{10}$)	0.08	0 (-$9.8x10^{7}$, 0)	-491 (1, 291)	0.62	1 (1, 291)	11555 (21, 100000)	0.08
Median GCF Titres									
P. gingivalis	23 (12, 33.7)	33 (12, 83)	0.21	3 (0, 14.5)	3 (-5, 47)	0.8	24.5 (13, 59)	35 (13, 162)	0.13
P. intermedia	7 (5, 14.75)	6 (5, 8)	0.65	0 (-10, 1)	0 (-1, 2)	0.33	6.5 (1.25, 10)	6 (6, 8)	0.79
B. forsythus	0 (0, 0)	1 (0, 1)	<0.001	0 (0, 0)	0 (-1, 0)	0.09	0 (0, 0.75)	1 (0, 1)	0.006
A. actinomycetem	0 (0, 31)	0 (0, 7)	0.6	2 (0, 33.2)	0 (0, 7)	0.16	14.5 (0, 36.2)	0 (0, 23.5)	0.33
T. denticola	1 (0, 2)	5 (0, 15)	0.27	0 (-1, 1)	0 (-5, 3)	0.27	0.5 (0, 2)	3 (0, 9)	0.04
Mean Avidity									
P. gingivalis	0.9 (±0.38)	1.4 (±0.73)	0.023	0.11 (±0.23)	0.26 (±0.56)	0.32	0.79 (±0.24)	1.15 (±0.52)	0.018
P. intermedia	0.82 (±0.26)	0.87 (±0.48)	0.79	0.1 (±0.29)	0.11 (±0.22)	0.93	0.72 (±0.23)	0.76 (±0.26)	0.7
B. forsythus	0.94 (±0.58)	1.22 (±0.86)	0.42	0.05 (±0.23)	-0.11 (±0.78)	0.44	0.89 (±0.73)	1.33 (±0.7)	0.18
A. actinomycetem	0.64 (±0.37)	0.79 (±0.36)	0.33	0.08 (±0.23)	0.02 (±0.24)	0.55	0.72 (±0.28)	0.62 (±0.35)	0.46
T. denticola	0.57 (±0.08)	0.66 (±0.32)	0.23	-0.06 (±0.38)	0.06 (±0.57)	0.53	0.62 (±0.37)	0.6 (±0.45)	0.88

T. denticola avidities than smokers, but these were not statistically significant. In non-smokers *B. forsythus* avidity increased compared to a decrease in smokers. *T. denticola* avidity increased in smoker subjects, and *A. actinomycetemcomitans* avidity had the greater reduction in smokers. Following therapy, *P. gingivalis* avidity was still significantly higher in non-smokers, and *B. forsythus* avidities were also higher in non-smokers.

6.1.7 Smoking and serostatus

Smoker subjects had fewer high responder antibody titres than non-smokers (table 6.5). Non-smokers had significantly more patients with high responder titres for antibodies to *P. gingivalis* and *T. denticola.*

6.2 Effect of smoking on periodontal therapy: Discussion

The results compared smokers to non-smokers in all patients and both disease groups. No attempt was made to stratify the results by pack-years due to the small number of smokers. In addition, former smokers were incorporated into the non-smoking group. All former smokers had given up five years or more previously, and there is evidence that, in these patients after this time, responses to periodontal therapy become similar to never smokers (Grossi et al., 1997).

6.2.1 Clinical parameters

6.2.1.1 Baseline

The analysis of the clinical data of smokers and non-smokers shows that smokers have significantly lower scores for gingival index and BOP. Reports by Preber

Table 6.5 Relationship between smoking and serostatus. Percentage of high responder patients in smokers and non-smokers.

Serostatus	Smoker (%)	Non-smoker (%)	p value
P. gingivalis	25	85	0.002
P. intermedia	12.5	30	0.33
B. forsythus	37.5	70	0.11
A. actinomycetem	37.5	45	0.72
T. denticola	25	90	0.001

& Bergstrom (1985, 1986a) found lower gingival index scores in their study populations. The decreased BOP in relation to non-smokers is consistent with a number of previous studies (Preber & Bergstrom 1985, 1986a; Bergstrom & Flodeus-Myrhed, 1983; Preber et al., 1980; Feldman et al., 1983; Bergstrom, 1990; Ah et al., 1994). Hedin et al. (1981) and Kinane and Radvar (1997) reported lower GCF volumes in smokers compared to non-smokers, and although there was a lower mean GCF volume in smokers in this study, this was not significant. The lower gingival index score, BOP and GCF volume reflects the vasoconstrictive effects of nicotine and diminished peripheral blood flow as a result (Bounaneaux et al., 1988; Michel et al., 1988).

No significant differences were noted between plaque scores in smokers and non-smokers, and this supports the findings of Preber & Bergstrom (1986a). However it appears that generally smokers have poorer oral hygiene (Preber et al., 1980; Preber & Bergstrom 1986a; Locker, 1992; Eklund et al., 1994; Holm et al., 1994). The poorer levels of plaque control have been conjectured to be the reason for the differences in the severity of periodontal destruction between non-smokers and smokers (Preber et al., 1980; Preber & Bergstrom, 1985). However in this current patient group this did not seem to be the case, but it may be that in patient groups with poorer oral hygiene there is greater periodontal destruction.

Smoker subjects had slightly, but not significantly, lower pocket depths. Similar findings have been reported by other authors (Preber & Bergstrom, 1985, 1986a; Ah et al., 1994), but this disagrees with others that showed that smokers have

greater pocket depths (Ismail et al., 1983; Solomon et al., 1968; Feldman et al., 1987; Bergstrom, 1989; Goultschin et al., 1990; Haber et al., 1993).

Mullally et al. (1999) and Schenkein et al. (1995) suggested that GEOP smokers have greater periodontal destruction than non-smokers and AP smokers, but in our patient group this was not the case. The increased pocketing in GEOP smokers compared to AP smokers was in keeping with the increased severity of periodontal disease in GEOP subjects in general, and compared to GEOP non-smokers the mean pocket depth was slightly lower.

In the current study population there was no evidence for increased pocket depth in smokers, but gingival index, BOP and GCF volume were decreased, which is consistent with the vasoconstrictive effect of nicotine.

6.2.1.2 Effect of SRP

Post-treatment pocket depth was significantly lower in non-smokers (4.3 ±1.4 mm compared to 5.1 ±1.3 mm, $p=0.0001$) and non-smokers had also a significantly greater reduction in pocket depth (2.1 ±1.4 mm compared to 1.2 ±1.2 mm, $p<0.0001$). Previous studies have reported a poorer response in smokers to SRP and findings of this current study are in agreement (Ah et al., 1994; Preber & Bergstrom, 1986b; Preber et al., 1995; Kaldahl et al., 1996b; Kinane & Radvar, 1997; Grossi et al., 1997; Machtei et al., 1998). Smoker subjects had significantly greater attachment loss after treatment. Kaldahl et al. (1996b) and Ah et al. (1994) reported similar findings in their patient population.

Oral hygiene was significantly better in smokers after treatment and so the poorer response to therapy is probably not due to oral hygiene levels (Preber & Bergstrom, 1986b). There was a greater reduction in gingival index in non-smokers but this was not statistically significant. Non-smokers also showed significantly greater reduction in bleeding on probing. After treatment mean GCF volume in smokers remained lower than non-smokers and the increase in GCF volume was lower than non-smokers. These are consistent with both a diminished peripheral blood flow (leading to diminished GCF flow) and a reduced response to therapy in smoker subjects.

The poorer response of smokers to therapy is probably the result of the general effect of smoking on the host which compromises the response to periodontal treatment. The mechanisms of healing among smokers may be impaired, especially if fibroblast and osteoblast functions are suppressed, which may result in poorer tissue repair. Fibroblast function is impaired by tobacco use (Raulin et al., 1989; Peacock et al., 1993; Lenz et al., 1992), with a decrease in chemotaxis and proliferation (Nakamura et al., 1995). Fibroblasts can store and later release nicotine and this may impair their function (Hanes et al., 1991). Fang et al. (1991) showed suppression of osteoblast function and proliferation in smokers compared to non-smokers.

The greater reduction in pocket depth in non-smokers but similar attachment loss changes are perhaps due to the greater reduction in gingival swelling of non-smokers. Non-smokers have more gingival inflammation and when this resolves

it may lead to some recession, which when combined with greater decrease in pocket depth results in small AL changes. The less favourable healing could not be attributed to the plaque control level. This is in agreement with studies by Ismail et al. (1983) and Tonetti et al. (1995).

6.2.2 Microbiological parameters

6.2.2.1 Baseline

There were no significant differences in the prevalence of the five pathogens between smokers and non-smokers. The prevalences of *P. intermedia* and *B. forsythus* were slightly higher in smokers. This current study's findings are in agreement with Stoltenberg et al. (1993) and Preber et al. (1992). In a study of 83 smokers and 62 non-smokers by Preber et al. (1992), the prevalence of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* from subgingival plaque of smokers and non-smokers was not significantly different. Stoltenberg et al. (1993) found no significant differences in the prevalence of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *E. corrodens*, and *F. nucleatum* between smokers and non-smokers. These authors did not investigate the prevalence of *B. forsythus* and *T. denticola* in their patients. Zambon et al. (1996) reported in a study of 1, 426 subjects, of whom roughly 60% smoked, that there were significantly higher levels of *B. forsythus*, *A. actinomycetemcomitans* and *P. gingivalis* in smokers. Although the age range was similar to our study, they analysed all attachment loss levels rather than just concentrating on the deeper sites, and this may account for the differences reported.

When smokers and non-smokers were analysed in their respective disease groups, there was a significantly higher prevalence of *T. denticola* in AP smokers. However when corrected for multiple comparisons and the smaller sample numbers, these differences were not significant.

6.2.2.2 Effect of SRP

Following therapy *P. gingivalis* was detected in significantly fewer smoker than non-smoker patients. There were higher prevalences of *P. intermedia*, *B. forsythus* and *A. actinomycetemcomitans* in smokers. *A. actinomycetemcomitans* was eliminated in non-smokers. A slight increase in *P. gingivalis* was detected in non-smoker subjects post-SRP. The sites comparison showed lower prevalences for all the test organisms in non-smokers, which was significant for *P. intermedia*, *B. forsythus* and *A. actinomycetemcomitans*. Non-smokers had greater reductions in the number of positive sites and this was significant for *B. forsythus*. *P. gingivalis* was found to decrease in more smoker patients, but the prevalence of *A. actinomycetemcomitans* remained the same in this group.

The reduction in *B. forsythus* is similar to that found by Grossi et al. (1997). They reported a lower reduction in *B. forsythus* levels in smokers, but also for *P. gingivalis*. However the finding in this thesis of greater *P. gingivalis* reduction is supported by Haffajee et al. (1997a), who found similar results comparing smoker and non-smoker groups. The higher number of *P. gingivalis*

positive smoker sites but lower number of *P. gingivalis* positive smoker patients compared to non-smokers may suggest a sub-group of patients who harbour high levels of *P. gingivalis*, as Mombelli et al. (1994a) suggested may occur for *A. actinomycetemcomitans* in some patients. Preber et al. (1995) reported almost total eradication of *A. actinomycetemcomitans* but similar levels of reduction for smokers and non-smokers for *A. actinomycetemcomitans*, *P. gingivalis* and *P. intermedia* in response to a favourable clinical outcome after therapy. Renvert et al. (1998) reported slight decreases in the levels of *P. gingivalis* and *P. intermedia/P. nigrescens* after SRP. They suggested that the microbiological response found seemed to be in conformity with the clinical response with little influence of the smoking habits. However this study used culture to examine the flora, which may account for the differences.

The differences reported in the pocket depths between smokers and non-smokers may account for the differences in the microflora after treatment. The deeper pocket depths in smokers, which are more anaerobic than non-smokers (Loesche et al., 1983), may favour an anaerobic flora whereas the shallower less anaerobic pockets after treatment in non-smokers may not be a suitable environment for the persistence of an anaerobic flora. It is possible that the differences are due to the differing plaque control levels between smokers and non-smokers, but given that plaque control is poorer in non-smokers, who had the better improvement in the flora, this is unlikely.

The poorer host response against infectious agents in smokers may also account for the findings. The host immune response is adversely affected in smokers (Seymour, 1991; Lamster, 1992). In smokers there are significantly increased numbers of peripheral blood leucocytes especially neutrophils (Barbour et al., 1997). However, smoking has been shown to affect neutrophil chemotaxis, adherence, phagocytosis and function (Kenney et al., 1977; Kraal et al., 1977, 1979; MacFarlane et al., 1992; Noble et al., 1975; Eichel et al., 1969), both systemically and locally in the periodontium. Low levels of nicotine are chemotactic (Totti et al., 1994) whereas high levels inhibit phagocytosis (Ryder et al., 1994). There is an enhanced oxygen metabolism and dysfunctional regulation of extracellular proteases (Barbour et al., 1997). A detrimental effect on the respiratory burst has also been reported (Kalra et al., 1991; Codd et al., 1987). The proportion of circulating natural killer cells is decreased in smokers (Tollerud et al., 1991) and the cytotoxic activity is dose-dependently reduced (Hersey et al., 1983; Ginns et al., 1985; Hughes et al., 1985; Phillips et al., 1985). Systemically there appears to be an increase in macrophage numbers but a decrease in function (Hoogsteden et al., 1991). There is inhibition of phagocytosis (Ortega et al., 1994) and the oxidative burst (Skold et al., 1993) from these cells however. *In vitro* exposure to nicotine seems to suppress the ability of macrophages to kill oral pathogens (Pabst et al., 1995). Plasma vitamin C levels are reduced in smokers, possibly affecting the ability of phagocytes to control oral pathogens (Tribble et al., 1993; van Antwerpen et al., 1993). In addition there is impaired production of IgA, IgG and IgM (see below). These could all be potentially important factors in protecting the host

tissues from re-infection during the healing process. Perhaps also the lower GCF volume in smokers results in less "washing-out" of bacteria from the gingival sulcus. The combination of these may result in inadequate clearance of oral pathogens, and so account for the increased prevalences of the test bacteria in smokers compared to non-smokers after SRP.

6.2.3 Humoral immune response

In general smoker subjects had a lower percentage of high responder patients, lower median systemic and local antibody titres to the test bacteria, both before and after treatment. The general lack of response in antibody titres to treatment reported in this study was seen in both smokers and non-smokers, though smokers tended to respond less well than non-smokers. A similar pattern was evident in a comparison of antibody avidity. Smokers had lower avidity molarity, both before and after treatment. However the response to treatment was the same for both groups.

The poorer immune response in smokers may reflect the systemic and local effects of smoking and nicotine on the host. The vasoconstrictive effects of nicotine may reduce antigen presentation and ingress of defence cells. However, Sopori et al. (1989) reported similar proportions of B-lymphocytes in smokers and non-smokers, but there seems to be a general decrease in proliferative responses (Sopori et al., 1989; Savage et al., 1991; Goud et al., 1992). A decrease in proliferative responses could result in fewer plasma cells and hence lower systemic and local antibody titres. The effect of smoking on B-

lymphocytes and plasma cell proportions and numbers in the gingiva is not yet known. There are conflicting reports on the effect of tobacco use on T-lymphocytes. In some patients the numbers may increase and in others decrease (Ginns et al., 1982; Jezewska et al., 1989; Costabel et al., 1986). The effect may be race specific with a decrease in CD4+ cells reported in Negroid smokers (Tollerud et al., 1991) and an increase in Caucasian smokers (Jezewska et al., 1989). There is also a decrease in proliferative responses (Chang et al., 1990), but the effect of smoking on cytokine production is unclear, although there may be an increase in IL-4 (Byron et al., 1994) and IL-2 production (Pedro et al., 1992). The impairment of T-cell function and cytokine production could have an effect on antibody production.

A general reduction in immunoglobulin production has been shown (Holt et al., 1987; Johnson et al., 1990; Finklfea et al., 1971). The concentration of serum IgG is reduced (Ferson et al., 1979; Hersey et al., 1983; Quinn et al., 1996). The results of this study support the finding of reduced serum IgG in smoker subjects. There are conflicting reports regarding the levels of IgM and IgA (Hersey et al., 1983; Ferson et al., 1979) and a reduction in salivary IgA has been reported (Bennett & Read, 1982). Reductions in serum IgG levels to the periodontopathogens, especially *P. intermedia* and *F. nucleatum*, have been reported (Haber, 1994). The reduction of the IgG response to *P. intermedia* is similar to that found in this study. Why titres to these organisms in particular are reduced is unclear and requires further investigation. Quinn et al. (1998) reported that the influence of smoking on IgG subclasses was dependent on race.

All but one of the AP patients in this study were Caucasian, so this could not be confirmed or refuted.

Smoking impairs the function of other host cells such as neutrophils and macrophages, as discussed above. Similar impairment of B-lymphocytes and plasma cells may again result in decreased antibody titres and also avidity, although the similar response to inoculation in both groups in this study argues against this.

Antibody titres are generally thought to be protective and absence or decrease in titres, especially locally, may result in disease. Therefore it seems reasonable to suggest that reduced antibody titres and avidity in smokers may contribute to the greater destruction observed in these patients. This strengthens the hypothesis that it is the host response rather than a difference in the flora that is responsible for the greater periodontal destruction.

CHAPTER 7

METHODOLGICAL CONSIDERATIONS AND CONCLUSIONS

This chapter deals with methodological issues of this thesis in the first part and, in the second part, brings together the results and discussions from the previous chapters in a conclusion

7.1 Methodological considerations

7.1.1 Site Selection

Four sites per patient were selected to sample in order to provide a representative sample and to minimise false negatives (Haffajee & Socransky, 1992). Gunsolley et al. (1992) reported large variation in microbial composition between sites in the same subject. In order to detect *P. gingivalis*, *P. intermedia*, and *B. forsythus* using IFA or DNA probes, they and other authors have suggested a minimum of 2-4 sites over 5mm (Gunsolley et al., 1992; Savitt et al., 1991; Christersson, 1992). To detect *A. actinomycetemcomitans*, which was not related to disease, required a greater number of sites: up to 25 (Gunsolley et al., 1992; Christersson, 1992).

Curettes can collect as much as 90% of the subgingival plaque and is the best technique to use if only a few organisms are available (Tanner & Goodson, 1986). PCR is more sensitive than IFA or DNA probes and it was the aim only to detect not to quantitate. Therefore it seemed possible to detect a representative sample of *A. actinomycetemcomitans* using four deep sites per patient.

In retrospect given the low prevalence of *A. actinomycetemcomitans* in AP, both by PCR and Checkerboard, perhaps a larger number of sites should have been used. However the prevalence of *A. actinomycetemcomitans* in GEOP is similar to that found in other studies. The use of four sites per patient appeared to provide representative samples of *P. gingivalis, P. intermedia, B. forsythus* and *A. actinomycetemcomitans* especially if the deepest pocket in each quadrant was used (Mombelli et al., 1991b).

7.1.2 Sample size

The study investigated the changes in the microflora and humoral immune response with SRP. In order to gain a reliable result with sufficient power, sample size was based on similar studies previously published.

Thirty patients were deemed necessary for each group (Badersten et al., 1984a, b; Persson et al., 1995). In the event it proved harder to recruit 30 suitable GEOP subjects of suitable age and medical condition. Initially 27 were recruited but 3 were excluded shortly after on medical grounds especially antibiotic consumption, or their lack of motivation (e.g. failed appointments).

Thirty-three AP patients were recruited, slightly more than thirty in an attempt to have a post-treatment sample size of 30 and account for drop outs. Twenty eight patients were sampled after treatment and the reasons for exclusion of the five drop-outs were similar to those above.

7.1.3 Sampling procedures

Sterile scaling instruments, either curettes or Gracey curettes, were used to collect subgingival plaque samples in a single vertical stroke. Much of the variation in microflora between studies is due to sampling procedures (Dahlén & Wikström, 1988). It was important to standardise procedures and to obtain a representative sample (Tanner & Goodson, 1986).

Possible methods of collecting subgingival plaque include curettes, paper points, capillary tubes and barbed broaches (Tanner & Goodson, 1986). The choice of method was influenced by the fact that after sampling the subgingival flora would be disturbed by scaling and so it was not necessary to leave an intact flora. Curettes may remove up to 90% of the subgingival plaque and may remove up to 10^7 organisms, which is much higher than other techniques (Tanner & Goodson, 1986). Such a sample size enables a representative sample to be collected and is the preferred technique if the candidate organism is present in low numbers. Although paper points have been shown to be as good as curettes (Renvert et al., 1992; Moore et al., 1985; Dahlén et al., 1989a), there are concerns about whether a representative sample is produced (Baker et al., 1991) and if they can reach the base of the pocket (Dahlén & Wikström, 1988).

One criticism of curettes is that they may not reach the base of the pocket especially in healing sites after treatment. This did not appear to be a problem as samples were removed after probing had expanded the pocket.

7.1.4 Periodontal probing

The most common and simplest method of assessing periodontal disease is by manual probing, be it pocket depth (PD) or attachment loss (AL). However there are a number of problems and errors inherent in probing measurements especially when determining change in pocket depth. Variation in the probing force may occur in the measurement of sites between examinations by the same examiner (Hassell et al., 1973) and between different examiners (Gabathular & Hassell, 1971). The thickness of the probe and its positioning around the tooth may influence the PD. Anatomical features such as tooth contour may not allow correct probe positioning. The depth measured depends on the probing force used and the severity of inflammation. The greater the probing force, the greater the PD measured (van der Welden & der Vries, 1978; Mombelli & Graf, 1986). The difference between the probing measurement and the histologic "true" pocket depth may range from fractions of a millimetre to several millimetres. The greater the degree of inflammation the greater the pocket depth as the probe penetrates the basal connective tissue of the pocket to touch bone (Listgarten, 1980; Listgarten et al., 1976; Magnusson & Listgarten, 1980; Fowler et al., 1982). Measurement variation increases as the time interval between replicate measurements increases and as the severity of the disease increases (Clark et al., 1992).

The use of the same thickness probe, a fixed reference point and a constant probing force improve the accuracy and reproducibility of periodontal probing. The introduction of pressure-sensitive automated probes aimed to overcome

some of these problems. These probes all use a constant force, each is a constant diameter, and read to a fraction of a millimetre obviating the need to round up or down. They allow the use of a fixed reference point if used with a stent or disc probe (Jeffcoat, 1991). A number of different types are available: Florida probe (Gibbs et al., 1988; Magnusson et al., 1988a), Interprobe (Rams & Slots, 1993), Toronto probe (Karim et al., 1990; Birek et al., 1987), Periprobe (Quirynen et al., 1993), Brodontic pressure probe (Walsh & Saxby, 1989), Vine Valley probe (Vine Valley corporation, USA) and even a probe that determined the position of the CEJ (Jeffcoat et al., 1986). In this study the Florida probe was used for all measurements.

Studies comparing the Florida probe with conventional probing have shown that there is a high correlation between manual and electronic probing (Magnusson et al., 1988a). However in deeper sites the Florida probe consistently recorded lower pocket depths than manual probing, but this may be a reflection of rounding up or down when using a manual probe (Osborn et al., 1992). This consistent under-measurement may also be due to difficulty in Florida probe placement due to the sleeve, compressibility of the soft tissue by the sleeve and the effect of multiple probing on the same site. The last seems unlikely as multiple probing of the same site usually results in increased pocket depth. Similar findings have also been reported with other electronic probes (Watts, 1987; Kalkwarf et al., 1986) with one report suggesting that manual probing is also more reproducible (Quirynen et al., 1993). However using the Florida probe, Magnusson et al. (1988b) reported that probing depth did not influence

reproducibility and that the reproducibility was significantly superior (Magnusson et al., 1988a). Florida probe measurements are much more reproducible than conventional measurements when each site is measured twice and the difference between measurements is no more than a millimetre (Osborn et al., 1990). However a single pass is no better than a single pass with a manual probe. Measuring attachment levels, the standard deviation of the measurement differences were lower than conventional probing, which when using the criteria of Haffajee et al. (1983) results in a lower threshold of change with the Florida probe. This allows the earlier identification of loser sites and also permits a smaller number of subjects in a study (Clark et al., 1992).

The Florida probe offers several advantages over conventional probing:

1) It reduces measurement error.

2) There is no examiner bias as the result is displayed on a screen which can be turned away from the examiner.

3) Resolution at 0.1mm or 0.2mm removes the need for rounding up or down, and so avoids the introduction of unpredictable error distributions.

4) Hence normal distributions are more closely approximated which are likely to reflect changes in measurement over time and therefore decisions about whether a site has lost or gained attachment made at lower cut off points.

In their studies of the Florida probe Osborn et al. (1990, 1992) stressed the need that although the Florida probe is much more reproducible than conventional probing, the same examiners should measure the same subjects in a longitudinal

clinical trial. On the basis of these studies each site was measured at least twice in the same group of patients by the same operator, with the difference between measurements no greater than 1mm. In addition the same operator always measured the same sites (IBD for AP and PJH for GEOP patients).

7.1.5 GCF sampling for ELISA

Methods for collecting GCF include capillary tubes and paper strips. Previous studies that have used capillary tubes have reported difficulty in collection and estimation of volume measurements (Ebersole et al., 1984a). Whatman filter paper has been shown to be more suitable compared to those supplied by Harco (Griffiths et al., 1988). Cut into strips of dimensions 2x8 mm, they are compatible with the dimensions of the Periotron electrodes. Recovery of human serum proteins including antibodies exceeds 90% (Ebersole et al., 1984a; Griffiths et al., 1988). This method is very suitable for sampling local antibody titres.

7.1.6 Inter-operator variability

Throughout this thesis two different operators examined the patients studied (IBD for AP patients and PJH for GEOP patients). As no inter-operator calibration was performed, some of the differences between operators may be due to operator variability rather than true differences. This is especially true for the more subjective parameters such as MGI and PLI. Although BOP and Supp are dichotomous scores, there are still differences in the way these are recorded between operators. As all these parameters may differ in the way they are

recorded between operators, few inter-operator comparisons have been made and any significant differences treated with caution.

GCF measurements tend to be more objective and provide a continuous variable that relates to degree of gingival inflammation. The GCF samples were taken on identical paper strips, cut from the same sheet of paper, for the same duration, measured in the same machine, and calibrated on the same curve. In addition, the same chair in the same clinic was used at all times by both operators when sampling or treating patients. Therefore, any differences in GCF volume are more likely to be true differences.

Pocket depth and attachment loss were recorded in both patient groups using the same probes and computer interface at the same dedicated chair. The standard deviation, which is a measure of probing variation/error, of the two PD measurements for IBD at baseline was 0.46mm and for PJH was 0.43mm. Therefore, the difference of 1mm in PD between AP and GEOP patients at baseline is likely to be a true difference, and it would not be unreasonable to base statistical comparisons on this parameter.

7.2 Conclusions

7.2.1 Comparison of AP and GEOP subjects

A major part of this research project was to compare AP and GEOP patients and the effect of SRP on clinical and microbial parameters in these subjects. Initially GEOP patients had deeper pockets, and lower BOP and GCF scores, which

could be due to the increased proportion of smokers in this patient group. GEOP subjects also showed greater prevalences for *B. forsythus* and *A. actinomycetemcomitans*, which may indicate greater involvement of these organisms in these GEOP subjects or the deeper pocketing. The higher prevalence of *T. denticola* compared to other studies in both groups perhaps reflects the use of PCR to determine the microbial prevalence.

In both groups, SRP was effective in reducing clinical parameters: the reduction in PD and AL was in keeping with other published studies. The slight increases in GCF may reflect resolution of inflammation or the healing process. SRP produced few significant changes in the microbial prevalence in AP subjects especially *P. gingivalis* which responded poorly. Again this reflects the use of PCR rather than a poor response to treatment. GEOP subjects had much better reductions in the flora which compared well with previous studies. The greater reduction in flora could reflect the greater reduction in pocket depth recorded in GEOP subjects. It suggests that it may also be harder to reduce bacterial numbers in AP patients.

An analysis of the relationship of pocket depth and clinical and microbial parameters showed that increased BOP, suppuration and bacterial prevalences were related to increased pocket depth in AP patients. In addition, deeper pocketing was linked to greater change in pocket depth following SRP. There were also lower reductions in the microflora in deeper sites. Deeper pocketing in GEOP subjects produced fewer significant differences in clinical and microbial

parameters. Post-SRP, *B. forsythus* and *T. denticola* were associated with deep pocketing in AP subjects and *P. intermedia* in GEOP subjects.

The relationship between the flora and clinical parameters was also examined. Deeper pocketing was related to the presence of *P. gingivalis* and suppuration to the presence of *T. denticola* in AP subjects. There were no significant patterns in GEOP patients, suggesting differences in the way in which AP and GEOP patients interact with the microflora.

In both patient categories bacterial groupings were investigated. In AP subjects the presence of one or more of *P. gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola* indicated increased prevalences for the other organisms. By contrast, in GEOP subjects, there were no clear patterns but *B. forsythus* was always found at *T. denticola* positive sites. A greater number of AP sites had no detectable bacteria than GEOP sites, and in both groups the number of these sites decreased as pocket depth increased.

7.2.2 Comparison of PCR and Checkerboard

This thesis also examined the efficacy of PCR and the Checkerboard technique in periodontal microbial diagnosis. PCR has been suggested as the "gold standard". Both these techniques are quicker, cheaper, less labour intensive and more accurate than culture. They also have lower detection limits. A comparison of the two techniques, PCR and the Checkerboard technique, showed roughly 60% agreement. The generally higher frequencies of detection

for PCR may reflect its lower detection limit. However, there were some problems with the Checkerboard technique and the analysis was not performed under optimal conditions. In addition, the probes for *P. intermedia* were very faint, even against the control bacteria, and the higher detection frequency for *B. forsythus* by the Checkerboard technique may be due to cross-reactivity or non-specific binding. Therefore no conclusions could be drawn from the comparison of these two techniques. In spite of these problems, both PCR and the Checkerboard technique appear to be good tools for periodontal microbial diagnosis. The Checkerboard technique offers advantages over PCR in that a greater number of samples can be analysed and a greater number of different bacteria detected, whereas PCR is much more accurate and useful for detecting specific DNA sequences.

7.2.3 Humoral immune response

Although serum titres appeared relatively high, there were generally low local antibody titres and low antibody avidity. SRP produced little change in antibody titres, which may reflect a poor response and suggests that the poor response may have been a factor in the onset of the disease.

An analysis of serostatus showed similar or higher percentages of high responder patients compared to previous studies. However, unlike previous studies, serostatus had little effect on clinical parameters and response of these parameters to SRP. The poor response was in keeping with the poor host response to therapy in general. *P. gingivalis* was found more commonly in high

responder *P. gingivalis* antibody titre patients. These patients also had deeper pockets compared to low responder subjects and the increased prevalence may reflect the deeper pocketing. Generally there were no significant differences in microbial prevalences between high responder and low responder patients, which may indicate that serostatus has little effect on microbial colonisation in the patients studied. High responder patients had generally higher serum and GCF titres, indicating a better general response than low responder patients, but similar clinical parameters. This suggests that there are other underlying mechanisms that are involved in the disease process in these patients.

P. gingivalis serum and GCF titres were increased when the organism was present and also related to pocket depth. This suggests that *P. gingivalis* is pathogenic in these patients but may reflect the increased number of organisms in deeper sites. *P. gingivalis*, *A. actinomycetemcomitans* and *T. denticola* systemic and local titres correlated suggesting greater systemic input to local titres, whereas *P. intermedia* and *B. forsythus* titres may have greater local production or destruction. The failure of the local response to *B. forsythus* may allow it to be pathogenic.

7.2.4 Effect of smoking

Smoker subjects recorded lower MGI and BOP scores than non-smokers at baseline, which is in keeping with the vasoconstrictive effects of nicotine, but similar pocket depths. A lower reduction in pocket depth was noted in smokers, which probably reflects the general deleterious effects of smoking on the host

and is similar to previous studies. There were no differences between smokers and non-smokers in the microflora at baseline. However, there was a greater decrease in the microflora in non-smokers following SRP and smokers had higher prevalences of *P. intermedia*, *B. forsythus* and *A. actinomycetemcomitans* post-SRP. This may reflect the deeper, more anaerobic pocketing in smokers post-SRP or poorer host clearance due to diminished host response. Smokers in general had lower antibody titres and avidity, and this again reflects the systemic and local effect of smoking and nicotine on the immune system. Both smokers and non-smokers showed the same non-responsiveness in titres to SRP. The reduced titres and avidity may have contributed to the periodontal destruction in smoker subjects and suggests that differences in host response rather than differences in flora between smokers and non-smokers are responsible for the greater destruction seen in some smoker patients.

7.2.5 Overall conclusions

This study showed that SRP is effective in producing clinical improvement in both AP and GEOP subjects, though there were differences between these groups in microflora reductions. The results suggest that the five test organisms may have different roles in the disease processes in the two groups. Smokers had similar clinical and microbial parameters to non-smokers at baseline, but responded less well to treatment than non-smokers reflecting the deleterious effects of smoking on the host. In terms of the humoral immune response, there was little change in antibody titre and avidity following SRP. No differences were found between high responder and low responder patients in their response

to treatment. However, smoking was associated with reduced antibody titres and avidity. It was concluded that PCR and the Checkerboard technique are effective tools for examining the periodontal microflora. The differing frequencies of detection between the two methods probably reflects the higher sensitivity of the PCR technique.

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List of Publications

The following paper is directly related to the work presented in this thesis:

Darby IB, Hodge PJ, Riggio MP, Kinane DF. Microbial comparisons of smoker and non-smokers adult and early-onset periodontitis patients using polymerase chain reaction. J Clin Periodontol 2000; 6: (in press).

Abstract presentations:

Darby IB, Hodge PJ, Riggio MP, Kinane DF. Comparative microflora of adult and early-onset periodontitis. J Dent Res 1998; 78 (Spec Iss): 1518.

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Slaney JM, Darby IB, Fawell S, Aduse-Opoku J, Rangarajan M, Curtis M. Serum antibody response to proteases of *Porphyromonas gingivalis*. J Dent Res 1998; 78 (Spec Iss): 3198.

Microbial comparison of smoker and non-smoker adult and early-onset periodontitis patients by polymerase chain reaction

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Abstract

A number of bacterial species are involved in the aetiology of periodontitis and include *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus* and *Treponema denticola*. Several studies have shown differences in the microflora between the various forms of periodontal disease. It is recognised that smoking is a risk factor for periodontal disease, but there are conflicting reports on whether or not smoking has an effect on the periodontal microflora. We utilised the polymerase chain reaction to determine the presence of *A.actinomycetemcomitans*, *P.gingivalis*, *P. intermedia*, *B. forsythus* and *T. denticola* in subgingival plaque samples in 33 Adult Periodontitis (AP) patients and 24 Generalized early-onset periodontitis (GEOP) patients prior to treatment. When GEOP and AP patients were compared there were significant differences in the number of positive patients and sites for both *A. actinomycetemcomitans* and *B. forsythus* ($p = 0.0023$ and 0.00001 respectively). No statistically significant differences in the prevalence of these organisms were found between smoker and non-smoker groups. These results confirm that AP and GEOP sites harbour varied microflora, but show that *B. forsythus* and *A. actinomycetemcomitans* were detected to a significantly greater extent in this group of GEOP than in the AP patients investigated. Our findings do not support the hypothesis that smokers have significant differences in the prevalence of periodontal pathogens from non-smokers.

Key words: micro-organism; adult periodontitis; generalized early-onset periodontitis; polymerase chain reaction; smoking

Introduction

Periodontal disease affects the supporting structures of the teeth in 10-15% of the population, and is characterised by alveolar bone destruction leading eventually to tooth loss. The two main groups are early onset periodontitis (EOP) and adult periodontitis (AP). The EOP forms of the disease are further subdivided into prepubertal (PP), localised early-onset periodontitis (LEOP), and generalised early-onset periodontitis (GEOP). AP affects subjects over 35 years of age, whereas the EOP variants of the disease can present in the early teens.

The 1996 World Workshop (Zambon 1996) implicated a number of bacteria in the aetiology of periodontitis, namely *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Bacteroides forsythus* and *Treponema denticola*. It has been suggested that the microflora differs between GEOP and AP (Moore & Moore 1994, Haffajee & Socransky 1994). *A. actinomycetemcomitans* has been found with greater frequency and quantity in LEOP patients (Zambon *et al* 1983, Zambon 1985, Haffajee *et al* 1984, Slots *et al* 1990, Savitt *et al* 1991), and has been detected in GEOP patients by a number of authors (Kamma *et al* 1995, van Winkelhoff *et al* 1989). It has also been shown to be present in AP lesions, but less frequently and in lower numbers, with other organisms being more prevalent and in greater numbers (Slots *et al* 1990, Rodenberg *et al* 1990). *P. gingivalis* has been associated with AP (Savitt *et al* 1991, Rodenberg *et al* 1990), and also with active disease (Dzink *et al* 1988, Moore *et al* 1991), and in recurrent lesions (Choi *et al* 1990). A number of studies have associated *P. gingivalis* with GEOP (Kamma *et al* 1995, Sasaki *et al* 1989, Kamagata *et al* 1989, Vandesteen *et al* 1984). In both AP and GEOP, *P. intermedia* has been demonstrated with increased frequency

and numbers (Vandesteen *et al* 1984, Kamma *et al* 1994, Slots & Genco 1984, Slots *et al* 1986). *B. forsythus* has been detected in AP periodontal sites (Gmur *et al* 1989, Haffajee *et al* 1997), and in GEOP sites (Kamma 1995, Kamma *et al* 1994, Listgarten *et al* 1995). Dzink *et al* (1988) and Lai *et al* (1987) demonstrated increased frequency and higher numbers in active periodontal lesions. *T. denticola* has been found in increased numbers in deep pockets of AP patients compared to healthy subjects (Riviere *et al* 1992), but to date there is little information about its detection in GEOP. Smoking is increasingly accepted as a risk factor for periodontitis and has been shown to affect various aspects of the host immune response (Barbour, *et al* 1997). Smoking may have an adverse effect on fibroblast function (Raulin, *et al* 1988), chemotaxis and phagocytosis (Kenney, *et al* 1977, Kraal, *et al* 1977), immunoglobulin production (Holt, *et al* 1987, Johnson, *et al* 1990), induction of peripheral vasoconstriction (Clarke, *et al* 1981), and on the outcome of treatment (Kinane and Radvar 1997). Epidemiological evidence indicates that cigarette smoking is a stronger risk indicator for periodontitis than the presence of certain suspected periodontal pathogens (Stoltenberg *et al* 1993). However, few studies have compared the oral microflora of smokers and non-smokers. No significant difference in the prevalence of various plaque bacteria was found between cultured dental plaque of smokers and non-smokers (Colman, *et al* 1976, Preber, *et al* 1992), or in an immunoassay of similar samples (Stoltenberg, *et al* 1993). Recently Zambon *et al* (1996), using immunofluorescence, found that smokers harboured significantly higher levels of *B. forsythus*, *A. actinomycetemcomitans* and *P. gingivalis.* Significantly higher levels of *Capnocytophaga* species and *E. saburreum* were demonstrated in non-smokers.

The PCR technique is a rapid and sensitive method for the detection of bacterial DNA sequences. The sensitivity of PCR allows detection of periodontal pathogens in subgingival plaque samples below the normal level of detection of culture methods, immunofluorescence, enzyme-based tests and DNA probes (Ashimoto *et al* 1995, Ashimoto *et al* 1996, Riggio *et al* 1996, Riggio *et al* 1998, Watanabe & Frommel 1996). Riggio *et al* (1996) concluded that PCR is much more sensitive than conventional culture methods for identification of periodontal pathogens. The aims of the present study were to use PCR to determine if the prevalence of the accepted periodontal pathogens, *P. gingivalis*, *P. intermedia*, *B. forsythus*, *A. actinomycetemcomitans* and *T. denticola*, differed between AP and EOP patients, and between smokers and non-smokers.

Materials and methods

Patient selection

Fifty-seven patients with untreated periodontal disease, with no history of systemic disease nor antibiotic therapy within the last three months, were recruited consecutively from new referrals to Glasgow Dental Hospital and School between January 1997 and July 1998. Thirty-three patients were designated as severe adult periodontitis (AP) patients, based on pocket depths greater than 6mm in all sextants. Twenty-four patients were designated as generalised early-onset periodontitis (GEOP) patients based on the criteria of Hart *et al* (1991). The mean age and standard deviation of the AP group was 46.6 ± 7.15 years and the EOP group 33.21 ± 3.41

years. The demographic details of these patients are presented in Table 1. The study protocol demanded that each patient had for the purposes of sampling at least two non-adjacent sites per quadrant with pocket depths of at least 5mm. This study was approved by the Glasgow Dental Hospital Ethics Committee. All patients gave informed consent.

Clinical measurements

In each patient, four interproximal sites with pocket depths of at least 5mm were selected, where possible one site in each quadrant, and with no furcation involvement. At each site the modified gingival index (MGI) (Lobene, *et al* 1986), plaque index (PI) (Silness and Loe, 1964), bleeding on probing (BOP), suppuration (Supp), and pocket depth (PD) and attachment level (AL) were recorded. Each tooth was air-dried, MGI was assessed, and a pocket charting probe (PCP 12) was used to determine PI. Supragingival plaque was then removed and gingival crevicular fluid (GCF) collected using filter paper strips for 30 seconds (Adonogianaki *et al* 1991). The volume of fluid was determined using a Periotron 6000 and a previously constructed calibration curve. Pocket depth and attachment level were measured at each site using the Florida probe, and each site was measured twice to assess the variability of the probing measurements. BOP and Suppuration were recorded between pocket depth measurements. The Florida probes were cleaned with 70% isopropyl alcohol between measurements to reduce bacterial contamination of the sites during probing.

Collection of subgingival plaque samples

After the clinical measurements were recorded, a subgingival plaque sample was taken from each of the four selected sites using separate sterile curettes and a single

vertical stroke. Each sample was immediately placed in a sterile microcentrifuge tube containing 0.5ml TE buffer (10mM Tris HCl pH7.6, 1mM EDTA pH8.0). Samples were stored on ice before being transported to the laboratory where they were vortexed and stored at -20°C until analysed.

Polymerase chain reaction

For polymerase chain reaction (PCR) analysis, 90µl of vortex-mixed subgingival plaque was added to 10µl of 10x lysis buffer (100mM Tris-HCl pH 8.0, 10mM ethylenediamine tetra-acetic acid, 10% Triton X-100) and boiled for 5 minutes. 10µl of this lysate was used in each PCR reaction.

The primers used for the various PCR analyses are shown in Table 2 with the size of the amplification product, target and references.

PCR amplification was carried out in a reaction volume of 100µl consisting of 10µl sample lysate and 90µl of reaction mixture containing 1xPCR buffer (10mM Tris-HCl pH8.8, 1.5mM $MgCl_2$, 50mM KCl, 0.1%Triton X-100), 2 units of Dynazyme DNA polymerase (Flowgen, Lichfield, England, UK), 0.2mM dNTPs (dATP, dCTP, dGTP, dTTP) and 50 pmol of each primer. The primers were separated from the other components of the reaction mixture by a layer of wax ("hot start" PCR) preventing the reaction from starting until the wax had melted upon commencement of PCR cycling. PCR cycling was carried out in an OmniGene thermal cycler (Hybaid, Teddington, England, UK). The cycling conditions for *A. actinomycetemcomitans* and *P. intermedia* comprised an initial denaturation for 5 minutes at 95°C, 35 amplification cycles of denaturation at 95°C for 1 minute, annealing of primers at 55°C for 1 minute and primer extension at 72°C for 1.5 minutes, followed by a final extension step at 72

°C for 10 minutes. The cycling conditions for the other organisms were the same except that in the amplification cycles the primer extension step lasted for 1 minute. The reaction products were either stored at -20°C or analysed immediately. Negative and positive controls were included in each batch of samples being analysed by PCR. The negative control was a 90µl reaction mixture with the sample replaced by 10µl of sterile water. The positive control contained 100ng of genomic DNA from the relevant organism in 90µl of reaction mixture, with sterile water added to bring the volume to 100µl.

Analysis of PCR products

20µl of each reaction product was fractionated on a 2% agarose gel containing ethidium bromide (0.5µg/ml), using a 100bp DNA ladder (Pharmacia Biotech, St.Albans, UK) as a size marker, and visualized and photographed using an ImageMaster video documentation system (Pharmacia Biotech).

Data Analysis

The clinical and microbiological data for each site and patient was statistically analysed using the Minitab statistical package (Minitab, release 9.2, Minitab Inc., State College, PA). The data was analysed for differences between AP and EOP groups and non-smokers and smokers, on a patient and site basis. For the patient based analysis the clinical measurements were averaged, and the patient was positive for a microorganism if one or more sites were positive for that organism. The Mann-Whitney test was used to analyse MGI and Plaque Index scores, and two sample t-tests for PD and GCF volumes. The differences between BOP, Supp and all

microorganisms were assessed using the Chi squared test, except when expected counts were less than five where the Fisher's exact test was used. The duplicate recordings of probing depth and attachment level measurements were averaged for each site. Statistical tests were accepted as statistically significant when the p value was < 0.05.

Results

Table 3 shows the average clinical measurements of the selected sites between the two groups. The MGI scores were 2.46 (±0.593) for the AP group (33 patients, 132 sites) compared to 1.92 (±0.833) for EOP group (24 patients, 96 sites), and were significantly different (p=0.006). PI and Supp scores were not significantly different between the two patients groups. Average pocket depth measurements were significantly different (p=0.0002) with 5.9 (±1.004) mm for AP patients and 6.8 (± 0.631) mm for EOP patients. Gingival crevicular fluid volumes were significantly different (p=0.015) with the average AP volume of 403 (± 199.5)nl/30s and average EOP volume 298.2 (±114.5)nl/30s.

The percentage of positive patients for each organism are shown in Table 4. *P. gingivalis* was detected in 54.5% of AP patients compared to 62.5% of EOP patients. *P. intermedia* was detected in 72.7% of AP patients and 79.2% of EOP patients. *B. forsythus* was found in 63.6% of AP patients and 91.7% of EOP patients. 3% of AP patients were positive for *A. actinomycetemcomitans* compared to 20.8% of EOP patients. *T. denticola* was detected in 54.5% of AP patients and 45.8% of EOP

patients. There were significant differences between the groups in the detection of *B. forsythus* (p=0.049) and *A. actinomycetemcomitans* (p=0.038).

The comparison of the percentage of positive sites is shown in Table 5. *P. gingivalis* was detected in 43.9% of AP sites and 54.2% of EOP sites. 56.8% of AP sites were positive for *P. intermedia* and 51.0% of EOP sites. *B. forsythus* was found in 57.6% of AP sites and 83.3% of EOP sites. *A. actinomycetemcomitans* was detected in 1.5% of AP sites and 12.5% of EOP sites. 37.1% of AP sites and 35.4% of EOP were positive for *T. denticola.* There were significant differences again between the two groups for the detection of *B. forsythus* (p=0.01) and *A. actinomycetemcomitans* (p=0.001).

Table 6 compares the clinical measurements between non-smoker and smoker patients. A Chi-squared analysis of the number of smokers in AP and GEOP groups returned a non-significant p value (p>0.05). MGI scores for non-smokers and smokers were 2.37 (±0.78) and 2.01 (±1.09) respectively, and these were significantly different (p=0.0052). The BOP scores for each group were 0.90 (±0.94) and 0.66 (± 0.48) respectively, and were significantly different (P=0.001). However the standard deviation for each BOP group is large (AP ±0.94 and GEOP ±0.48). There were no other significant differences between the two groups.

Table 7 shows the comparison of the percentage of positive non-smoker and smoker patients for each micro-organism. Although there were differences in prevalence between the groups, none were statistically significant. The comparison of the percentage of positive sites for non-smokers and smokers for each organism is shown in Table 8. Again although there were some differences between groups, none were significant.

Table 6 also shows the comparison of the clinical data for AP smokers and non-smokers. The only significant difference between the groups were that smokers have significantly less BOP (p=0.049). The comparison of the microflora for these AP groups is also shown in Tables 7 and 8. When the difference in the prevalence of *T. denticola* between AP smokers and non-smokers was corrected using the Bonferroni correction, it was not found to be significant.

The clinical data for EOP non-smoker and smoker groups are shown in Table 6, and the microbiological data in Tables 7 and 8. The only significant difference in the clinical measurements between the two groups is that smokers again have significantly lower BOP (P=0.049). When the difference in *B. forsythus* prevalence between EOP smokers and non-smokers is Bonferroni corrected, it is not significant.

Discussion

There were a number of significant differences between the two patient categories with respect to their clinical measurements. AP patients appeared to exhibit significantly more gingival inflammation than GEOP patients. GEOP patients were found to have significantly lower BOP scores than AP patients, and similarly significantly lower GCF volumes. As the BOP score may relate to probing pressure and technique, it is possible that operator variation is responsible for the differences between AP and GEOP subjects. GCF measurements tend to be more objective and provide a continuous variable which relates to degree of gingival inflammation. The GCF samples were taken using identical paper strips, for the same duration, measured

using the same Periotron machine and calibrated on the same curve. The differences in clinical inflammation noted between the two groups are therefore more likely to be due to the higher numbers of smokers in the GEOP group. Alternatively it could be hypothesised that GEOP patients have less gingival inflammation.

In AP subjects the patient based analysis showed that the most common organism was *P. intermedia* (72.7%), followed by *B. forsythus* (63.6%), *P. gingivalis* and *T. denticola* (both 54.4%) and lastly *A. actinomycetemcomitans* (3%). The order was slightly different in the site based analysis. *B. forsythus* was the most common (57.6%), then *P. intermedia* (56.8%), *P. gingivalis* (43.9%), *T. denticola* (37.1%), and *A. actinomycetemcomitans* (1.5%).

B. forsythus was the most commonly detected organism in GEOP patients (91.7%), followed by *P. intermedia* (79.2%), *P. gingivalis* (62.5%), *T. denticola* (45.8%) and *A. actinomycetemcomitans* (20.8%). The site based analysis gave a slightly different order. Again *B. forsythus* was the most prevalent (83.3%), but followed by *P. gingivalis* (54.2%), *P. intermedia* (51.0%), *T. denticola* (35.4%) and *A. actinomycetemcomitans* (12.5%). The higher frequency of detection of *B. forsythus* in both patients and sites compared to the other organisms suggests that it may be more closely associated with GEOP.

Both the patient and site analysis of the microflora between the two groups revealed significant differences between the prevalences of *B. forsythus* and *A. actinomycetemcomitans* in AP and GEOP subjects. GEOP patients have a significantly higher prevalence of both of these organisms, which may be due to the deeper pockets noted in our GEOP patients or perhaps their greater involvement in the GEOP disease process. Studies by Dzink, *et al* (1985) and Christersson, *et al* (1992)

have previously reported that *B. forsythus* is associated with deeper pockets. *P. gingivalis* and *P. intermedia* are also found at a higher prevalence in GEOP than AP patients, but this was not statistically significantly different, and may also reflect the deeper pocketing. The prevalence of *T. denticola* was similar between the two groups.

Other studies that have used PCR to detect the presence of periodontopathogens have reported different detection rates than found in this study. Riggio *et al* (1996) reported 24% of 43 AP patients positive for both *P. gingivalis* and *A. actinomycetemcomitans*. In this study we found *P. gingivalis* in 54.5% and *A. actinomycetemcomtians* in 3% of AP patients. In a subsequent study, Riggio *et al* (1998) found 39% of sites and 52% of AP patients PCR-positive for *P. intermedia.* Mooney *et al* (1995) reported that 29% of AP patients were positive for *P. gingivalis* and 47% for *A. actinomycetemcomitans*. Similarly varying prevalences have been reported by other authors (Christersson 1992, Haffajee 1988, Dzink 1985, Ashimoto 1996, Moore 1982) indicating that there is considerable variation in the detection of pathogenic flora between different studies. The high levels of positive sites and patients for the organisms investigated in this study supports the polymicrobial nature of periodontal disease.

The level of *T. denticola* detection was similar between both disease groups (37.1% of AP sites and 35.4% of GEOP sites), but much higher than previously reported (Moore, *et al* 1982, Riviere, *et al* 1995). Riviere *et al* (1995) detected high levels of spirochetes in AP patients, but only 16% of sites were positive for *T. denticola.* The higher prevalences reported in the present study may reflect a more accurate and sensitive diagnostic technique. The difficulty in culturing *T. denticola* may have

masked its role in periodontal disease. Further studies using non-culture techniques could indicate that *T. denticola* may have an important role in both AP and GEOP. Kamma *et al* (1994) examined a number of periodontopathogens from GEOP patients by culture. It was reported that in pockets over 6mm in depth, *P. gingivalis* was detected in 91.7%, *B. forsythus* in 53.4%, and *A. actinomycetemcomitans* in10.9% of sites. In that study the prevalence of *P. gingivalis* was higher, with *B. forsythus* and *A. actinomycetemcomitans* lower when compared with our study. *P. gingivalis* was the more prevalent micro-organism in the study of Kamma *et al* (1994), whereas *B. forsythus* was the more prevalent micro-organism in our present study. The fact that two pathogens can be detected at different levels in two similar groups of patients again supports the variable and polymicrobial nature of periodontal disease. Christersson *et al* (1992) found a correlation between *B. forsythus* and *P. gingivalis* and deep pockets, but none for *P. intermedia* and *A. actinomycetemcomitans*.

The analysis of the clinical data of smokers and non-smokers shows that smokers have significantly lower scores of gingival index and bleeding on probing. The differences between the other measurements were not significant. Preber and Bergstrom (1985, 1986) reported similar findings, although they also reported that smokers have higher plaque scores. Hedin *et al* (1981) and Kinane and Radvar (1997) reported lower GCF volumes in smokers compared to non-smokers, and although the present study also found a lower GCF volume, this was not statistically significant. An analysis of the AP smokers and non-smokers, and the GEOP smokers and non-smokers, found that in both disease groups smokers had significantly lower bleeding scores. All other measurements were not significantly different.

There were no significant differences in the prevalence of the five pathogens between smokers and non-smokers. Our findings are in agreement with Stoltenberg *et al* (1993) and Preber and Bergstrom (1992). In a study of 83 smokers and 62 non-smokers by Preber and Bergstrom (1992) the prevalence of *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* from subgingival plaque of smokers and non-smokers was not significantly different. Stoltenberg *et al* (1993) found no significant differences in the prevalence of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *E. corrodens*, and *F. nucleatum* between smokers and non-smokers. Zambon *et al* (1996) reported in a study of 1426 subjects, of whom approximately 60% smoked, that there were significantly higher levels of *B. forsythus*, *A. actinomycetemcomitans* and *P. gingivalis* in smokers. Although the age range was similar to our study, they analysed all attachment loss levels rather than just concentrating on the deeper sites, and this may account for the differences reported. When smokers and non-smokers were analysed in their respective disease groups, there was a significantly higher prevalence of *T. denticola* in AP smokers and *B. forsythus* in EOP smokers. However when corrected for multiple comparisons and the smaller sample numbers, these differences were not significant.

These results confirm that AP and GEOP sites harbour varied microflora, but show that *B. forsythus* and *A. actinomycetemcomitans* are detected to a significantly greater extent in GEOP than in AP patients. Our findings do not support the hypothesis that smokers have significant differences in the prevalence of specific periodontal pathogens from non-smokers.

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	Number (Sites)	Mean Age	Min. Age	Max. Age	Male	Female	Smokers
AP	33 (132)	46.64	35	66	13	20	10
GEOP	24 (96)	33.21	26	35	9	15	12

Table 1 Number, Age, Sex and Smoking distribution of sample population.

Primer Pairs (5'-3')	Amplicon length (bp)	Target	Reference
P.gingivalis		Fimbrillin gene	Watanabe &
ATAATGGAGAACAGCAGGGAA			Frommel (1993)
TCTTGCCAACCAGTTCCATTGC	131		
P.intermedia		16S RNA	Riggio, *et al* (1997)
CCTAATACCCGATGTTGTCCACA			
AAGGAGTCAACATCTCTGTATCC	855		
A.actinomycetemcomitans		Leukotoxin gene	Goncharoff, *et al*
GGAATTCCTAGGTATTGCGAAACAAT			(1993)
GGAATTCCTGAAATTAAGCTGG	262		
B.forsythus		16S RNA	Slots, *et al* (1995)
GCGTATGTAACCTGCCCGCA			
TGCTTCAGTGTCAGTTATACCT	641		
T.denticola		16S RNA	Slots, *et al* (1995)
TAATACCGAATGTGCTCATTTACAT			
TCAAAGAAGCATTCCCTCTTCTTCTTA	316		

Table 2. Sequences, expected product size, target and references for primers.

Clinical Measurement (n)	AP (SD) (132)	GEOP (SD) (96)	p value
MGI	2.46 (±0.593)	1.92 (±0.833)	0.006 *
PI	1.47 (±0.723)	1.13 (±0.726)	0.1104
BOP	0.86 (±0.1876)	0.74 (±0.2707)	0.049*
Supp	0.27 (±0.2791)	0.34 (±0.3521)	0.95
PD (mm)	5.924 (±1.004)	6.791(±0.631)	0.0002 *
GCF (nl/30s)	403.0 (±199.5)	298.2 (±114.5)	0.015 *

Table 3 Average clinical measurements for the selected sites in each patient group and statistical significance.

Microorganism	AP (132)	GEOP (96)	p value
P. gingivalis	54.5 (18)	62.5 (15)	0.95
P. intermedia	72.7 (24)	79.2 (19)	0.95
B. forsythus	63.6 (21)	91.7 (22)	0.049*
A. actinomycetemcomitans	3 (1)	20.8 (5)	0.038*
T. denticola	54.5 (18)	45.8 (11)	0.95

Table 4 Percentage (and in brackets number) of positive patients for each organism and statistical significance.

Microorganism	AP (132)	GEOP (96)	p value
P. gingivalis	43.94 (58)	54.17 (52)	0.95
P. intermedia	56.82 (75)	51.04 (49)	0.95
B. forsythus	57.58 (76)	83.33 (80)	0.01*
A. actinomycetemcomitans	1.52 (2)	12.5 (12)	0.001*
T. denticola	37.12 (49)	35.42 (34)	0.95

Table 5 Percentage (and in brackets number) of positive sites for each organism at AP and EOP sites, with statistical significance.

Clinical Measurement	Non-Smoker (SD)	Smoker (SD)	AP NS	AP SM	GEOP NS	GEOP SM
MGI	2.3714 (±0.7805)	2.011 (±1.088) *	2.42 (±0.546)	2.55 (±0.715)	2.27 (±0.734)	1.56 (±0.799)
PI	1.3571 (±0.9450)	1.273 (±1.047)	1.44 (±0.688)	1.55 (±0.832)	1.2 (±0.656)	1.04 (±0.811)
BOP	0.90 (±0.9450)	0.6591 (±0.4767)+	0.91 (±0.1217)	0.73 (±0.2486) ^	0.88 (±0.1685)	0.6 (±0.2911) ^
Supp	0.3143 (±0.4659)	0.2727 (±0.4479)	0.3 (±0.2816)	0.175 (±0.2648)	0.33 (±0.3077)	0.35 (±0.405)
PD (mm)	6.346 (±1.253)	6.199 (±1.340)	5.995 (±0.945)	5.763 (±1.166)	7.019 (±0.522)	6.563 (±0.669)
Periotron (nl/30s)	377.4 (±317.3)	324.6 (±294.1)	410.1 (±219.7)	388.9 (±149.4)	320.5 (±114.7)	276.0 (±114.8)

Table 6 Comparison of average clinical measurements for the selected sites in non-smoker (NS) and smoker (SM) patients, and for each disease group. * statistically significantly different $p<0.001$, + significant $p=0.001$, ^ significant $p=0.01$.

	All Patients		Adult Periodonitis		GEOP	
Pathogen	NS (35)	SM (22)	NS (23)	SM (10)	NS (12)	SM (12)
P. gingivalis	60 (21)	54.54 (12)	56.5 (13)	50 (5)	66.66 (8)	58.3 (7)
P. intermedia	71.43 (25)	81.81 (18)	73.9 (17)	70 (7)	66.66 (8)	91.7 (11)
B. forsythus	71.43 (25)	81.81 (18)	65.2 (15)	60 (6)	83.33 (10)	100 (12)
A. actinomycetemcomitans	11.43 (4)	9.09 (2)	4.3 (1)	0 (0)	25 (3)	16.7 (2)
T. denticola	51.43 (18)	50 (11)	52.2 (12)	60 (6)	50 (6)	41.7 (5)

Table 7 Patient based analysis of percentage of positive patients for each organism in smokers (SM) and non-smokers (NS) for each disease category and all patients.

	All Patients		Adult Periodontitis		GEOP	
Pathogen	NS (140)	SM (88)	NS (92)	SM (40)	NS (48)	SM (48)
P. gingivalis	48.57 (68)	47.73 (42)	41 (38)	50 (20)	62.5 (30)	45.83 (22)
P. intermedia	51.43 (72)	59.09 (52)	55 (51)	60 (24)	43.75 (21)	58.33 (28)
B. forsythus	67.14 (94)	71.59 (63)	62 (57)	47.5 (19)	77 (37) *	89.58 (44)*
A. actinomycetemcomitans	5.71 (8)	6.81 (6)	2 (2)	0 (0)	12.5 (6)	5.71 (8)
T. denticola	35 (49)	37.5 (33)	32 (28) *	50 (20) *	43.75 (21)	35 (49)

Table 8 Site based analysis of percentage of positive sites for each organism in smokers (SM) and non-smokers (NS) for each disease category and all patients. * significant difference $p<0.05$, but >0.01. The Bonferroni correction for multiple comparisons would require $p<0.01$ for statistical significance.

Comparative Microflora of Adult and Early-onset Periodontitis.

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Although a large number of species of microorganism are present in the oral cavity, only a few have been consistently implicated in the pathogenesis of periodontal disease, and include *Actinobacillus actinomycetemcomitans* (Aa), *Porphyromonas gingivalis* (Pg), *Prevotella intermedia* (Pi), *Bacteroides forsythus* (Bf), and *Treponema denticola* (Td). A number of studies have shown a difference in the microflora between the various forms of periodontal disease. The majority of these studies have used culture techniques for identification of the suspected periodontal pathogens, but some organisms are difficult to culture (Bf) or cannot be cultured (Td). Polymerase chain reaction (PCR) is a molecular biological approach to identifying organisms regardless of their ability to be cultured. We used PCR to examine the presence of Aa, Pg, Pi, Bf and Td in subgingival plaque samples in 25 Adult Periodontitis (AP) patients and 18 Early-onset periodontitis (EOP) patients before any treatment had been undertaken. Aa was found at 15.8% of EOP sites compared to 1.9% of AP sites, Pg 52.6% to 43.3%, Pi 42.1% to 48%, Bf 97.4% to 61.5%, and Td 44.7% to 40.4% respectively. There were significant differences in the number of positive sites for Aa and Bf ($p = 0.0023$ and 0.00001 respectively). These differences could not be attributed to differences in the clinical measurements, however the level of Bf detection was related to the smoking status of the patients in the EOP group. These findings support the fact that Bf and Aa may be more associated with EOP than AP.

The effect of periodontal therapy on the humoral immune response in patients with chronic periodontal disease. M. PODMORE,* I.B. DARBY & D.F. KINANE. (University of Glasgow Dental School, UK)

Numerous bacteria and the host humoral immune response to their antigens have been implicated in the pathogenesis of periodontal disease. The aim of this study was to assess the effect of initial periodontal therapy on 25 untreated adult periodontitis patients, and specifically assess the patient's serum antibody titres to a range of bacteria and specific antigens. The antigenic targets investigated were the whole fixed bacteria of P.g, A.a, P.i, T.d and B.f, crude outermembrane protein preparations of P.g, A.a, P.i and B.f and various purified antigens thought to play a role; Leukotoxin (A.a), Lipopolysaccharide (P.g), Heat shock protein 60 (P.g) and RIA protease (p.g). Antibody titres were assayed by ELISAs. Following the initial therapy the average reduction in pocket depth was 2.1 mm ($\pm$ 0.9). In addition, specific antibody titres increased after treatment for all whole bacteria and antigens tested, for example, the antibody titre to P.g whole cells before treatment was 0.510 ELISA units ($\pm$ 0.180) and after treatment was 0.927 ELISA units ($\pm$ 0.334). This increase was statistically significant ($P<0.05$). Interestingly, P.g outer membrane protein preparations had a 119% increase in titre whereas antibodies to Lipopolysaccharide from P.g increased by only 25.1%.

In conclusion, periodontal therapy influenced the magnitude of the humoral immune response to all of the putative periodontal pathogens and specific antigens tested. Initial periodontal treatment does not increase antibody titres to certain periodontal pathogens and their antigens to the same extent and this may indicate the relevance of certain candidate antigens in the disease process.

Serum antibody response to proteases of *Porphyromonas gingivalis*.
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Proteases of *Porphyromonas gingivalis* are considered to be important microbial determinants in the periodontal diseases. The specific immune response to these factors may therefore play a critical role in the host defence to this organism. In the present study the serum IgG specific antibody response in adult periodontal patients (25) was measured by ELISA to *P. gingivalis* W50 whole organism and compared to antibody responses to the arginine protease RIA, to a recombinant form of this enzyme expressed in *E. coli* as a $(his)_6$-tagged fusion protein and to lipopolysaccharide (LPS) of this organism. Purification of the $(his)_6$-tagged fusion protein was performed by nickel affinity chromatography. The IgG antibody response to *P. gingivalis* whole cells was significantly correlated with the response to RIA ($p=0.0001$) but showed no correlation with the response to the recombinant enzyme. Futhermore there was no relationship between the responses to the wild type and recombinant enzymes. However there was a strong correlation between the IgG antibody response to RIA and to LPS ($p=0.0001$). Treatment of RIA with trifluoromethane sulphonic acid to remove covalently linked carbohydrate residues caused a significant reduction in the IgG recognition of the enzyme by on Western blots and also abolished the reactivity of RIA with anti-LPS monoclonal antibodies. We conclude that the IgG antibody response of adult periodontal patients to *P. gingivalis* RIA is directed primarily towards covalently-linked carbohydrate residues which are cross-reactive with the LPS of this organism. Glycosylation of this enzyme may represent an immune shielding device to hinder recognition of the enzyme polypeptide chain.